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Characterization of BmaI methylase from *Bacillus macerans*

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*Bacillus macerans*의 BmaI methylase의 특성

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ABSTRACT: The isolation and characterization of a new type II methylase, BmaI methylase, from *Bacillus macerans* ATCC 8244 were described. BmaI methylase was isolated by procedures of ammonium sulfate fractionation, DEAE-cellulose chromatography and phosphocellulose chromatography. Two types of methylases were present in this strain and only one of the two was a site specific BmaI methylase. The pBR322 DNA methylated by BmaI methylase was not cleaved by BmaI endonuclease, and pBR322 DNA cleaved by BmaI endonuclease was not methylated by BmaI methylase. The optimal pH for the BmaI methylase activity was 7.5, and optimal NaCl concentration was about 50 mM. BmaI methylase could methylate single-stranded M13mp18 DNA.
KEY WORDS□Type II methylase, BmaI methylase

Many microorganisms contain restriction-modification system. Type II restriction enzyme recognizes a specific sequence of a certain DNA and makes a double strand cleavage within the sequence. Its cognate modification enzyme recognizes and modifies the same sequence and protects it against restriction endonuclease (Roberts, 1985).

The methylation product of type II methylase is usually either N^6-methyladenosine or 5-methylcytosine. Methylation of guanine or thymine has not been detected.

Many of the type II methylases were isolated and characterized (Yoo and Agarwal, 1980; Kim and Rho, 1984). Recently clonings of restriction-modification genes were also reported (Mann *et al.*, 1978; Walder *et al.*, 1981; Jun *et al.*, 1987).

We have isolated and characterized a new type II restriction endonuclease, BmaI endonuclease, from *Bacillus macerans* ATCC 8244 (Kwon *et al.*, 1988). This enzyme was an isoschisomer of PvuI endonuclease whose recognition sequence is 5'-CGATCG-3'. In this report to study the restriction-modification system of *Bacillus macerans* ATCC 8244, the isolation and characterization of BmaI methylase from this strain were described.

MATERIALS AND METHODS

Chemicals

DEAE-cellulose (DE-22) and phosphocellulose (P-11) were purchased from Whatman. S-Adenosyl-(^{3}H-methyl)-methionine (73.8 mCi/mmol) was purchased from New England Nuclear. Other chemicals were reagent grade.

Bacterial strains

Bacillus macerans ATCC 8244 was 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 DNA (Bolivar *et al.*, 1977) was isolated by procedures of Birnboim and Doly (1979). BmaI endonuclease was isolated as described elsewhere (Kwon *et al.*, 1988). Other restriction enzymes were purchased from New England Biolabs (NEB) and Bethesda Research Raboratories (BRL).

Preparation of resins

Whatman DEAE-cellulose (DE-22) and phosphocellulose (P-11) were precycled and equilibrated by the procedures of Green *et al.* (1978).

Calculation of concentration of protein and DNA

The protein concentration was determined by the method of Lowry *et al.* (1951) and Bradford (1976). DNA concentration was measured by spectrophotometer.

Assay of BmaI methylase

The assay for the BmaI methylase activity measures the transfer of ^{3}H-methyl group to pBR 322 DNA. The reaction (50 u*l*) contained 50 mM Tris.Cl (pH 7.5), 5 mM EDTA, 50 ug/m*l* BSA, 10 mM 2-mercaptoethanol, 0.6 ug of pBR322 DNA, 0.07 uM ^{3}H-AdoMet and 2 u*l* of enzyme solution. After incubation for 1 hour at 37°C, 30 u*l* samples were spotted on DE-81 paper and washed with 0.2 M NH_4HCO_3 solution, twice with ethanol, once with ether and dried for 30 minutes at 60°C. The radioactivity was determined with Beckman Scintillation Counter after addition of 5 m*l* toluene scintillant. One unit of methylase activity transfers 1 pmol of ^{3}H-methyl group to λDNA in 1 hour at optimal condition.

Methylation of pBR322 DNA with BmaI methylase following the digestion with BmaI endonuclease

For the digestion of pBR322 DNA with BmaI endonuclease the reaction mixture contained 10 mM Tris. Cl(pH 7.5), 6 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 100 mM NaCl, 50 ug/m*l* BSA, 500 ng of pBR322 DNA and 1 unit of BmaI endonuclease. After incubation at 37°C for 1 hour, 0.07 uM ^{3}H-AdoMet was added to reaction mixture and then 10 units of BmaI methylase.

Digestion of pBR322 DNA with BmaI endonuclease following the methylation with BmaI methylase

For the methylation of pBR322 DNA with BmaI methylase the reaction mixture (50 u*l*) contained 50 mM Tris. Cl (pH 7.5), 10 mM 2-mercaptoethanol, 0.2 mM EDTA, 0.07 uM AdoMet, 500 ng of pBR322 DNA and 10 units of BmaI methylase. After incubation at 37°C for 1 hour, $MgCl_2$ was added to 10 mM and then 1 unit of BmaI endonuclease.

