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Purification and Characterization of A Thermotolerable Restriction Endonuclease from *Streptomyces violochromogenes* D2-5

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A thermotolerable restriction endonuclease. *Svi*I, found in *Streptomyces violochromogenes* D2-5 was purified. For the purification, streptomycin sulfate and ammonium sulfate precipitation was used. Phosphocellulose P-11, DEAE-Cellulose and Sephacryl-S200 HR colum chromatography were also performed. The purified enzyme was found to be homogeneous and the molecular weight of the enzyme estimated by polyacrylamide gel electrophoresis containing 0.1% SDS was about 32,000 daltons. The recognition sequence and cleavage site of the enzyme were determined to be 5'-TT↓CGAA-3' which is the same sequence as that of *Asu*II. Unlike *Asu*II, however, the *Svi*I shows high thermal stability.

Because of sequence speciticity of type II endonucleases and the different enzymatic properties of their isoschizomers, type II endonucleases have become one of the most important enzyme classes in the field of molecular biology and genetic manipulation (8), and are constantly in demand and under study.

An isolate from a compost in Kyeongido was selected to research a thermotolerable strain and the strain was identified, using TAXON program, numerically to be a member of *Streptomyces violochromogenes* (2). This organism was found to harbor a restriction endonuclease, designated *Svi*I. Therefore, an efficient purification method of *Svi*I endonuclease was studied in order to investigate its biochemical and enzymatic properties. The cleavage specificity of the enzyme was also determined.

MATERIALS AND METHODS

Materials and Reagents

DEAE-cellulose, molecular weight standard protein, N,N'- methylenebisacrylamide, TEMED, agarose and ethidium bromide were purchased from Sigma Chem. Co. Phosphocellulose P11 was purchased from Whatman, Sephacryl S-200 HR from Pharmacia, lambda DNA and restriction endonucleases from KOSCO, [α-^{35}S] dATP from Amersham, and other deoxyribo-, nucleotides and dideoxyribonucleotides from Bio-Rad. All other reagents used were of reaqent grade.

Preparation and Purification of *Svi*I.

Streptomyces violochromogenes D2-5 was grown in 3 l of nutrient liquid medium at 50°C for 48 hours and collected by centrifugation at 5000×g at 4°C. The cell mass was disrupted with a bead beater in buffer A (10 mM potassium phosphate, 10 mM 2-mercaptoethanol and 5% glycerol, pH 6.5) at ice-cold temperature. The supernatant enzyme solution was prepared by the ultracentrifuge (100,000×g) for 90 minutes.

The supernatant, after streptomycin sulfate precipitation for one hour at 2% final concentration of the volume, was again precipitated with 45-80% ammonium sulfate saturation and collected by centrifugation at 15,000×g for 30 min.

The precipitate was dissolved in the 10 ml of buffer A and dialysed against the same buffer A for 16 hours. The crude enzyme solution dialysed was then chromatographed with a Phosphocellulose P11 column (2.5×30 cm), and the active fractions were further purified with DEAE-cellulose columns as described in the results.

Finally, the active fractions were concentrated by Centriprep (Amicon) and rechromatographed by Sephacryl S-200 HR column (1×100 cm).

Determination of the Restriction Endonuclease, *Svi*I, Activity

One unit of the restriction endonuclease was defined as the amount of the enzyme required to cleave completely, 1 μg DNA per hour at 50°C. Enzyme reactions were conducted, and the activity of the en-

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zyme was examined in the solution of Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl and 0.5 μg lambda DNA (5 μl) at 50°C.

Enzyme reactions were completed by the treatment of the solution at 65°C for 5 minutes with the addition of a loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in H_2O).

The reaction products of the enzyme were confirmed by agarose gel electrophoresis. The DNA bands on the agarose plates were treated with 1 μg/μl ethidium bromide for 5 minutes and examined under UV light.

Determination of the Molecular Weight of *Svi*I

For the determination of the molecular weight of the purified enzyme, polyacrylamide (10%) gel electrophoresis containing 0.1% sodium dodecyl sulfate (SDS-PAGE) in Tris-glycine buffer (pH 8.3) was carried out according to the method of Laemmli (6).

SDS-PAGE was performed for 3 hours at a constant current of 20 mA. Then, the gel was stained with 0.1% coomassie brillient blue R solution for 16 hours and destained under gentle stir in distilled water for 5 hours.

