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A New Restriction Endonuclease from *Clostridium thermocellum*

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*Clostridium thermocellum*으로 부터 새로운 type II 제한효소 Cth I의 분리

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The isolation and characterization of type II restriction endonuclease from *Clostridium thermocellum* ATCC 27405 were described. This enzyme (Cth I endonuclease) is an isoschizomer of Bcl I endonuclease recognizing 5'-TGATCA-3'. Cth I endonuclease requires Mg^{2+} ion for its activity and is maximally active at pH 7.5 to 10.5 in the presence of 0 to 10mM NaCl. Cth I endonuclease is heat stable and has an optimum temperature of 60°C. The activity of Cth I enzyme is sensitive to *dam* methylation.

Type II restriction endonucleases, which cleave DNA at defined nucleotide sequences, are extensively used in the structural analysis of DNA molecules, and for the construction of recombinant DNA species *in vitro*. Also restriction endonucleases are investigated as a potentially useful model system for the study of site specific protein-DNA interaction. New specificities are continually required in order to increase the number of ways in which DNA can be manipulated *in vitro*. Finding of isoschizomers from various microorganisms is also necessary because the new enzymes can have advantages in purification processes and catalytic properties over the known enzymes. This paper describes the partial purification and characterization of Cth I endonuclease from *Clostridium thermocellum* which is an isoschizomer of Bcl I endonuclease (for Bcl I, 1).

Materials and methods

Materials

The strain of *Clostridium thermocellum* was from American Type of Culture Collection (ATCC 27405). Restriction enzymes, Bcl I, and Lambda DNA (N^6-methyl adenine free) were purchased from New England BioLabs (USA). Adenovirus 2 DNA was the generous gift from Genetic Engineering Center, Korea. 2-Mercaptoethanol was purchased from Aldrich Chemical Company, Inc. (USA). Yeast extract was from Difco Laboratories (Detroit, Mich., USA). Ammonium sulfate, reagent grade glycerol and sodium chloride were from Kanto Chemical Company (Japan). Electrophoresis grade agarose, EDTA, Trizma base, cellobiose, cysteine and ethidium bromide were purchased from Sigma Chemical Company. Phosphocellulose

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(P-11), DEAE-Cellulose (A-50) were delivered by Whatman. All other chemicals used during the isolation procedure are reagent grade.

Culture of cells

Clostridium thermocellum ATCC 27405 was grown in 2 liters of the culture medium (per liter; 1.5 g K_2HPO_4, 2.5 g KH_2PO_4, 1 g cysteine, 5 g yeast extract, 0.002 g resazurin, 0.006 g $FeSO_4$, 5 g D(+)-cellobiose, pH 7.6) anaerobically at 60 °C. Cells were harvested at late exponential phase ($A_{525}=1.3$) and adjusted to 50% (w/w) glycerol. The cell paste was stored at −20 °C.

Enzyme assays

Cth I endonuclease activity was assayed by monitoring the cleavage of lambda DNA (N^6-methyl adenine free) by agarose gel electrophoresis. Reaction mixtures contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.8 μg of bacteriophage lambda DNA, and 1 μl of enzyme solution. The mixtures were incubated at 60 °C for 30 minutes, and were terminated by adding one fifth volume of the dye solution which contains 60 mM EDTA, 0.6% bromophenol blue, and 60% glycerol.

The recognition sequence of Cth I endonuclease

Lambda DNA and Adenovirus 2 DNA were used to compare the digestion patterns with various restriction endonucleases. Endonuclease reactions were performed at the buffer coditions recommended by the company by using 0.8 ug of DNA substrates, and cleavage patterns were monitored by electrophoreses on 0.8% agarose gels.

Results and Discussion

Purification of Cth I

The restrction enzyme Cth I was isolated from 2 g (wet weight) of *Clostridium thermocellum*. All steps were performed at 0 to 4 °C. Buffers used during the purification procedure were as follows: Buffer A; 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 5% (v/v) glycerol, and 0.1 mM EDTA, Buffer B; 20 mM sodium phosphate (pH 7.6), 20 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.1 mM EDTA.

Preparation of cell extracts

2 g of cell paste was suspended in 10 m*l* of Buffer A. The suspended cells were sonicated using Sonifier Cell Disrupter (VWR Scientific Inc.) equipped with a macrotip. Temperature was maintained below 4 °C with ice water bath during the disruption steps. The extract was clarified by centrifugation at 35,000 rpm in Beckman Type 65 roter for 60 minutes (Fraction I; 10 m*l*).