RESULTS AND DISCUSSION

The summary of the isolation of BmaI methylase from 20 g of cells is presented in Table 1. All procedures 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; The 20g of cells were suspended in 60 m*l* of Buffer A. The suspension was treated with 150 ug/m*l* of lysozyme on ice for 30 minutes and was sonicated ten

Table I. *Summary of the isolation of BmaI methylase.*

Fraction	Volume (m*l*)	Total protein (mg)	Total unit ($\times 10^{-3}$)	Specific activity units/mg ($\times 10^{-2}$)
I Crude extract	110	1130	—	—
II Ammonium sulfate	80	760	—	—
III DEAE-cellulose	25	50	5.0	1
IV Phosphocellulose	1.4	0.9	1.4	16

times for 30 sec. The sonicated suspension was clarified by centrifugation. The resulting supernatant was diluted with Buffer A to protein concentration of 10 mg/ml (Fraction I; 110 ml).

Step 2: Ammonium sulfate fractionation; Ammonium sulfate fractionation was performed at a range of 40 to 60%. The precipitate collected by centrifugation was suspended in 30 ml of Buffer B and dialysed against Buffer B. The dialysed sample was diluted to protein concentration of 10 mg/ml (Fraction II; 80 ml).

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 enzyme was eluted with a 300 ml linear gradient of NaCl (0 to 0.6 N) in Buffer B at a flow rate of 15 ml/hour. Every other fractions were assayed for methylase activities. As shown in Fig. 1, there were two peaks of methylases on DEAE-cellulose chromatography. Methylase peak I was eluted between 0.2 and 0.22 N NaCl and methylase peak II between 0.4 and 0.42 N NaCl. They were pooled and dialysed against Buffer C respectively (Fraction III for methylase peak I; 25 ml, Fraction III' for methylase peak II; 30 ml).

Step 4: Phosphocellulose chromatography of methylase peak I: Dialysed sample of methylase peak I was applied to phosphocellulose column (1.5 × 7 cm). The column was washed with 5 column volumes of Buffer C. The enzyme was eluted with

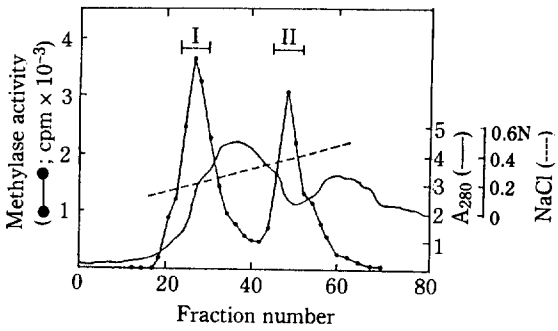

Fig. 1. *DEAE-cellulose chromatography. There were two peaks of methylases on DEAE-cellulose chromatography.*

Methylase peak I was eluted between 0.2 and 0.22 N NaCl and methylase peak II between 0.4 and 0.42 N NaCl.

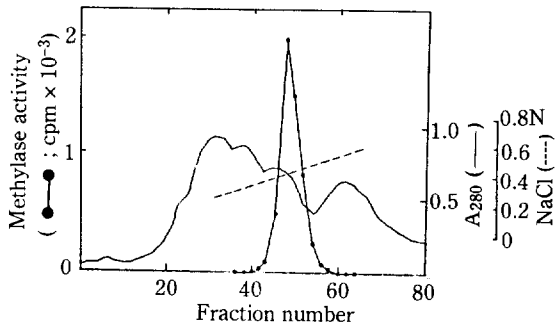

Fig. 2. *Phosphocellulose chromatography of methylase peak I.*

The column (1.5 × 7 cm) was eluted with a 120 ml linear NaCl gradient (0 to 0.6 N) in Buffer C at a flow rate of 5 ml/hour. The enzyme was eluted at 0.45 N NaCl.

120 ml linear NaCl gradient (0 to 0.6 N) in Buffer C at a flow rate of 5 ml/hour. As shown in Fig. 2, the enzyme was eluted at 0.45 N NaCl. The active fractions were pooled and dialysed against 50% glycerol in Buffer C (Fraction IV; 1.4 ml).

Step 5: Phosphocellulose chromatography of methylase peak II; The column was run in similar conditions to that of methylase peak I. The enzyme was eluted at 0.25 N NaCl (Fig. 3). The active fractions were pooled and dialysed against 50% glycerol in Buffer C (Fraction IV'; 2 ml).