Determination of Recogmition Site of *Svi*I on DNA

The recognition site of *Svi*I on DNA sequence was determined by the method of Sanger *et al* (9) using the pGEM-7Zf(+) vector. After alkaline denaturation, pGEM-7Zf(+) was annealed with SP6 primer, and elongated in the presence of all four dNTP's and α-^{35}S] dATP. The product of the primed synthesis was subsequently cleaved by the restriction endonuclease, *Svi*I. To determine protruding ends generated by cleavage, half of the digested fragment was filled in to make blunt ends. The cleavage site of the enzyme was demonstrated by the migration of the released fragment on a 8.3 M urea, 8% polyacrylamide DNA-sequencing gel.

RESULTS AND DISCUSSION

Detection of Thermotolerable Endonuclease Activity

From a large number of streptomyces strains isolated from various places in Korea, 73 strains were selected as thermotolerable organisms to grow at 50°C. For the detection of restriction endonuclease activity, every strain was cultured at 50°C on a rotary shaker and examined to select strains producing restriction endonuclease, according to the methods described by Bae *et al.* (1). Among them, the strain D2-5, which was identified to be a *Streptomyces violochromogenes* (2) was found to harbor a restriction endonuclease which is active at 50°C. The thermotorelable endonuclease was designated as *Svi*I according to the name of the genus and species identified.

Purification of *Svi*I

The crude enzyme after treatment of streptomycin sulfate and ammonium sulfate following cell breakage was applied to Phosphocellulose P 11 column previouly equillibrated with buffer A. The restriction enzyme was eluted out with linear gradient from 0.15 to 0.38 M NaCl in the buffer A. The elution profile and the enzyme activity of corresponding fractions is shown in Fig. 1. Specific activity of the enzyme on this column was 27,700 unit/mg protein (Table 1). Active fractions (Fr. 205-250) eluted from the column were collected, dialyzed in buffer B (buffer A adjusted to pH 7.5 with dil-NaOH) for 16 hours, and concentrated by means of Centri-prep.

The concentrated enzyme solution was applied to a DEAE-cellulose column and eluted with linear gradient from 0 to 1.0 M NaCl. The active enzyme was eluted out at a range of 0.22 to 0.45 M NaCl (Fr. 52-72). The elution profile from the column is shown in Fig. 2. The active fractions were collected and con-

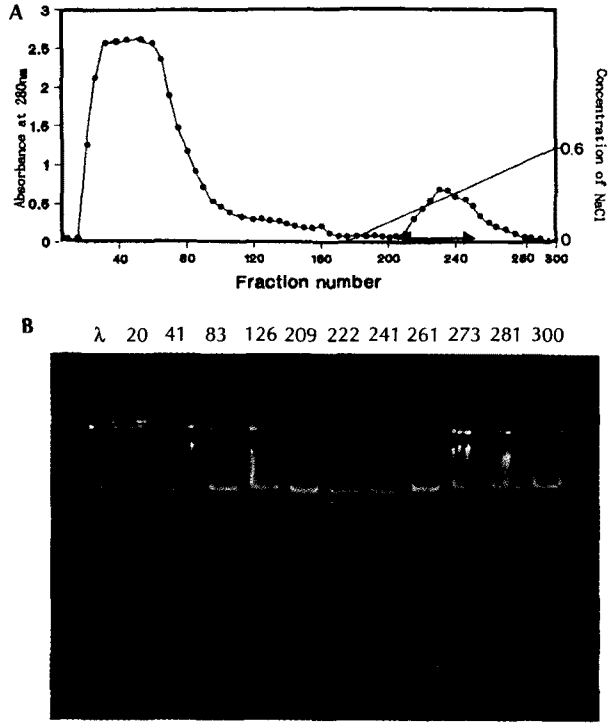

Fig. 1. The Phosphocellulose P11 column chromatography (2.5×30 cm) of ammonium sulfate precipitant and enzyme activity of corresponding fractions.

(A) Numbers in abscissa indicate fraction numbers. Protein concentration measured as absorbance at 280 nm (●—●): Active fraction (◀—▶), (B) An aliquot (5 μl) from each Phosphocellulose column fraction was incubated at 50°C for 1 hour with 30 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, and 0.5 μg of lambda DNA. The electrophoresis of the cleavage products was performed on a 0.8% agarose gel.

lane λ: lambda DNA marker. lane 20-300: # 20-300 of fraction number.

centrated with PEG 10,000 after dialysis in buffer C (buffer B containing 0.2 M NaCl). The concentrated enzyme solution was finally applied to a Sephacryl S-200 HR gel filtration column equilibrated with buffer C and eluted with the same buffer. The active enzyme was eluted out at a range of Fraction No. 28-35 when elution rate was 4 ml/h as shown in Fig. 3. The specific activity of the enzyme after the chromatography was 203, 340 unit/mg of protein (Table 1). In Fig. 1B, 2B, and 3B only two restriction fragments of lambda DNA showed up in the lanes treated with active aliquots, which was due to the low amount of DNA loaded on agarose gel. For clear restriction pattern of lambda DNA cut with *Svi*I, see Fig.

