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Characterization of BmaI endonuclease from *Bacillus macerans* ATCC 8244

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*Bacillus macerans*의 BmaI endonuclease의 특성에 대한 연구

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ABSTRACT: The isolation and characterization of a new type II restriction endonuclease, BmaI, from *Bacillus macerans* ATCC 8244 were described. BmaI endonuclease was partially purified by procedures of ammonium sulfate fractionation, DEAE-cellulose and phosphocellulose chromatographies. This enzyme recognized one site on pBR322 DNA, two sites on Bluescribe DNA, three sites on λDNA and no site on SV 40 DNA. The same cleavage patterns for various DNAs as PvuI indicated that BmaI is an isoschisomer of PvuI whose recognition sequence is 5′-CGATCG-3′. The optimal pH for the BmaI endonuclease activity was about 7.0 and optimal NaCl concentration was about 100 mM. Manganese ion could partially replace magnesium as a cofactor, but calcium could not at all.
KEY WORDS □ type II restriction endonuclease, BmaI endonuclease, *Bacillus macerans*.

Since the first type II restriction endonuclease, Hind II, was isolated and characterized from *Haemophilus influenzae* (Kelly and Smith, 1970), many restriction endonucleases have been isolated. According to recent report, 515 different restriction endonucleases from 397 bacterial strains were listed. Among them there are at least 116 different cleavage specificities (Roberts, 1985).

Type II restriction enzymes recognize the specific sequence of a DNA and make a double strand cleavage within the sequence. Therefore, to use a certain strain as a host in recombinant DNA technology, it is necessary to study the restriction-modification system of the strain.

In this report a new type II restriction endonuclease, BmaI endonuclease, from *Bacillus macerans* ATCC 8244 was partially purified and characterized.

MATERIALS AND METHODS

Chemicals

DEAE-cellulose (DE-22) and phosphocellulose (P-11) were purchased from Whatman. They were precycled and equilibrated by the procedures of Green *et al.* (1978). Other chemicals were reagent grade.

Bacterial strains

Bacillus macerans ATCC 8244 and other strains were provided by Y.H. Kho (KAIST, Korea). Cells were grown at 37°C in LB medium (10g of Bactotrypton, 5g of Yeast extract and 10g of NaCl per liter) until OD_{600} of the culture reached 1.0.

DNAs and Enzymes

Plasmid pBR322 (Bolivar *et al.*, 1977) was isolated by procedures of Birnboim and Doly (1979). λ DNA was prepared from *E. coli* W 3110 according to the methods of Maniatis *et al.* (1982). PvuI endonuclease was provided by Dr. U.J. Yoo (KAIST, Korea). Other restriction enzymes were purchased from New England Biolabs (NEB) and Bethesda Research Laboratories (BRL). The protein concentration was measured by the methods of Lowry *et al.* (1951) and Bradford (1976). DNA concentration was determined by spectrophotometer.

Screening of restriction enzyme activity

The 100 m*l* culture was harvested by centrifugation and suspended in 1 m*l* of a sonication buffer consisting of 50 mM Tris. Cl (pH 8.0), 10 mM 2-mercaptoethanol and 1 mM EDTA. The suspension was treated with 200 ug/m*l* lysozyme on ice for 30 minutes and sonicated ten times for 10 sec. The sonicated sample was clarified by Eppendorf centrifuge for 30 minutes at 4°C. A 2 u*l* serially diluted sample was used to screen the restriction activity. The 20 u*l* reaction mixture contained 33 mM Tris. Cl (pH 7.0), 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol. The reaction was terminated by adding 5X stopping buffer (0.1 mM EDTA, 0.01% bromophenol blue and 40% sucrose). Then SDS was added to final concentration of 0.2% and incubated at 67°C. The mixture was analysed by agarose gel electrophoresis.

Assay of BmaI endonuclease

BmaI endonuclease activity was assayed by monitoring the cleavage of pBR322 DNA by agarose gel electrophoresis. The 20 u*l* reaction mixture contained 10 mM Tris. Cl (pH 7.5), 6 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 100 mM NaCl, 100 ug/m*l* BSA, 500 ng of DNA and 1 u*l* of enzyme solution. The mixtures were incubated at 37°C for 30 minutes and terminated by adding stopping buffer. One unit is the enzyme activity that completely digest 1 ug of λDNA for 1 hour in optimal condition.

