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Purification and Characterization of Stu I Endonuclease from *Streptomyces Tubercidicus*

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*Streptomyces tubercidicus*에 존재하는 Stu I endonuclease의 정제와 특징

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ABSTRACT: Stu I, a type II restriction endonuclease, has been purified to homogeneity from *Streptomyces tubercidicus* (ATCC 25502), and its catalytic properties have been studied. For the purification of Stu I endonuclease free of nonspecific nucleases, DEAE-Sephadex (A-50), QAE-Sephadex (A-50) and Heparin-agarose column chromatography have been performed after ammonium sulfate fractionation of the crude extract. The enzyme was further purified by gel filtration using Sephadex G-100 column to obtain homogeneous form of protein.

The single polypeptide species of Stu I endonuclease has a subunit molecular weight of 34,000 ± 1,000 daltons as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Stu I endonuclease requires Mg^{2+} ion for its activity and is maximally active at neutral pH (7.0-8.0) in the absence of NaCl.

KEY WORDS□Type II restriction endonuclease, Stu I, homogenous preparation.

The importance of restriction endonucleases as tools in genetic research is now well established. The type II restriction endonucleases, which cleave DNA at defined nucleotide sequences with very high fidelity, have become indispensable in the structural analysis of DNA molecules, and recombinant DNA research. Despite their widespread uses, the detailed biochemical investigation of these enzymes has not been well performed. In order to detailed structural and mechanistic studies, restriction endonucleases must be purified in homogeneous form and characterized thoroughly. Shimotsu *et al.* (1980) reported Stu I endonuclease activity in the strain of *Streptomyces tubercidicus,* obtained from H. Takahashi. They also identified that Stu I endonuclease recognize the nucleotide sequence 5'-d(AGG↓CCT)-3' and cut at the site indicated by the arrow. Since the purification procedures reported by these authors do not yield homogeneous enzyme in large amount, we have developed an efficient procedure for the isolation of Stu I endonuclease in homogeneous form. In this report, we describe the isolation procedure for Stu I endonuclease from the ATCC strain of *Streptomyces tubercidicus* (ATCC 25502), together with its physical and enzymatic properties.

MATERIALS AND METHODS

Materials

The strain of *Streptomyces tubercidicus* was

from American Type Culture Collection (ATCC 25502). DEAE-sephadex (A-50), QAE-sephadex (A-50), and Sepharose 6B were perchased from Pharmacia Fine Chemicals. Coomassie brilliant blue (G-250), electrophoresis grade agarose, heparin, Tris (hydroxy-methyl aminomethane) were from Sigma Chemical Company. 2-mercaptoethanol was from Aldrich chemical company. Cyanogen bromide was from Merck. Bacteriophage lambda DNA was from New England BioLabs. All other chemicals used during the isolation procedure are reagent grade. Heparin agarose was prepared by the procedure of Davidson *et al.* (1979).

Culture of Cells

Streptomyces tubercidicus was grown in medium (0.3% nutrient broth, 0.1% yeast extract, 0.2% casamino acid, and 1% glucose, pH 7.2) at 28°C with shaking and aeration. Cells were harvested in the late exponential phase ($A_{560} = 4.2$) and adjusted to 50% (w/w) glycerol. The cell paste was stored at -20°C.

Enzyme Assay

Stu I endonuclease activity was assayed by monitoring the cleavage of DNA by agarose gel electrophoresis. Reaction mixtures containing 10 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol and 0.2 ug of bacteriophage lambda DNA. After incubation for 30 minutes at 37°C, the reaction was stopped by addition of 2 ul of the gel loading buffer which contained 120 mM EDTA, 60% (v/v) glycerol, and 0.1% bromophenol blue. The products were analyzed by agarose gel electrophoresis.

Protein determination

The concentration of proteins were determined according to the method of Marion, M. Bradford (1976).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was carried out according to the method of Weber and Osborn (1969) on 10% acrylamide vertical gels (16 × 18 × 0.15 cm). The protein bands were visualized by staining with 0.25% Coomassie brilliant blue in methanol: acetic acid: water (5:1:5) for 2 to 3 hours and destaining in 7.5% acetic acid, 5% methanol for 5 to 20 hours.

RESULTS AND DISCUSSIONS

The Purification of Stu I endonuclease

A summary of the purification of restriction endonuclease Stu I from 100g (wet weight) cells of *Streptomyces tubercidicus* is presented in Table 1. 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.