Table 1. The purification of restriction endonuclease from *Streptomyces violochromogenes* D2-5.

Purification step	Total Protein (mg*)	Total unit**	Specific activity (unit/mg)	Purification fold	Yield (%)
Crude extract	250.40	5.47×10^5	2184	1	100
Streptomycin sulfate supernatant	249.2	5.43×10^5	2176	1	99
Ammonium sulfate precipitation	207.3	4.76×10^5	2299	1.1	87
Phosphocellulose P11 column	7.87	2.18×10^5	27700	13	40
DEAE-cellulose column	2.50	1.94×10^5	80165	37	37
Sephacryl S-200 HR column	0.354	7.2×10^4	203340	93	13

*Protein concentration is determined by the Dye-binding method (Bradford, 1976). **One unit protein is defined as I μg DNA completely digested in 1 h at 50°C.

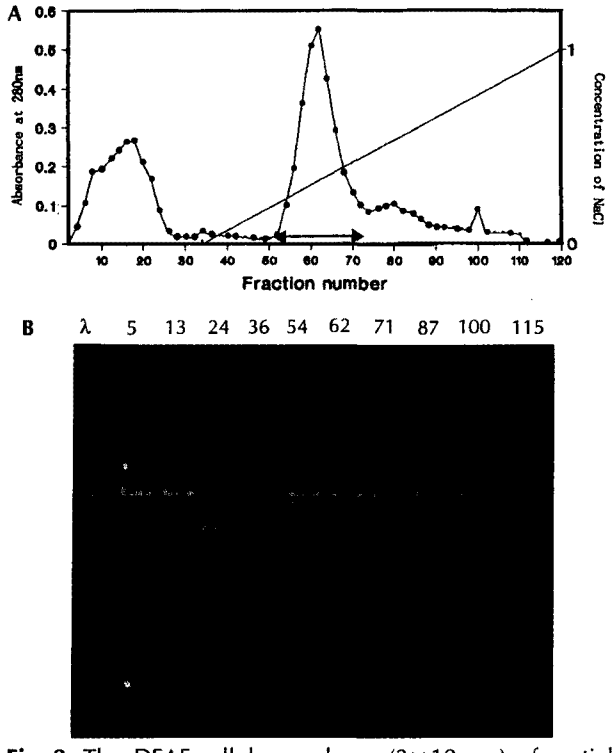

Fig. 2. The DEAE-cellulose column (2×10 cm) of partial purified enzyme with Phosphocellulose P11 column chromatography and the enzymatic activity of corresponding fractions.

(A) Numbers in abscissa indicate fraction numbers. Protein concentration measured as absorbance at 280 nm (●—●): Active fractions (◀—▶), (B) An aliquot (5 μl) from each fraction was incubated at 50°C for 1 hour with 30 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, and 0.5 μg of lambda DNA. The electrophoresis of the cleavage products was performed on a 0.8% agarose gel.

lane λ: lambda DNA marker. lane 5-115 : # 5-115 of fraction number.

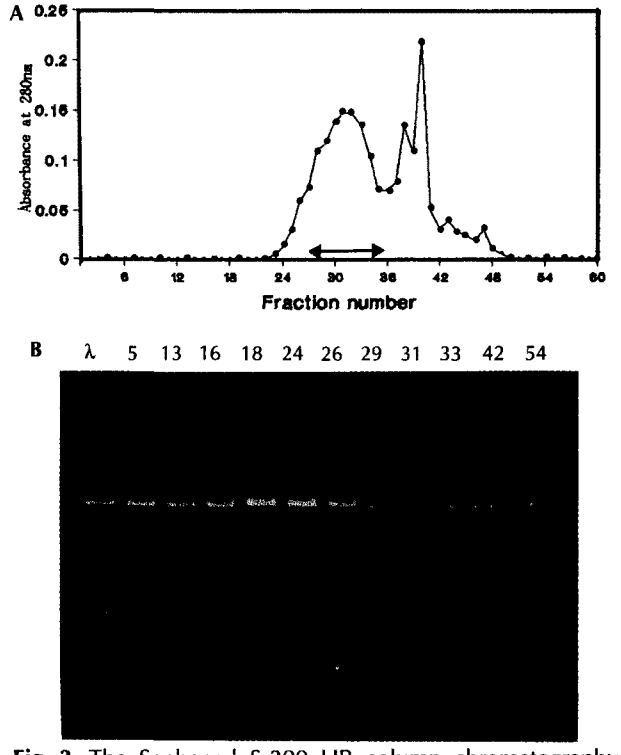

Fig. 3. The Sephacryl S-200 HR column chromatography (1×100 cm) of partial purified enzyme with DEAE-cellulose column and the enzymatic activity of corresponding fraction.

