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Isolation and Characterization of a Newly Identified Type II Restriction Endonuclease from a Local *Streptomyces sp.* in Taiwan

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ABSTRACT

Streptomyces chusanensis ZS-2, isolated from a soil sample in Chusan in Taiwan, was found to produce a new Type II restriction endonuclease. This restriction enzyme was designated as *Sch*I. The purified enzyme was characterized as having a subunit mol wt of 28 kDa, and was apparently free from exonuclease activities. It cleaves the phosphodiester bond between the fourth C and the fifth G on the 5′-CCGCGG-3′ sequence of DNAs, leaving a 2-nucleotide protruding end at its 3′ site. This data suggests that *Sch*I is an isoschizomer of *Sac*II. In addition, based on the comparison between *Sch*I and *Sac*II regarding reaction parameters, it seems that *Sch*I is a better choice of restriction enzyme for genetic analysis and mapping.

Index Entries: *Streptomyces chusanensis;* restriction endonuclease; *Sch*I; *Sac*II; isoschizomer.

Abbreviations: bp, base pair(s); conc., concentration; EtBr, ethidium bromide; FPLC, fast protein liquid chromatography; kDa, kilodalton(s); PAGE, polyacrylamide-gel electrophoresis; PEI, polyethyleneimine; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; U, unit(s).

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INTRODUCTION

Restriction endonucleases are indispensable tools for gene analysis and recombinant DNA technology. They are characterized by their ability to cleave DNA at, or close to, their recognition sequences. Over 2500 different restriction endonucleases have been identified among various microorganisms, and they can be categorized into three different types *(1)*. The authors have recently found a new restriction endonuclease-producing strain from a local *Streptomyces sp.* at Chusan in central Taiwan. This strain was named *Streptomyces chusanensis* ZS-2, and the restriction endonuclease it produced was designated as *Sch*I. Presented here is the isolation and initial characterization of this newly identified restriction enzyme. Based on the data gathered, *Sch*I was found to be an isoschizomer of *Sac*II, which is produced by *Streptomyces achromogenes (2)*. Comparison of the properties between these two restriction enzymes is also discussed.

MATERIALS AND METHODS

Growth of *Streptomyces chusanensis* ZS-2

A *Streptomyces sp.* strain ZS-2 was isolated and identified from a soil sample in Chusan, Taiwan. Its physiological properties (Table 1), including utilization of carbon sources (Table 2), were examined by the method of Pridham and Gottlieb *(3)*. It was found to be a new Type II restriction enzyme-producing strain. It was grown aerobically at 28°C in YD broth (yeast extract 1%, dextrose 1%, pH 7.5) for 48 h. Cells were recovered by centrifugation. One liter of culture medium yielded 10 g of wet cell paste, which was stored at −70°C until use.

Substrate DNA and *Sch*I Assay

In the beginning, enzyme assays were carried out in 50 μL 10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$, and 7 m*M* 2-mercaptoethanol at 37°C for 1 h. One μg bacteriophage λDNA (Boehringer Mannheim, Mannheim, Germany) was used as the substrate for the activity assay during the enzyme purification. Reaction products were separated on a 0.8% agarose gel in Tris-borate buffer. The gels were stained with ethidium bromide (EtBr) (1 μg/mL), and photographed to examine the activity of the enzyme.

Purification of *Sch*I

The purification processes were all carried out at 4°C. During each step, the fractions containing *Sch*I activity were identified using the assay

Table 1
Physiological Properties of *S. chusanensis* ZS-2

Melanin formation	+[a]
Tyrosinase reaction	+
H_2S production	−
Nitrate reduction	−
Liquefaction of gelatin	+
Hydrolysis of starch	+
Hydrolysis of casein	+
Peptonization of milk	+
Growth temperature	15–40°C

[a] +, growth or with reaction; −, no growth or without reaction

Table 2
Comparison of Carbon Sources Utilized by *S. chusanensis* ZS-2 and *S. achromogenes*

Carbon source	*S. chusanensis*	*S. achromogenes*
D-Glucose	+[a]	+
L-Arabinose	±	+
Cellulose	−	−
D-Fructose	+	+
Inositol	+	+
Raffinose	+	−
D-Mannitol	+	+
L-Rhamnose	+	+
D-Xylose	+	+
Sucrose	+	−

[a] +, good utilization; ±, doubtful utilization; −, no utilization

method described above. Protein was measured by the Bradford method *(4)*, using bovine serum albumin as a reference standard.

