PURIFICATION OF THE RESTRICTION ENDONUCLEASE Sac I, FREE OF Sac II AND Sac III CONTAMINANTS

✉Raysa Vázquez, José Brito and Maribel Guerra

Division of Industrial Biotechnology, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba.

ABSTRACT

The restriction endonuclease Sac I was isolated from *Streptomyces achromogenes* and was purified to homogeneity, until no contaminant nuclease activities were detected. On the basis of ion exchange chromatography (Q-Sepharose and phosphocellulose P-11), Sac I can be obtained with a high level of purity and used in molecular cloning. The practical utitlity of Sac I enzyme is given by a high stability, high yield, easy handling of producing cells, and the ability to recognize new sequences, such as GAGCTC. The molecular weight (MW) of this enzyme was estimated by High Performance Liquid Chromatography and SDS polyacrylamide gel electrophoresis, being of about 50 kDa approximately. According to the results obtained from the accelerated stability study, the enzyme preparation is stable for at least 20 months.

Key words: **Restriction endonucleases, purification, Sac I**

Biotecnología Aplicada 1996;13:21-24

RESUMEN

La enzima de restricción Sac I se aisló del microorganismo *Streptomyces achromogenes* y se purificó a homogenidad, libre de nucleasas contaminantes. Empleando la cromatografía de intercambio iónico (Q-sefarosa y fosfocelulosa P-11), la Sac I pudo ser purificada con alto grado de pureza y usada en experimentos de clonaje. La utilidad práctica de esta enzima es su gran estabilidad, alto rendimiento, fácil manipulación de las células productoras y la habilidad de reconocer secuencias, tales como GAGCTC. El peso molecular de esta enzima se estimó por cromatografía líquida de alta resolución y por electroforesis en geles de acrilamida, siendo de alrededor de 50 kDa. La preparación enzimática fue estable al menos 20 meses, según el ensayo de estabilidad acelerado.

Palabras claves: **Endonucleasas de restricción, purificación, Sac I**

Introduction

Restriction endonucleases are enzymes, isolated chiefly from prokaryotes, that recognize specific nucleotide sequences within double-stranded DNA and cleaves it at these sites.

These so-called restriction endonucleases facilitate the specific fragmentation of double-stranded DNA and are very useful for DNA sequence analysis (1).

These enzymes can be classified into three groups. Type-I and type III enzymes carry a modification (methylation) and an ATP-requiring restriction (cleavage) activity within the same protein.

Both types of enzymes identify unmethylated sequences in substrate DNA, but type-1 enzymes cleave randomly, whereas type-III enzymes cut DNA at specific sites.

Type-II restriction/modification systems consist of a separate restriction endonuclease and a modification methylase. In the last 20 years, a great number of type-II restriction enzymes have been isolated, many of them useful in molecular cloning.

These enzymes cut DNA within or near their particular recognition sequences, which are typically four to six nucleotides long with two axis of symmetry. Among these enzymes, Sac I is isolated from Streptomyces achromogenes. This micro-organism produces two other restriction enzymes, Sac II and Sac III. Sac I recognizes the hexanucleotide sequence 5'...GAGCT↓C...3' and cleaves it at the position marked by the arrow (2). A restriction enzyme preparation must be invariably free of any undesirable nuclease, and must also be stable (3).

The accelerated degradation assay is the most widely used method for predicting the stability of a biological product (4).

The objective of the assay is to use the fitted Arrhenius equation in order to predict the degradation rate of the restriction endonuclease at the temperature at which it is commonly stored. A high level of stability is essential in any biological standard and this is also desirable in restriction endonucleases.

The Sac I enzyme is commercially available, but its purification has not yet been reported.

Here, we report the purification and partial characterization of the specific endonuclease Sac I involved in a restriction-modification system (5, 6).

Materials and Methods

Strains

Streptomyces achromogenes strain ATCC 12767 was grown at 28 °C in a 50 liter fermenter, ATCC medium # 5 [0,33 g/L of Yeast extract, 0,33/L g of Lab-Lemco, 0,67 g/L of Tryptone and 3,33 g/L of Glucose] and the growth was stopped at the stationary phase.