Isolation of BmaI endonuclease

All procedures described below were performed at 4°C and centrifugation was done with Sorvall RC-5B SS34 rotor at 12,000 g for 1 hour. Buffers used in the isolation were as follows: Buffer A; 20 mM Tris. Cl (pH 8.0), 10 mM 2-mercaptoethanol and 1 mM EDTA. Buffer B; 20 mM Tris. Cl (pH 8.0), 7 mM 2-mercaptoethanol, 0.2 mM EDTA and 10% glycerol. Buffer C; 20 mM sodium phosphate (pH 7.4), 7 mM 2-mercaptoethanol, 0.2 mM EDTA and 10% glycerol.

Step 1: Preparation of crude extract; 20 g cells were suspended in 60 m*l* of Buffer A. Lysozyme (150 ug/m*l*) was added and stirred on ice for 30 minutes. The suspension was sonicated on ice ten times for 30 sec. The sonicated suspension was clarified by centrifugation for 1 hour. The resulting supernatant was diluted with Buffer A to protein concentration of 10 mg/m*l* (Fraction I).

Step 2: Ammonium sulfate fractionation; Powdered ammonium sulfate was added to Fraction I to 30% saturation with stirring on ice for 30 minutes. After further stirring for 30 minutes, the supernatant was collected by centrifugation and another ammonium sulfate was added to 70% saturation. After further stirring for 30 minutes, the precipitate collected by centrifugation was suspended in 30 m*l* of Buffer B and dialysed against 2 liter of Buffer B with three changes for 16 hours. The dialysed sample was diluted with Buffer B to protein concentration of 10 mg/m*l* (Fraction II).

Step 3: DEAE-cellulose chromatography; Fraction II was applied to DEAE-cellulose column (2 × 16 cm). The column was washed with Buffer B until A_{280} of the effluent reached 0.05. The column was eluted with a 300 m*l* linear gradient of NaCl (0 to 0.6 N) in Buffer B at a flow rate of 15 m*l*/hour. Small aliquot (1 u*l*) from every other fractions was used for BmaI endonuclease assays. Active fractions were pooled and dialysed against Buffer C (Fraction III).

Step 4: Phosphocellulose chromatography; Fraction III was applied to phosphocellulose column (1.5 × 6.8 cm). The column was washed with 5 column volumes of Buffer C and eluted with a 120 m*l* linear NaCl gradient (0 to 0.6 N) in Buffer C at a flow rate of 5 m*l*/hour. The enzyme was pooled and dialysed against 50% glycerol in Buffer B

(Fraction IV).

RESULTS AND DISCUSSION

Screening of restriction activity for various *Bacillus* strains

Restriction activity was screened for various Bacillus strains. As shown in Fig. 1, *Bacillus macerans* ATCC 8244 apparently showed restriction activity.

Isolation of BmaI endonuclease

A summary of the isolation of BmaI endonuclease from 20 g of cells is presented in Table I. Fig. 2 shows DEAE-cellulose chromatography of BmaI endonuclease. The enzyme was eluted between

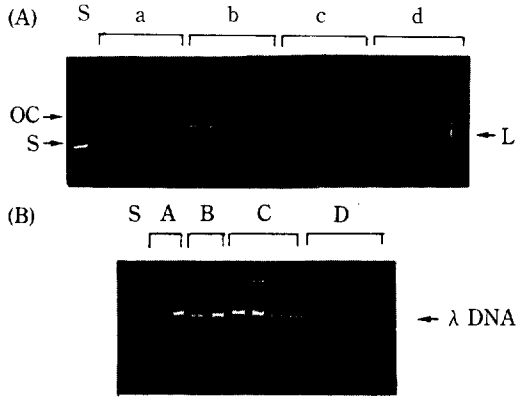

Fig. 1. *Screening of restriction activity for various Bacillus strains. The 500 ng of pBR322 DNA (A) and λ DNA (B) were digested with serially diluted samples of various Bacillus strains as described in METHODS.*
Lanes a and A; *B. polymixa* KCTC 1761. lanes b and B; *B. macerans* ATCC 8244. lanes c and C; *B. thermoglucosidasius* KCTC 1828. lanes d and D; *B. licheniformis* ATCC 21415. OC; open circular pBR322 DNA. S; supercoiled pBR322 DNA. L; linear pBR322 DNA.