1. Preparation of cell extract: Frozen cells (30 g) were suspended in 100 mL TEM buffer (20 m*M* Tris-Cl, 0.5 m*M* EDTA, 7 m*M* 2-mercaptoethanol, pH 7.5) containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by sonication (Cell Disruptor Model W-225R, HEAT Systems-Ultrasonics, Farmingdale, NY) in an ice bath at 200 W with bursts of 30 s spaced by 1-min cooling. Samples were sonicated for a total of 10 min. Cell debris was removed by centrifugation at 4°C for 1 h at 27,000*g*.

2. Initial purification steps: The cell extract was treated with 0.1 M NaCl and 1% neutralized PEI (Polymin-P; Gibco-BRL; Gaithersburg, MD), and centrifuged at 15,000 g for 10 min. The supernatant (40 mL) was collected and concentrated by precipitation with 70% ammonium sulfate, and dialyzed overnight against TEM buffer.
3. DEAE-Sepharose chromatography: The dialyzed sample (7.5 mL) was clarified by passing through a Millipore (Bedford, MA) membrane filter (0.45 mm) and applied into Pharmacia (Uppsala, Sweden) fast protein liquid chromatography (FPLC) system, using a DEAE-Sepharose fast flow (Pharmacia LKB Biotechnology) column (20 × 2 cm) pre-equilibrated with TEM buffer. The loaded column was washed with 650 mL TEM buffer, and the enzyme was eluted with a linear gradient of 240 mL TEM buffer containing 0.0–1.0 M NaCl at a flow rate of 2 mL/min. The endonuclease activity was recovered in fractions corresponding to 0.1–0.2 M NaCl. The active fractions were combined and dialyzed overnight against TEM buffer.
4. Storage of the enzyme: The final enzyme pool (320 mL) was concentrated to 6 mL with a Millipore CX-10 ultrafiltration unit, and adjusted to a glycerol concentration of 50%. Aliquots of 0.5 mL were stored at −20°C. No loss of activity was detectable after 3.5 yr of storage. The purified enzyme was analyzed by SDS-PAGE. The endonuclease activity of the purified enzyme was tested by reacting with the three DNA substrates of ϕX174 RF I, Ad-2, and λ DNA.

Determination of the Cleavage Site for *Sch*I

From preliminary characterization data, it was possible that *Sch*I may be an isoschizomer of *Sac*II. In order to test this observation, recombinant DNA was constructed that containing a unique *Sac*II site, using the M13/dideoxy DNA cloning-sequencing system *(5)*. Briefly, bacteriophage M13mp18 RF DNA was digested with *Sma*I and dephosphorylated by alkaline phosphatase (from calf intestine; Boehringer Mannheim, Mannheim, Germany), and finally ligated with a *Sac*II linker (5′-pGCCGCGGC-3′, Clontech, Palo Alto, CA) *(6)*. The recombinant M13mp18/*Sac*II DNA was transformed into *Escherichia coli* JM107 cells. The M13mp18/*Sac*II DNA transformants were verified, as described by J. Messing *(7,8)*. The recombinant M13mp18/*Sac*II DNA was digested with *Sac*II, to provide a linear molecule yielding the diagnostic fragment of 7258 bp.

Then, a primer extension experiment *(9)* was performed to determine the cleavage site of *Sch*I. Briefly, 2 μg M13mp18/*Sac*II DNA was digested with *Sch*I, followed by precipitation with ethanol. The pellet (restricted

Table 3
Summary of *Sch*I Purification from *S. chusanensis* ZS-2

	Volume (mL)	Total activity[a] (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield %
Crude extract	100	3,600,000	1650	2200	1	100
PEI eluate	40	2,300,000	128	18000	8	63
$(NH_4)_2SO_4$	7.5	2,200,000	93.8	23000	11	60
DEAE-Sepharose Fast flow	6.0	550,000	7.2	76000	34	15

[a] One unit is defined as 1 μg/h of substrate DNA cleaved under standard conditions, as described in Materials and Methods.

DNA) was redissolved in 4 μL H_2O, into which 3 μL primer solution containing 3 ng M13 universal primer (5′-GTAAAACGACGGCCAGT-3′) was added. The resulting DNA–primer mix was heated at 93°C for 3 min, and chilled on ice for 5 min. Then, 2 μL dNTP solution (0.3 *M* each of dCTP, dGTP, and dTTP) and 1 μL α-[^{35}S]dATP (600 Ci/mmol, 8 mCi/mL, Amersham, Arlington Heights, IL) were added, and the primer extension reaction was carried out with Klenow enzyme (0.6 U, Boehringer Mannheim). The single-stranded template of M13mp18/*Sac*II DNA, annealed with the same M13 universal primer, was used to generate the sequencing ladder, by the Sanger dideoxy method *(10)*.