Ammonium sulfate fractionation

In order to make 85% saturation, 5.59 g of powdered ammonium sulfate was added to Fraction I with gentle stirring for 3 hours, the precipitate was collected by centrifugation at 35,000 rpm in Beckman Type 65 roter for 60 minutes. The precipitate was resuspended in 10 m*l* of Buffer B, and dialyzed against 20 volumes of Buffer B with three buffer changes for 12 hours. The dialyzed sample was diluted with 5 m*l* of Buffer B to yield 20 of absorbance at 280 nm (Fraction II; 15 m*l*).

DEAE-cellulose (A-50) column chromatography

Fraction II was applied at 15 m*l*/hour to DEAE-Cellulose column (30×2.5 cm dia.) equilibrated with Buffer B. The column was washed with 200 m*l* of Buffer B and then eluted with 500 m*l* linear gradient of NaCl (0.0-1.0 M) in Buffer B. Aliquot (1 μl) from column fractions was assayed. Fractions

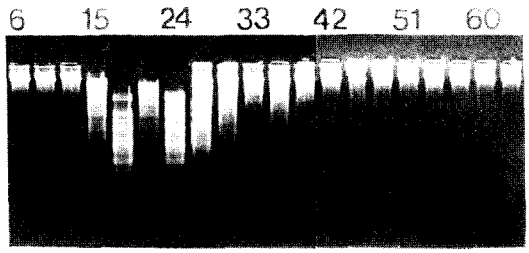

Fig. 1. DEAE-cellulose column chromatography.
Cth I endonuclease activity was assayed by monitoring the cleavage of lambda DNA (N^6-methyl adenine free) by the electrophoresis on 0.8% agarose gel. Reaction mixtures containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.8 ug of bacteriophage lambda DNA, and 1 ul of enzyme solution were incubated at 60 °C for 30 minutes. Fraction numbers are indicated on the top of the gel.

containing sequence specific endonucleases, which is eluted between 0.15 and 0.30 M NaCl (Fig. 1), were collected and dialyzed against 20 volumes of Buffer B for 10 hours with two buffer changes (Fraction III; 50 m*l*).

Phosphocellulose (P-11) column chromatography

Fraction III was applied at 21 m*l*/hour to phosphocellulose column (30×1.5 cm dia) equilibrated with Buffer B. The column was washed with 90 m*l* of Buffer B and then eluted with 200 m*l* of linear gradient of (NaCl) (0.0-1.0 M) in Buffer B. Cth I endonuclease activity appeared between 0.50-0.58 M NaCl (Fig. 2). Another activity of type II restriction endonuclease (Cth II) was also appeared at late fractions (Fig. 2), indicating that the fraction III contained two sequence specific endonucleases. The fractions containing Cth I activity were pooled and dialyzed against Buffer B supplemented with 50% (v/v) glycerol (Fraction V; 0.4 m*l*). This enzyme lost little activity (<4%) over a period of at least 10 months at −20°C.

Catalytic properties

Cth I endonuclease showed the maximum activity on the following conditions: pH 7.5 to 10.5, 5 to 20 mM $MgCl_2$, 0 to 10 mM NaCl at 60°C. Bovine serum albumin and sulfhydryl compounds had no influences on the activity. Cth I enzyme was active even after the incubation at 80°C for 10 minutes.

The Specificity of Cth I endonuclease

Restriction patterns of Cth I endonuclease by using DNAs of lambda and adenovirus 2 were analyzed. As shown in Fig. 3, the patterns obtained were identical to those obtained by using Bcl I endonuclease. Considering the fact that the combination of lambda and adenovirus 2 DNAs are good enough substrate to characterize restriction endonucleases recognozing hexanucleotide sequences, the identity of cleavage patterns by Cth I and Bcl I enzymes certainly indicates that Cth I endonuclease is recognizing same DNA sequence as Bcl I endonuclease.