(A) Numbers in abscissa indicate fraction numbers. Protein concentration measured as absorbance at 280 nm (●—●): Active fractions (◀—▶), (B) An aliquot (5 μl) of each column fraction was incubated at 50°C for 1 hour with 30 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, and 0.5 μg of lambda DNA. The electrophoresis of the cleavage products was performed on a 0.8% agarose gel.

lane λ: lambda DNA marker. lane 5-60: # 5-60 of fraction number.

5 (lane 2).

Estimation of Molecular Weight of the Enzyme Protein

The enzyme preparation in the final stage was homogeneous as judged by SDS-PAGE. The molecular weight of the subunit of the purified restriction enzyme was estimated to be 32,000 dalton by SDS-PAGE (Fig. 4). However, it remains to determine how many subunits compose the active enzyme because it has been reported that the restriction endonuclease type II is usually composed of similar size of a few subunits (3).

Cleavage Site of *Svi*I on DNA

The enzyme *Svi*I cuts 7 sites of lambda DNA but it does not cut pBR322, pUC18 and pBS, and it was predictable that this enzyme would be similar with *Asu*II from *Anabaena cylindrica* by the digestion pattern. The cleavage patterns of lambda DNA by both *Svi*I and *Asu*II were then compared with those of the digestion by each single enzyme together with digestion by *Hin*dIII (Fig. 5). The same restriction patterns of lambda DNA by *Svi*I and *Asu*II also suggests that *Svi*I would be another isoschizomer of *Asu*II in addition to *Sca*1, *Acp*1, *Cbi*1, *Csp*45.I, and *Sfu*1 (4, 5, 7, 9, 10, 11).

The recognition sequence of *Svi*I was confirmed by applying the enzymatic sequencing method as described in Materials and Method (Fig. 6). This enzyme cleaves after the second T (lane 1 of Fig. 6) and produces a 2-base 5'-extension (lane 2) is demonstrated by the migration of the released fragment on a polyacrylamide DNA sequencing gel. From the sequencing reactions, the specificity of *Svi*I was concluded as

5'-TT ↓ CGAA-3'
3'-AAGC ↑ TT-5'

Effect of Temperature on Actvity

The activity of the enzyme was measured at various

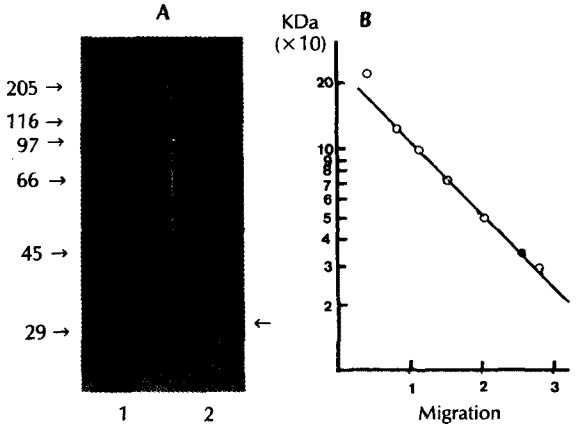

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified *Svi*I (A) and the subunit molecular weight (closed circle) of *Svi*I (B).
A. lane 1: Molecular weight size marker
lane 2: Protein by Sephacryl S-200 HR column chromatography.

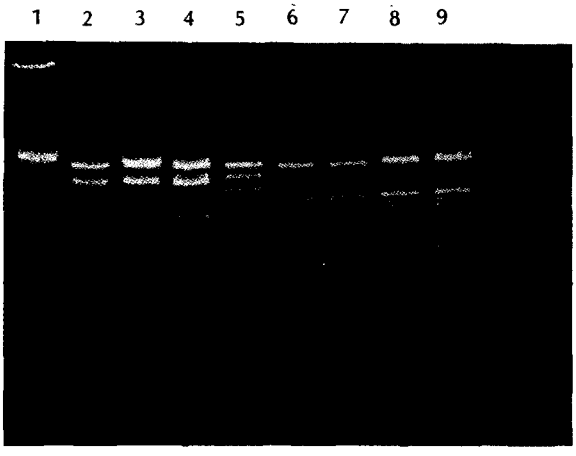

Fig. 5. The comparison of *Svi*I and *Sfu*I activity.
lane 1: lambda DNA. lane 2: lambda DNA + *Svi*I. lane 3: lambda DNA + *Asu*II. lane 4: lambda DNA + *Svi*I + *Asu*II. lane 5: lambda DNA/*Hin*dIII. lane 6: lambda DNA/*Hin*dIII + *Svi*I. lane 7: lambda DNA/*Hin*dIII + *Asu*II. lane 8: lambda DNA/*Hin*dIII + *Svi*I + *Asu*II.