RESULTS

Purification of *Sch*I

Isolation of the restriction endonuclease *Sch*I from *S. chusanensis* ZS-2 is summarized in Table 3. The specific activity of the isolated enzyme was calculated to be 76,000 U/mg protein. The relative molecular mass of the purified *Sch*I is 28 kDa, as determined by SDS-PAGE, and is shown in Fig. 1. Gel filtration chromatography using Superose 6™ prepacked HR 10/30 column (Pharmacia) showed the mol wt of *Sch*I was ca. 56 kDa (data not shown). These data indicated that the purified enzyme may be a homodimer with a relative subunit molecular mass of 28 kDa.

Properties of *Sch*I

Several parameters, such as pH, temperature, and the concentrations of mono- and divalent cations, were investigated to obtain the optimal reaction conditions for *Sch*I endonuclease (Fig. 2). Basically, the enzyme is stable from pH 7.5 to 9.5, with a peak at pH 8.5 (Fig. 2A). Although it dis-

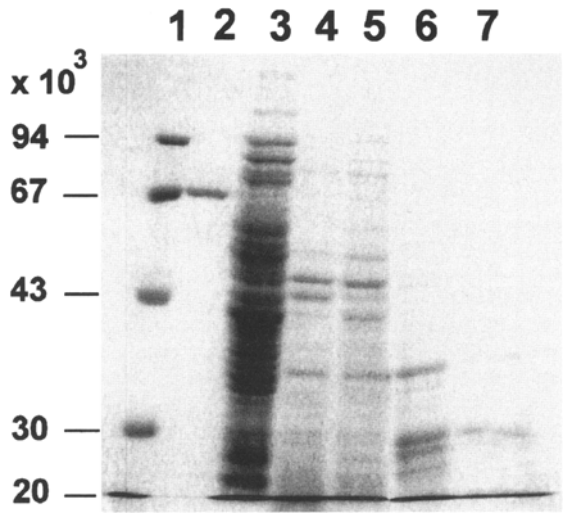

Fig. 1. SDS-PAGE analysis of crude and purified *Sch*I endonuclease from *S. chusanensis* ZS-2. The relative molecular mass of the purified enzyme was measured by 7.5% polyacrylamide gel. The gel was stained with Coomassie blue. Lane 1, mol wt standards (Pharmacia); lane 2, *Sac*II (New England BioLabs, Beverly, MA); lane 3, crude extract (approx 100 μg); lane 4–5, PEI treatment (25 μg); lane 6, ammonium sulfate precipitation (25 μg); lane 7, DEAE-Sepharose column (5 μg).

played optimal activity at 37°C, *Sch*I remained nearly as active up to 40°C (Fig. 2B). The enzyme was not inhibited by 0–200 m*M* NaCl (Fig. 2C). However, higher concentration of NaCl dramatically reduced its activity. Apparently, NaCl is not required for the enzyme reaction. In addition, it strictly required $MgCl_2$ for the enzyme reaction. However, concentrations of Mg^2+ higher than 50 m*M* inhibited enzyme activity (Fig. 2D).

To test for contaminating activity (e.g., exonuclease, and so on), 1 μg of λDNA was digested with 10 U of enzyme for 16 h at 37°C. Upon gel electrophoresis, no alteration in the characteristic banding pattern was observed. In addition, the endonuclease activity of the purified *Sch*I was tested, and is shown in Fig. 3 by cleaving ϕX174 RF I, Ad-2, and λDNA substrates at 1, >22, and 4 sites, respectively.

Cutting Site

The restriction enzyme *Sac*II cleaves after the fourth C residue of its recognition sequence, as previously reported *(1,2)*. Following cleavage of the substrate DNA with *Sac*II, a template is produced which contains, at its 5′ end, the dinucleotide GG from the *Sac*II recognition sequence, and at its 3′ end, the tetranucleotide sequence CCGC, which represents the first four

