A New Restriction Endonuclease from *Xanthomonas citri*

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새로운 TYPE II 제한효소 Xci I의 분리

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Abstract: The isolation and charaterization of a type II restriction endonuclease from *Xanthomonas citri* IFO 3835 were described. This enzyme (Xci I endonuclease) is an isoschizomer of Sal I endonuclease recognizing 5'-GTCGAC-3' and cleaving at the site indicated by the arrow. Unlike Sal I endonuclease, Xci I endonuclease requries a NaCl concentration of 50 mM for its maximum activity.

Key words: type II endonuclease, Xci I, isoschizomer, Sal I, cleavage site.

Restriction endonucleases are widely used in the characterization and restricting of DNA molecules and they are being increasingly used as model systems to study DNA-protein interactions. 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 Xci I endonuclease from *Xanthomonas citri* which is an isoschizomer of Sal I endonuclease.

MATERIALS AND METHODS

Chemicals and Enzymes

Restriction enzymes, T4 polynucleotide kinase, ligase, and hydroxylapatite were purchased from New England BioLabs. Lambda DNA was obtained from Bethesda Research Laboratories. Heparin and DEAE-sephadex were delivered by Pharmacia. Heparin agarose was prepared by coupling with CNBr (Davison, *et. al.*, 1979). Whatman phosphocellulose P-11 was precycled and equilibrated according to the procedure of Green, *et. al.* (1978). Plasmid DNAs were prepared by the method of Clewell and Helinski (1972). α -^{32}P-dATP (3,000 Ci/m mole) was purchased from Amersham.

Culture of Xanthomonas citri

Xanthomonas citri was grown at 30 ℃ in 20 liter of the culture medium (Rhee, *et. al.*, 1980). Cells were harvested at late exponential phase (A_{600}=2.5), and adjusted to 50% (w/v) glycerol.

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Enzyme Assays

Xci I endonuclease activity was assayed by monitoring the cleavage of lambda DNA by agarose gel electrophoresis. The reaction mixtures contained 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.2 ug of lambda DNA, and 1 μl of enzyme solution. The mixtures were incubated at 37 ℃ for 15 minutes, and were terminated by adding one fifth volume of the dye solution which containing 60 mM EDTA, 0.6% bromophenol blue, and 60% glycerol.

The protein content

The protein content of the partially purified fractions was determined by the method of Schaffner and Weissman (1973).

The recognition sequence and the cleavage site of Xci I endonuclease

Lambda, Ade 2 and pUC9 DNA were used to compare the digestion patterns with various restriction endonucleases. Among the tested endonucleases, Sal I endonuclease which showed identical cleavage patterns to Xci I endonuclease was used to compare the cleavage site with the isolated enzyme.

To determine the cleavage site, the second strand of M13 DNA was synthesized by using universal primer and α -^{32}P-dATP. The size of the released DNA fragment was measured on the DNA sequencing gel before and after filling up the 3' recessive end which has been generated by the enzyme. The result obtained here was compared with that obtained with Sal I endonuclease.

RESULTS AND DISCUSSION

A summary of the purification of Xci I endonuclease from 100 g (wet weight) of *Xanthomonas citri* is presented in Table 1. All steps were performed at 0 to 4 ℃, and centrifugation was at 12,000 g for 30 minutes in Sorvall GSA or GS3 rotor unless otherwise indicated. Buffers used in the purification were described previously (Yoon, *et. al.*, 1985).

Preparation of the crude extract

100g of the cells was suspended in 300 ml of buffer A with gentle stirring for 4 hours at 4 ℃. Cells were disrupted by two passages through French Press at 15,000 p.s.i. The extract was centrifuged at 35,000 rpm (type 42.1 rotor, Beckman) for 60 minutes, and the resulting supernatant was diluted with buffer A to A_{280} of 60 (fraction I, 350 ml).