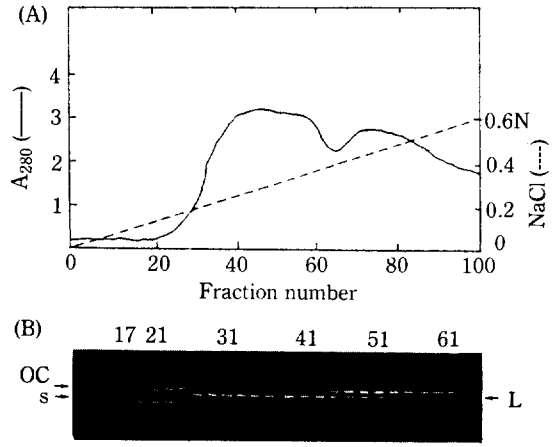

Fig. 2. *(A); Protein profile of DEAE-cellulose chromatography. (B); Agarose gel electrophoresis of pBR322 DNA digested with fractions of DEAE-cellulose chromatography.*
The enzyme was eluted between 0.17 and 0.21 N NaCl. OC; open circular pBR322 DNA. S; supercoiled pBR322 DNA. L; linear pBR322 DNA.

0.17 and 0.21 N NaCl. Fig. 3 shows phosphocellulose chromatography of BmaI endonuclease. The enzyme was eluted at 0.4 N NaCl.

The enzyme was stable for at least 4 months at -20 °C in 50% glycerol. The yield of BmaI endonuclease was 80 units per gram cells. Nonspecific endonuclease of the enzyme was assayed by incubation of 1 ug of λ DNA with 10 units of BmaI endonuclease for overnight. Exonuclease activity was assayed by ligation and recutting. These contaminating activities were negligible for general cloning purposes (Data not shown).

Characterization of BmaI endonuclease

To study the properties of BmaI, various DNAs were digested with BmaI. As shown in Fig. 4 this enzyme recognized one site on pBR322

Table 1. *Summary of the isolation of BmaI endonuclease.*

Fraction	Volume (ml)	Total protein (mg)	Total unit ($\times 10^{-3}$)	Specific activity units/mg ($\times 10^{-2}$)
I Crude extract	100	970	-	-
II Ammonium sulfate	80	790	-	-
III DEAE-cellulose	15	30	7.5	2.5
IV Phosphocellulose	1.6	0.8	1.6	20

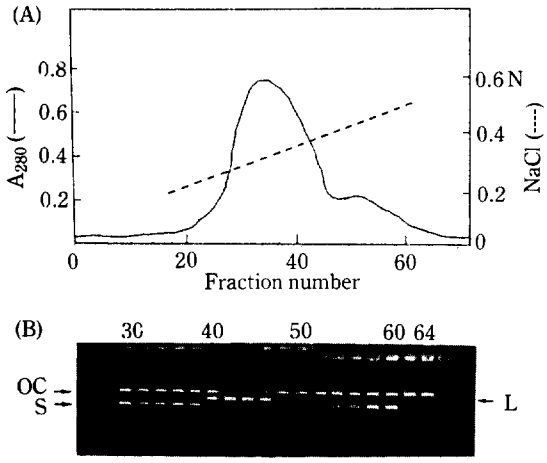

Fig. 3. *(A); Protein profile of phosphocellulose chromatography. (B); Agarose gel electrophoresis of pBR322 DNA digested with fractions of phosphocellulose chromatography.*
The enzyme was eluted at 0.4 N NaCl. OC; open circular pBR322 DNA. S; supercoiled pBR322 DNA. L; linear pBR322 DNA.

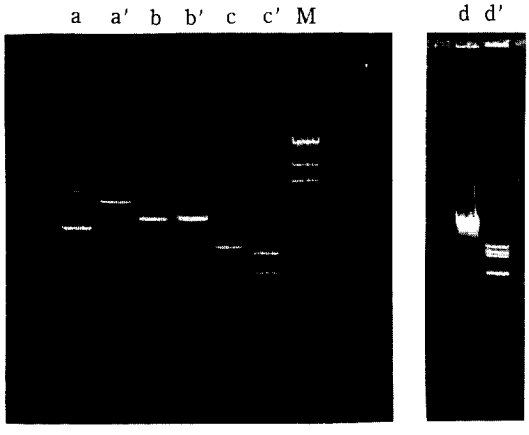

Fig. 4. *Cleavage patterns of various DNAs with BmaI endonuclease.*
Lane a; pBR322 DNA. a'; pBR322 + BmaI. b; SV 40 DNA. b'; SV 40 DNA + BmaI. c; Bluescribe DNA. c'; Bluescribe DNA + BmaI. d; λ DNA. d'; λ DNA + BmaI. M; λ DNA + Hind III.