Step 1: **Preparation of the crude extracts;** 100g (wet weight) of *Streptomyces tubercidicus* cell paste containing 50% glycerol was suspended in 300 m*l* of Buffer A with gentle stirring for 5 hours at 4°C. Cells were disrupted in 100 m*l* portions by sonication using Artek sonicator equipped with a macrotip (30 sec. × 30 times). Temperature was maintained at 4°C or less with ice water bath during the

Table 1. *The progress of the purification from 100g cells of Streptomyces tubercidicus*

	Total protein (mg)	Total units	Specific activity (units/mg protein)	Percent recovery (%)	Purification fold
I. Crude extract	1,558	–			
II. Ammonium sulfate	954	9×10^6	9.5×10^3	100	1
III. DEAE-Sephadex A-50	85	5.3×10^6	6.2×10^4	59	6.6
IV. QAE-Sephadex A-50	70	4.6×10^6	6.6×10^4	51	7
V. Heparin Agarose	1.2	1.0×10^6	8.3×10^5	11	88.3

a. Protein concentrations were determined by the method of Marion, M. Bradford (1976).

disruption steps. The extract was clarified by centrifugation at 35,000 rpm in Type 42.1 rotor (Beckman) for 90 minutes. The supernatant was diluted with Buffer A to yield on $A_{280} = 60$ (Fraction I; 540 m*l*).

Step 2: **Ammonium sulfate fractionation;** Powdered ammonium sulfate (278.64g) was added to Fraction I with gentle stirring over a period of 60 minutes at 4°C to make 80% saturation. After stirring for 4 hours, the precipitate was collected by centrifugation at 15,000 rpm in type 42.1 rotor (Beckman) for 60 minutes.Pellet was suspended in 80 m*l* of Buffer B, and dialyzed against 10 volumes of Buffer B for 16 hours with four buffer changes. The dialyzed sample was diluted with 350 m*l* of Buffer B to yield 20 of absorbance at 280 nm (Fraction II; 450 m*l*).

Step 3: **DEAE-Sephadex (A-50) column chromatography;** Fraction II was applied to a DEAE-Sephadex (A-50) column (30 cm × 2.5 cm) equilibrated with Buffer B containing 50 mM NaCl. The column was washed with Buffer B (50 mM NaCl) and then eluted with 500 m*l* linear gradient of NaCl (0.2-1.0 M) in Buffer B. Fractions containing Stu I endonuclease activity, which eluted at 0.30-0.56 M NaCl, were collected and dialyzed against Buffer B (50 mM NaCl). (Fraction III; 77 m*l*).

Step 4: **QAE-Sephadex (A-50) column chromatography;** Fraction III was applied to a QAE-Sephadex (A-50) column (30 cm × 1.3 cm) equilibrated with Buffer B (50 mM NaCl). After washing the column with Buffer B (50 mM NaCl), the enzyme was eluted with 200 m*l* linear gradient of NaCl (0.1-1.0 M) in Buffer B. Active fractions, which eluted at 0.30-0.58 M NaCl, were pooled and dialyzed against Buffer B (50 mM NaCl). (Fraction IV; 65 m*l*).

Step 5: **Heparin-agarose column Chromatography;** Fraction IV was applied to Heparin-agarose column (25 cm × 1.3 cm) equilibrated with Buffer B (50 mM NaCl), the enzyme eluted with 200 m*l* linear gradient of NaCl (0.1-1.5 M) in Buffer B. Stu I endonuclease activity eluted between 0.44 M and 0.72 M NaCl. Active fractions were pooled and then dialyzed against Buffer B supplemented with 50% (v/v) glycerol and stored at -20°C (Fraction V; 1 m*l*).

The overall yield of Stu I endonuclease from 100g of cells was 1.0×10^6 units. Stu I endonuclease thus purified was free of other contaminating nuclease activity as shown by incubation of 1 ug of DNA with 100 units of enzyme at 37°C for 48 hours followed by 0.8% agarose gel electrophoresis. No further cleavage of the DNA was detected under these conditions. Fraction V showed a single major protein band and other two minor bands when analyzed by SDS-polyacrylamide gel electrophoresis, therefore Fraction V was not pure as a protein (data not shown). Because there were wide differences in mobility between proteins in fraction V on the polyacrylamide gel electrophoresis, a gel filtration might be enabled us to achieve homogeneous preparation of Stu I endonuclease.