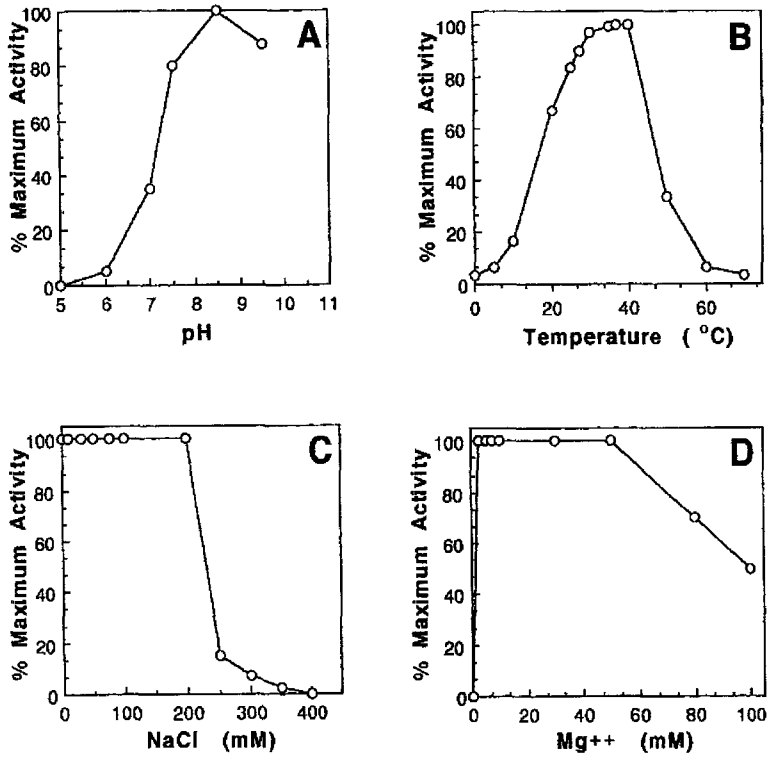

Fig. 2. Relative enzyme activities of *Sch*I with various reaction conditions. **(A)** pH; **(B)** temperature (°C); **(C)** NaCl (m*M*); **(D)** $MgCl_2$ (m*M*). Reaction conditions (except for the various parameters) were 10 m*M* Tris-Cl, pH 7.5, 50 m*M* NaCl, 10 m*M* $MgCl_2$, 37°C. Reaction products were analyzed by agarose gel electrophoresis and stained with EtBr. The reaction activities were determined by serial dilutions of the enzyme for each point of each parameter.

nucleotides of the *Sac*II recognition sequence. When carrying out a priming reaction on such a substrate, one will extend in the 5′ to 3′ direction, and will generate a product in which the primer is extended up to, but not beyond, the GG at the 5′ end of the template. That is, the extended product should contain the 3′ terminal sequence CC, which is exactly what the authors have observed (Fig. 4, lane 1). The position of the fragment generated by *Sch*I cleavage, followed by primer extension, corresponds to the second C of the *Sac*II recognition sequence 5′-CCGCGG-3′ (Fig. 4, lane 2). This result indicates that the site of cleavage by *Sch*I is between the fourth C and the fifth G, with 2-nucleotide 3′ overhang. Both *Sac*II- and *Sch*I-cleaved and primer-extended DNA fragments migrate to the same position as C within the *Sac*II site. Therefore, the *Sch*I cutting site is identical to that of *Sac*II *(1,2)*.

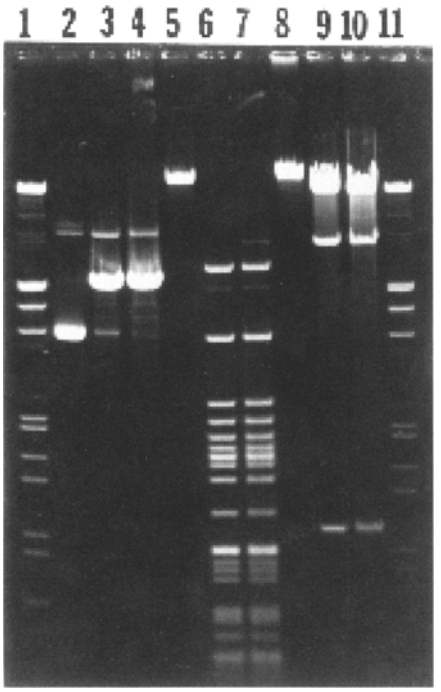

Fig. 3. Comparison of restriction patterns of λDNA, ϕX174 RF DNA and Ad-2 DNA digested with *Sac*II and *Sch*I endonucleases. Lanes 1 and 11; λDNA/*Eco*RI + *Hind*III; lane 2, uncut ϕX174 RF DNA; lane 3, ϕX174 RF DNA/*Sch*I; lane 4, ϕX174 RF DNA/*Sac*II; lane 5, uncut Ad-2 DNA; lane 6, Ad-2 DNA/*Sch*I; lane 7, Ad-2 DNA/*Sac*II; lane 8, uncut λDNA; lane 9, λDNA/*Sch*I; lane 10, λDNA/*Sac*II.