DNA, two sites on Bluescribe DNA, three sites on λ DNA and none on SV 40 DNA. Since BmaI has a single site on pBR322 DNA, the location of the cleavage site of BmaI was determined on pBR322 DNA (Fig. 5). When pBR322 DNA was codigest-

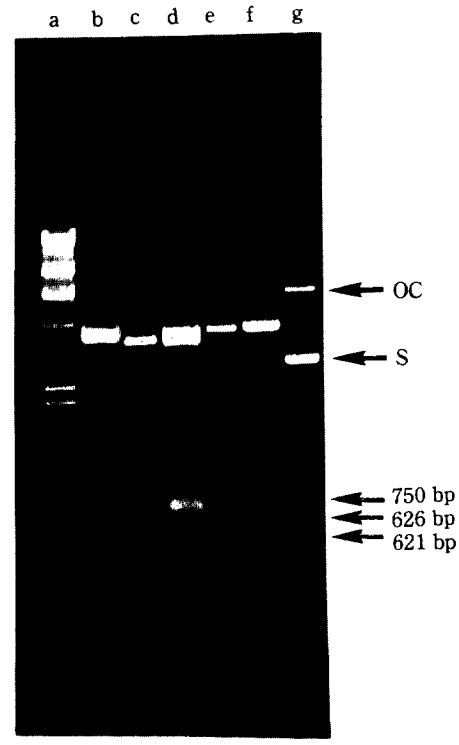

Fig. 5. *Determination of BmaI site on pBR322 DNA.*
Lane a; λ DNA + Hind III. b; pBR322 DNA + Hind III + Sal I. c; pBR322 DNA + BmaI + EcoRI. d; pBR322 DNA + PstI + EcoRI. e; pBR322 DNA + BmaI + PstI. f; pBR322 DNA + BmaI. g; pBR322 DNA. OC; open circular pBR 322 DNA. S; supercoiled pBR322 DNA.

ed with BmaI and PstI, the size of the large fragment was about 4.3 kb (lane e). This showed that BmaI site was in the proximity of PstI site. To determine BmaI site more precisely, pBR322 DNA was codigested with various restriction enzymes. The small fragment of pBR322 DNA codigested with BmaI and EcoRI (lane c) was smaller than 750 bp fragment of pBR322 DNA codigested with PstI and EcoRI (lane d), and was nearly the same as 621 bp fragment of pBR322 DNA codigested with Hind III and Sal I (lane b). This fact implies that BmaI site on pBR322 DNA is away from PstI site by 120-130 bp to EcoRI site. On pBR322 DNA PvuI has the same locus.

To determine whether BmaI is an isoschisomer of PvuI, the cleavage patterns of λ DNA for BmaI and PvuI were compared. As shown in Fig. 6, the

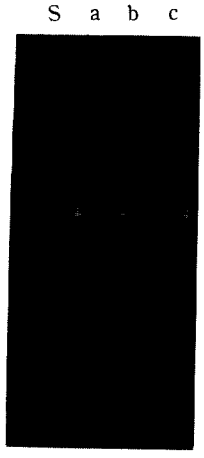

Fig. 6. *Comparison of cleavage patterns of λ DNA for BmaI and PvuI endonuclease.*
Lane S; λ DNA. lane a; λ DNA + BmaI. lane b; λ DNA + BmaI + PvuI. lane c; λ DNA + PvuI.

cleavage pattern of λ DNA with BmaI was precisely the same as that of PvuI. The fact that the cleavage patterns of various DNAs with BmaI were precisely the same as those of PvuI indicated that BmaI is an isoschisomer of PvuI whose recognition sequence is 5′-CGATCG-3′. The cutting mode of BmaI endonuclease is under study at present.

The optimal temperature for BmaI was about 37°C. The enzyme was inactivated after incubation at 65°C for 10 minutes. The optimal pH was about 7.0. The optimal NaCl concentration was about 100 mM. The enzyme was inactivated above 150 mM NaCl. Manganese ion could replace magnesium partially as a cofact, but calcium could not at all.

적 요

Bacillus macerans ATCC 8244로 부터 새로운 type II restriction endonuclease(Bma I)를 분리하여 그 특성을 연구하였다. 분리 방법으로는 ammonium sulfate fractionation, DEAE-cellulose chromatography, 그리고 phosphocellulose chromatography를 사용하였다. 여러가지 DNA에 대한 Bma I과 Pvu I의 cleavage pattern을 비교해본 결과, Bma I endonuclease는 5′-CGATCG-3′을 인지하는 Pvu I endonuclease의 isoschizomer임이 밝혀졌다. 이 효소의 cofactor로써 Mn^{++}는 Mg^{++}를 부분적으로 대치할 수 있었으나, Ca^{++}는 전혀 cofactor로써 작용하지 못하였다. 이 효소의 최적 활성 pH는 7.0이었으며, 최적 NaCl 농도는 100 mM 이었다.

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