Ammonium sulfate fractionation

Solid ammonium sulfate (36.1g) was added gradually to fraction I to reach the final concentration of 30% saturation. After removal of the precipitate by centrifugation, the concentration was adjusted to 70% over a period of 60 minutes with gentle stirring. The pellet was collected by centrifugation, resuspended in 50 ml of buffer B, and then dialyzed against 10 volumes of the same buffer for 16 hours with four buffer changes. The sample solution was diluted with buffer B to reach the A of 20

Table 1. *Purification of endonuclease Xci I from 100g of Xanthomonas citri IFO 3835.*

	Total protein (mg)	Total units	Specific activity	% Recovery	Purification fold
1) Crude extract	3,750	–	–	–	–
2) Ammonium sulfate fractionation	3,280	–	–	–	–
3) Heparin-agarose	30.18	3.85×10^4	1,275	100	1
4) DEAE-Sephadex (A-50)	15.76	2.60×10^4	1,650	67.5	1.29
5) Phosphocellulose (P-11)	0.52	1.40×10^4	26,900	36.4	21.1
6) Hydroxylapatite	0.12	8.0×10^3	67,000	20.7	52.5

A unit of enzyme activity was defined as the amount necessary for complete digestion of 1 μg of phage λ DNA for 1 hr under the optimal condition.

(fraction II, 410 m*l*).

Heparin-agarose chromatography

Fraction II was applied at 30 m/hour to a heparin-agarose column (2×30 cm) equilibrated with buffer B. The column was washed with 300 m*l* of buffer B, and the protein was then eluted with 500 m*l* of linear gradient of NaCl (0.02 M-1.0 M) in buffer B. 1 μl of every other fractions was used for assay of Xci I activity. It was eluted between 0.35 M and 0.55 M NaCl. The fractions were dialyzed against 10 volumes of buffer B for 12 hours with three buffer changes (Fraction III, 77 m*l*).

DEAE-sephadex A-50 chromatography

Fraction III was applied at 20 m*l*/hour to DEAE-sephadex A-50 column (2×15 cm) equilibrated with buffer B. The proteins were eluted with 200 m*l* of linear gradient of NaCl (0.02-1.0 M) in buffer B. Xci I endonuclease activity appeared between 0.2 to 0.3 M NaCl. The fractions containing Xci I endonuclease activity were pooled and dialyzed against 10 volumes of buffer B for 6 hours with three buffer changes (Fraction IV, 24 m*l*).

Phosphocellulose P—11 chromatography

Fraction IV was applied at 15 m*l*/hour to phosphocellulose column (0.7×15 cm) equilibrated with buffer B. From it, the proteins were eluted with 80 m*l* of linear gradient of NaCl (0.02-1.0 M) in buffer B. Xci I activity was eluted between 0.2 to 0.35 M NaCl. The fractions containing this activity were pooled and dialyzed against 10 volumes of buffer C for 6 hours with three buffer changes (Fraction V, 14 m*l*).

Hydroxylapatite chromatography

Fraction V was applied at 2 m*l*/hour to hydroxylapatite column (0.5×10 cm) equilibrated with buffer C. The proteins were eluted with 40 m*l* of linear gradient of potassium phosphate, pH 7.8 (0.02-0.8 M) in buffer C. Xci I activity was eluted between 0.2-0.26 M potassium phosphate. The fractions containing Xci I activity were pooled and dialyzed against buffer B supplemented with 50% (w/v) glycerol three times (Fraction VI, 1.0 m*l*). This enzyme lost little activity (<4%) over a period of at least 8 month at -20 ℃. The DNA fragment generated by treatment with the isolated enzyme was being ligated almostly completely by T4 DNA ligase showing the enzyme contains negligible amounts of exonuclease and nonspecific endonuclease (Fig.1).

Catalytic properties

Xci I endonuclease showed the maximum activity on the following conditions: pH 7.5 to 9.0, 1 to 2 mM $MgCl_2$, 20 to 50 mM NaCl at 37 ℃. Bovine serum albumin and sulfhydryl compounds had no influences on the activity. Considering that Sal I endonuclease requires 100 mM NaCl for its activity which should be reduced for the following reaction, it is likely that Xci I endonuclease would replace Sal I endonuclease because of the requirement of low salt concentration for its maximum activity.

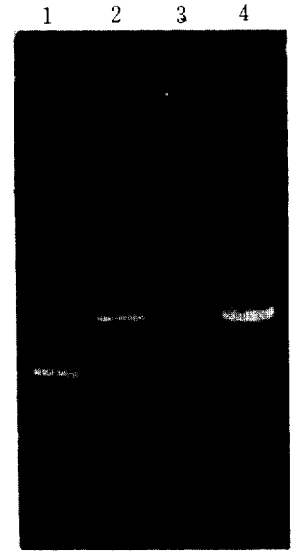

Fig.1. *Ligation and recutting test of Xci I endonuclease in order to identify whether the Xci I preparation is free of exonuclease and nonspecific endonuclease.*

Lane 1 : intact M13 mp8 RF DNA, lane 2 : M13 mp8 DNA digested overnight with Xci I of this preparation, lane 3 : M13 mp8 DNA digested with Xci I and ligated with T4 ligase, lane 4 : M13 mp8 DNA digested, ligated and recutted with Xci I.