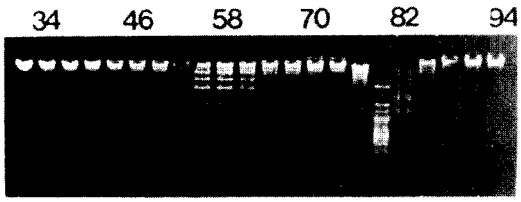

Fig. 2. Phosphocellulose (P-11) column chromatography.
Cth I endonuclease activity was assayed by monitoring the cleavage of lambda DNA (N^6-methyl adenine free) by the electrophoresis on 0.8% agarose gel. Reaction mixtures containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.8 ug of bacteriophage lambda DNA, and 1 ul of enzyme solution were incubated at 60°C for 30 minutes. Fraction numbers are indicated on the top of the gel.

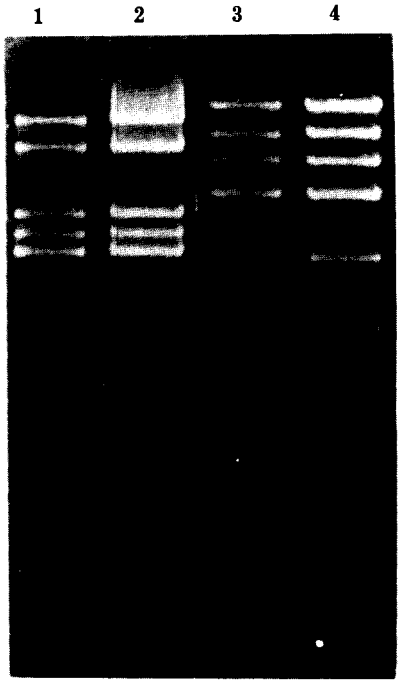

Fig. 3. Digestion patterns of lambda DNA and Adenovirus 2 DNA by Cth I and Bcl I endonuclease.
The reaction conditions for Cth I endonuclease were as follows. Reaction mixtures containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.8 ug of substrate DNA, and an appropriate amount of enzyme solution were incubated at 60°C for 1 hour. The reactions for Bcl I endonuclease were performed in the buffered solution of 75 mM KCl, 6 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol and by using 0.8 ug of substrate DNA and 10 unit (defined by the company, new England BioLabs) of enzyme. Incubation was at 50°C for 1 hour. Cleavage patterns were monitored by electrophoreses on 0.8% agarose gels.
lane 1: Adenovirus 2 DNA digested with Cth I,
lane 2: Adenovirus 2 DNA digested with Bcl I,
lane 3: lambda DNA digested with Cth I,
lane 4: lambda DNA digested with Bcl I.

An experiment for determining the cleavage site of Cth I endonuclease within its recognition sequence is under investigation. According to the progress data, it was confirmed that Cth I enzyme recognizes the sequence 5'-TGATCA-3' same as the sequence for Bcl I endonuclease (data not shown), and cleaves somewhere inside the recognition sequence.

Influence of *dam* methylation on the Cth I activity

The recognition sequence of Cth I endonuclease contains the sequence 5'-GATC-3' recognized by *dam* methylase which transfers a methyl group from S-adenosyl methionine to the N^6 position of the adenine residues (2, 3). Methylation by either *dam* or *dcm* methylases has been shown to inhibit cleavage of DNA by certain restriction endonucleases whose recognition sequence contain the recognition sequence of *dam* or *dcm* methylase (4). The activity of Cth I endonuclease was also sensitive to *dam* methylation as the activity of Bcl I enzyme.

요 약

새로운 제한효소인 Cth I을 *Clostridium thermocellum* ATCC 27405로 부터 분리하여, 그 효소의 생화학적 특성을 연구하였다. 이 효소는 Bcl I endonuclease의 isoschizomer로서 5'-TGATCA-3'를 인식한다. Cth I endonuclease는 섭씨 60도의 높은 온도에서 잘 작용하며, 10 mM까지의 저농도의 NaCl과 Mg^{2+}이온의 존재하에서 pH 7.5와 10.5사이에서 최적의 활성을 보여주었다. Cth I endonuclease의 활성은 DNA 기질이 *dam* methylation 되었을 때 저해를 받았다.

References

1. Bingham, A.H. A., T. Atkinson, D. Sciaky and R.J. Roberts: *Nucleic Acids Res.*, **5**, 3457 (1978).
2. Marinus, M.G. and N.R. Morris: *J. Bacteriol.*, **114**, 1143 (1973).
3. Geier, G.E. and P. Modrich: *J. Biol. Chem.*, **254**, 1408 (1979).
4. New England BioLabs, Catalog 1986/1987, p 112.

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