DISCUSSION

The authors have isolated a new restriction endonuclease, designated as *Sch*I, from a local *Streptomyces sp.* (named *S. chusanensis* ZS-2) found in Chusan, Taiwan. The purification was primarily achieved after the PEI step. In this step, and in the following ammonium sulfate step, most of the nonspecific nucleases were removed. The final product eluted from the DEAE-Sepharose column seemed to be nearly homogeneous and free from nonspecific nucleases. A similar one-step chromatographic procedure, used to purify restriction enzymes, has also been reported by Leung et al. *(11)* and Poch and Somkuti *(12).*

Both restriction patterns of *Sch*I and *Sac*II on ϕX174 RF I, Ad-2, and λDNA substrates are identical (Fig. 3). In addition, both *Sch*I and *Sac*II cleave at the phosphodiester bond between the fourth C and the fifth G on the 5′-CCGCGG-3′ sequence (Fig. 4), leaving a 2-nucleotide protruding end at its 3′ site. These data demonstrate that *Sch*I is an isoschizomer of

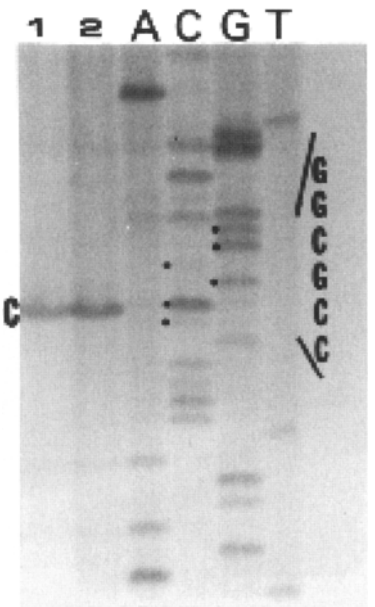

Fig. 4. Determination of the cleavage site for the *Sch*I endonuclease. Lane 1, [^{35}S]-labeled and extended product from the recombinant M13mp18/*Sac*II DNA cleaved by *Sac*II; lane 2, [^{35}S]-labeled and extended product from the recombinant M13mp18/*Sac*II DNA cleaved by *Sch*I; lanes A, C, G, and T, dideoxy sequencing ladder, with the *Sac*II site indicated on the right margin. In lane 1 and 2, both *Sac*II- and *Sch*I-cleaved and primer-extended DNA fragments migrate to the same position as C within the *Sac*II site.

*Sac*II. A comparison of the properties between *Sch*I and *Sac*II shows that *Sch*I could be a better endonuclease than *Sac*II, as far as the reaction parameters are concerned (Table 4). The *Sch*I endonuclease reacts over a broader pH spectrum, and over a broader range of temperature, 2-mercaptoethanol concentration, and salt concentration than the *Sac*II endonuclease. Furthermore, *Sch*I is very stable. The authors have stored the enzyme at −20°C for 3.5 yr without detectable loss of activity (Table 4). In addition, it can easily be inactivated by heat treatment at 65°C for 10 min (Fig. 2B). As a result, the *S. chusanensis* ZS-2 strain may be a good candidate for the production of restriction enzyme with the same specificity as *Sac*II and its isoschizomers. In order to substantiate the understanding and application of *Sch*I, cloning of the gene in *Escheridua coli*, and construction of an overproducing strain, is the major goal of our future study.

It has been shown that a number of restriction endonucleases demonstrate preferential cleavage of certain sites in the same substrate *(13–17)*. For example, *Sac*II has four cutting sites on λDNA. Three of the sites

Table 4
Comparison of Properties Between *Sch*I and *Sac*II

	*Sch*I	*Sac*II[a]
Temperature	30–40°C	37°C
pH (Tris-HCl)	7.5–9.5	7.5
Salt conc. (NaCl)	0–200 m*M*	0–60 m*M*
Mg^+ conc.	3–50 m*M*	6–10 m*M*
Buffer conc. (Tris-HCl)	0–100 m*M*	6–10 m*M*
2-Mercaptoethanol	0–100 m*M*	2–10 m*M*
Stability (−20°C)	>3.5 yr	not done

[a] Data obtained from the literature and all commercial product catalogs.

located near the center of λDNA are cleaved 50-fold faster than the remaining site at the right end of λDNA (at nucleotide 40,386). This site-preferential feature is also observed in *Sch*I (Kao, M. C. et al., data not shown). Three other restriction enzymes, *Nar*I, *Nae*I, and *Xma*III, have a more dramatic site preference. All these five enzymes recognize a sequence containing all G and C bases. However, there are other known GC hexanucleotide enzymes (*Sma*I and *Apa*I) *(1)* that do not show distinguishable site preferences. The reasons for the site preference remain to be investigated.

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