Sephadex G-100 column chromatography

In order to obtain homogeneous form of protein with Stu I endonuclease activity, one fifth volume of the Fraction V was subjected to Sephadex G-100 column chromatography. Sephadex G-100 beads were equilibrated with buffer B containing 5% glycerol and 200 mM NaCl, and packed into the column ($1.7\ cm^2 \times 75$ cm). The bed volume of the column was 135 m*l* and the void volume was 42 m*l*. A constant flow rate of 2.5 m*l*/hour was maintained. Stu I activity was eluted at 73 % of bed volume. Each fraction having Stu I activity was applied to SDS-polyacrylamide gel electrophoresis to monitor their purity as a protein. Two fractions in the center of Stu I activity peak were demonstrated to have only one species of protein with respectable amounts. Active fractions were dialyzed against Buffer B supplemented with 50% (v/v) glycerol and stored at -20°C. Recovery of Stu I endonuclease activity in sephadex G-100 column chromatography was considered greater than 80%.

The Properties of Stu I endonuclease

The concentrated Stu I endonuclease did not lose any enzyme activity over a period of at least 6 months at -20°C.

To determine the subunit molecular weight of

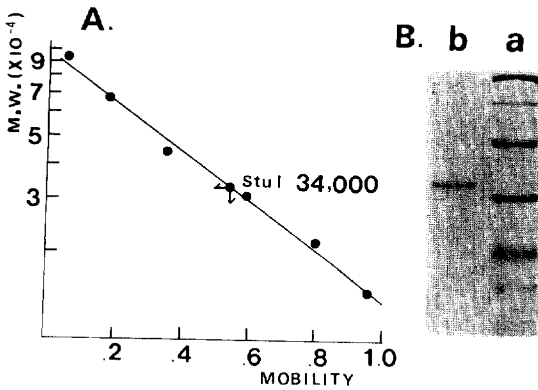

Fig. 1. *SDS-polyacrylamide gel electrophoresis of the sample from the Sephadex G-10C column chromatography.*
A: The subunit molecular weight of Stu I endonuclease.
B: lane a; standard proteins (from the top, phosphorylase B; 92,500, bovine serum albumin; 66,000, ovalbumin; 45,000, carbonic anhydrase; 31,000, soybean trypsin inhibitor; 21,500, lysozyme; 14,400) lane b; Stu I endonuclease from the fraction of Sephadex G-100 column chromatography.

Stu I endonuclease, 10% polyacrylamide gel electrophoresis was performed in the presence of 0.1% SDS. As shown in Fig. 1B, lane b, our preparation of Stu I endonuclease showed a single protein band and had the relative mobility of 0.53 which indicates that the single protein species has a molecular weight 34,000 ± 1,000 daltons (Fig. 1 A).

The elution pattern of Stu I enzyme in the sephadex G-100 column chromatography showed that the molecular weight of the native form of this enzyme was nearly two-fold of that judged by SDS-polyacrylamide gel electrophoresis. This implies that Stu I endonuclease may exist as a dimer form in solution as many other type II endonucleases.

Requirements for the best enzymatic activity of Stu I endonuclease were examined on various reaction conditions. The enzyme required magnesium ion (5 to 30 mM) for its activity and was maximally active in the absence of NaCl. Stu I did not essentially require sulfhydryl group compound for its activity.

적 요

Type II 제한효소 Stu I을 순수 정제하고 그 효소적 특성을 연구하였다. 100g(wet weight)의 *Streptomyces tubercidicus* (ATCC 25502)로 부터 얻은 crude extract를 ammonium sulfate fractionation한 후, DEAE-Sephadex(A-50), QAE-Sephadex(A-50) 그리고 Heparin-agarose의 순서로 column chromatography를 수행하여 1.2mg의 비특이성 nuclease가 없는 Stu I 제한효소를 얻었다. 이 시료에 포함되어 있는 다른 오염 단백질은 Sephadex G-100 column으로 gel filtration하여 제거함으로써, 순수한 Stu I 단백질을 얻을 수 있었다.

정제된 Stu I 제한효소는 10% SDS-polyacrylamide gel electrophoresis 결과 한 개의 band로 나타났으며, 그 분자량은 34,000± 1,000 dalton이었다.

이 효소는 Mg^{2+}이온 존재하에 중성의 pH(7.0-8.0)에서 최대의 활성을 나타내었다. NaCl은 이 효소의 활성에는 필요하지 않았다.

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