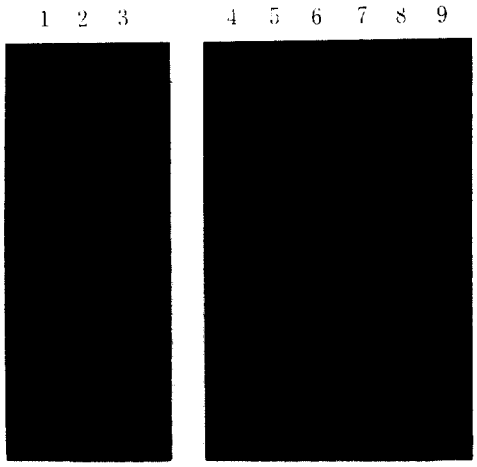

Fig.2. *Digestion of lambda DNA, Ade 2 DNA and pCU9 with Sal I and Xci I endonuclease (see MATERIALS AND METHODS).*

Lane 1 : intact lambda DNA, lane 2 : lambda DNA digested with Sal I, lane 3 : lambda DNA digested with Xci I, lane 4 : intact Ade 2 DNA, lane 5 : Ade 2 DNA digested with Sal I, lane 6 : Ade 2 DNA digested with Xci I, lane 7 : pUC9 DNA, lane 8 : pUC9 DNA digested with Sal I, and lane 9 : pUC9 DNA digested with Xci I.

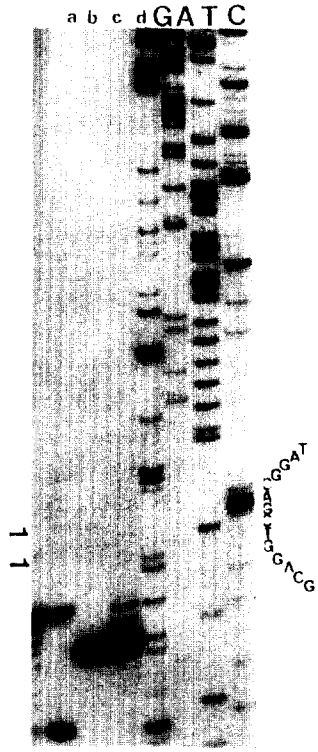

Fig.3. *Determination of the cleavage site of Xci I endonuclease by chain termination method(see MATERIALS AND METHODS).*

G, A, T, C: four lanes of dideoxy-squencing, a: the second M13 DNA digested with Sal I endonuclease and then treated with Klenow fragment, b: the second M13 DNA digested with Xci I endonuclease and then treated with Klenow fragment, c: the second M13 DNA digested with Sal I endonuclease, and d: the second M13 DNA digested with Xci I endonuclease.

The specificity of Xci I endonuclease

Restriction patterns of Xci I endonuclease by using lambda, Ade 2 and pUC9 DNA were analyzed. As shown in Fig.2, the patterns obtained were identical to those obtained by using Sal I endonuclease, indicating that Xci I endonuclease is recognizing the same DNA sequence as Sal I endonuclease.

The specific cleavage site of this enzyme was determined as described in MATERIALS AND METHODS. Fig.3 shows that Xci I endonuclease recognizes 5'-G↓TCGAC-3' and cuts at the site indicated by the arrow, which is identical to the result obtained from Sal I endonuclease.

적 요

새로운 제한 효소인 Xci I endonuclease를 *Xanthomonas citri* IFO 3835로부터 분리하고 그 효소의 생화학적 특성을 연구하였다. 100g의 cell로부터 얻은 crude extract를 ammonium sulfate fractionation을 거친 후 heparin-agarose, DEAE-sephadex, phosphocellulose 그리고 hydroxylapatite chromatography를 행하여 최종적으로 nonspecific endonculease와 exonuclease가 오염되어 있지 않은 0.12mg의 효소를 얻었다. 이 효소는 5'-G↓TCGAC-3'을 인식하여 G와 T 사이를 절단하는 효소임을 결정하였다. 이 결과로부터 Xci I endonuclease는 Sal I endonuclease의 isoschizomer임을 알 수 있었다. 그러나 Xci I endonuclease는 20에서의 50mM 사이의 NaCl 농도에서 최대 활성을 보여 주었다.

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(Received Dec. 1, 1986)