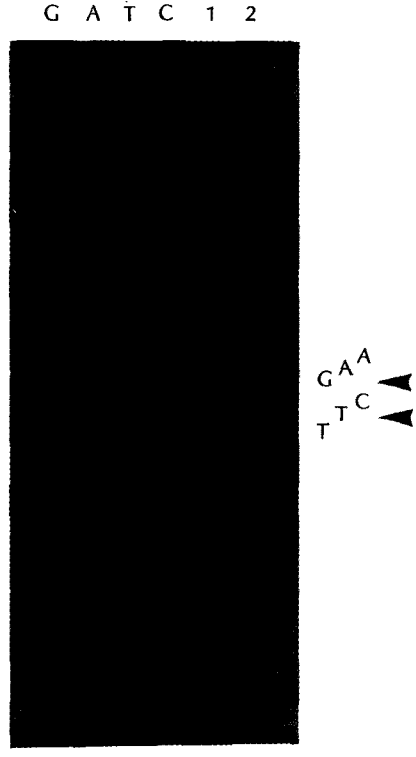

Fig. 6. Determination of *Svi*I cleavage Specificity.
G,A,T,C: four lane reactions of dideoxy chin termination. lane 1: [α-^{35}S] -internally labeled pGEM-7Zf(+) DNA + *Svi*I. lane 2: Klenow fragment and dNTP were added to reaction mixture of lane 1 for carrying out filling-in reaction.

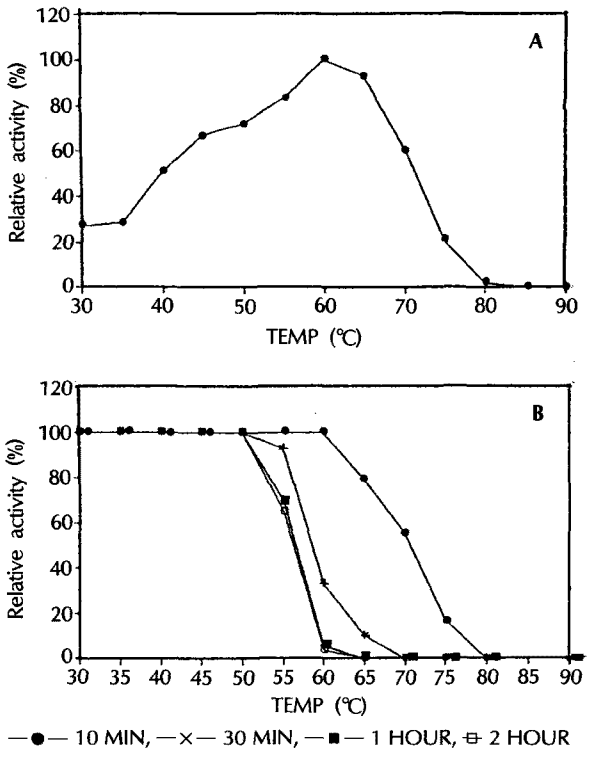

Fig 7. (A) The effect of temperature on the activity of restriction endonuclease *Svi*I; Enzyme solution (2 μl) was incubated at various temperature in reaction mixture (25 μl) containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, and 0.5 g of lambda DNA. (B) Thermal stability of the activity of restriction endonuclease *Svi*I; Enzyme solution (10 μl) was preincubated at 30°C-100°C for 10 min, 30 min, 1 hour, 2 hour, and assayed enzyme activities in the presence of 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl and 0.5 μg of lambda DNA at 50°C.

temperatures from 30° to 90°C. The optimum temperature for reaction of the enzyme was about 60°C as shown in Fig. 7A. The enzyme was stable up to 60°C for 10 min. In spite of treatment for 10 min at 70°C, 60% of the original actvity was maintained as shown in Fig. 7B. *Svi*I maintained the enzyme activity after treatment at 50°C for 2 hours, unlike *Asu*II (3).

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