These enzymes isolated above were stable for at least 4 months at -20°C in 50% glycerol. The yield of BmaI methylase (Fraction IV) was 70 units per gram cells. BmaI methylase was assayed for BmaI endonuclease and nonspecific nucleases,

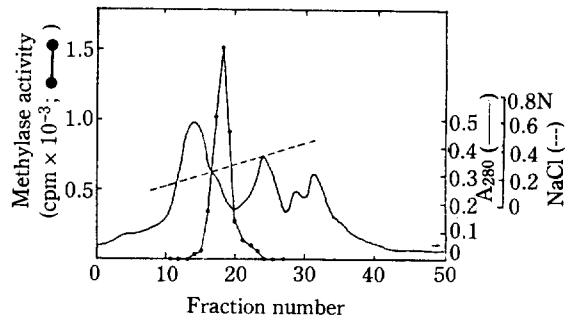

Fig. 3. *Phosphocellulose chromatography of methylase peak II.*

The column (1.5 × 8.0 cm) was eluted with a 150 ml linear gradient of NaCl (0 to 0.6 N) in Buffer C at a flow rate of 7 ml/hour. The enzyme was eluted at 0.25 N NaCl.

Table II. *Methylation of BmaI-cleaved pBR322 DNA with methylase peak I and peak II.*

Reaction	Bma I endonuclease	Methylase	^{3}H-cpm
A	+	I	134
B	–	I	4687
C	+	II	1696
D	–	II	1713

but these activities were negligible for general purposes.

Determination of specificity of BmaI methylase

To determine which of the two methylases, methylase peak I and peak II, is a site specific BmaI methylase, pBR322 DNA digested with BmaI endonuclease was methylated with methylase peak I and peak II respectively (Table II). The cpm value of reaction C which used pBR322 DNA digested with BmaI endonuclease as a substrate for methylase peak II was similar to that of reaction D which used pBR322 supercoiled DNA. But the cpm value of reaction A which used pBR322 DNA digested with BmaI endonuclease as a substrate for methylase peak I was control level to that of reaction B. This fact implies that methylase peak I is a site specific BmaI methylase. Furthermore as shown in Fig. 4, pBR322 DNA methylated with BmaI methylase was not digested with BmaI endonuclease. This fact indicates that BmaI methylase methylates BmaI site and protects it against BmaI endonuclease.

Methylation of single-stranded M13mp18 DNA

BmaI methylase methylated M13mp18 single-stranded DNA, although the degree of methylation was slightly lower than that of M13mp18 RF DNA (Table III). This fact indicates BmaI methylase recognizes single-stranded DNA as a substrate.

Catalytic properties of BmaI methylase

BmaI methylase was maximally active at pH 7.5 (Table IV). The optimal NaCl concentration was about 50 mM (Table V). The enzyme seemed not to require divalent cation. The optimal temperature of this enzyme was about 37°C. This enzyme was inactivated by the incubation at 60°C for 5 minutes.

E. coli dam methylase can methylate adenine of

Fig. 4. *Determination of specificity of BmaI methylase using pBR 322 DNA.*
Lane a; pBR322 DNA. lane b; BmaI methylase treatment followed by BmaI endonuclease treatment. lane c; BmaI endonuclease treatment. lane d; BmaI methylase treatment followed by BmaI and EcoRI endonuclease treatment. lane e; BmaI and EcoRI endonuclease treatment. lane M; size maker.

Table III. *Methylation of single-stranded M13mp18 DNA by BmaI methylase.*

Reaction	DNA	BmaI methylase	^{3}H-cpm
A	M13mp18 RF DNA	+	5542
B	M13mp18 single-stranded DNA	+	3678
C	M13mp18 RF DNA	–	47

Table IV. *pH dependence of BmaI methylase activity.*

Enzyme	pH							
	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
	^{3}H-cpm							
BmaI methylase	133	1100	5810	7136	6454	3193	1611	137

* The assay of methylase activity was performed as described in METHODS.

Table V. *Ionic strength dependence of BmaI methylase activity.*

Enzyme	Ionic strength (NaCl)				
	0 mM	50 mM	100 mM	150 mM	200 mM
	^{3}H-cpm				
BmaI methylase	7104	10984	3585	197	45

* The assay of methylase activity was performed as described in METHODS.

GATC within CGATCG which is the recognition sequence of BmaI endonuclease. BmaI methylase may be cytosine methylase since adenine methylated pBR322 DNA with *E. coli* dam methylase was cleaved by BmaI endonuclease.

Methylase peak II is thought to be nonspecific methylase judging from the fact that only one type of type II restriction endonuclease is detected in this strain and methylase peak II can not protect recognition sequence of BmaI endonuclease.

적 요

Bacillus macerans ATCC 8244로부터 BmaI methylase를 분리하여 그 특성을 연구하였다. 분리방법으로는 ammonium sulfate fractionation, DEAE-cellulose chromatography 그리고 phosphocellulose chromatography를 사용하였다. 이 균주에는 두 종류의 methylase가 존재하였으며 이 중 한가지만이 site-specific BmaI methylase였다. BmaI methylase는 BmaI endonuclease로 자른 pBR322 DNA를 methylation시키지 못하였으며, BmaI endonuclease는 BmaI methylase로 methylation시킨 pBR322 DNA를 자르지 못하였다. 또한 BmaI methylase의 최적활성 pH는 7.5였으며 최적 NaCl 농도는 50 mM이었다.

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