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✉Author's correspondence address

λ-DNA
λ-DNA was purified in our laboratory from the CSH-45 strain, (Cold Spring Harbor Collection), according to the procedure of Silhavy *et al.*, 1984 (7).

Plasmid DNA
Plasmid pCB121 was constructed in our laboratory (the plasmid is a pUC18 derivative, not cleaved by Sac I enzyme).

Oligonucleotides
DNAs were synthesized at CIGB (Havana, Cuba); deoxynucleoside triphosphates (dNTPs) were from Boehringer. [γ32P] ATP was purchased from Amersham.

Enzymes
T4 DNA Ligase was purified in our laboratory (8).

Chemicals
Q- Sepharose Fast Flow was purchased from Pharmacia (Sweden); Phosphocellulose P-11 was purchased from Whatman (England); Agarose was from Sigma Ltd (USA), and TSK-3 000 SW was from Toyo Soda (Japan). The other chemicals were purchased from Merck, Sigma, and B.D.H. Chemicals Ltd.

Purification of Sac I
S. achromogenes cells (80 g) were thawed and resuspended in two volumes of buffer Q [10 mM Tris HCl pH 8; 0,1 mM EDTA; 10 mM 2-mercaptoethanol; 5 % glycerol, and 0,02 mM benzamidine and 0,02 mM PMSF]. Then, the cells were disrupted by explosive decompression in a French press. As a result, a crude lysate, free of debris, was obtained by centrifugation at 40 000 rpm for 1 h, at 4 °C.

Proteins were later precipitated with $(NH_4)_2SO_4$ at 40 % saturation. The precipitate was collected by centrifugation and redissolved in a Q buffer (about 30 mL) and dialyzed against a Q buffer during 12 h, at 4 °C. The sample was applied to an 80 mL Q-Sepharose Fast Flow column, equilibrated with the same buffer. After washing with ten column volumes, bound proteins were eluted with a linear gradient combining pH and ionic strength of 100 mM NaCl pH 8-650 mM NaCl pH 7,4 (400 mL), at a flow rate of 80 mL/h.

The Sac I activity was pooled and dialyzed against buffer PC [10 mM KH_2PO_4 pH 7,5; 0,1 mM EDTA; 7 mM 2-mercaptoethanol; 5 % glycerol] during 12 h, at 4 °C. This pool was applied to a 36 mL phosphocellulose P-11 column, which had been previously equilibrated with the PC buffer. The column was then washed with ten column volumes of the PC buffer. A linear gradient from 0 to 1M NaCl was developed in PC buffer and applied to the column. The active enzyme Sac I eluted at about 400 mM NaCl.

The fractions containing the activity were pooled and the final preparation of the enzyme was concentrated against PEG 20 000, and then dialyzed against the storage buffer [20 mM Tris HCl pH 8; 150 mM KCl; 0,1 mM EDTA; 1 mM DTT; 50 % glycerol, and bovine serum albumin to a final concentration of 500 μg/mL].

A 7,5 x 600 mm column of TSK-3 000 SW, previously calibrated with several proteins of known molecular weight (bovine serum albumin 67 kDa; ovalbumin 43 kDa, trypsinogen 21 kDa and ribonuclease A 18 kDa), was used to estimate the molecular weight of the native protein. To do this, the column was equilibrated in buffer A [10 mM Tris-HCl pH 7,2; 0,1 mM EDTA; 10 mM 2-mercaptoethanol and 150 mM NaCl].

Assay for restriction enzyme activity
To quantify the enzyme activity at each step of the purification process, the standard technique of separating the cleaved DNA fragments by agarose gel electrophoresis was used, aliquots of 1 μL from each fraction were added to 20 μL of reaction mixtures containing the specific buffer and 1 μg of λ-DNA. After incubation for 1 h at 37 °C, the reactions were terminated by the addition of 2 μL of a solution containing 0,05 M Na_2EDTA (pH 8), 15 % glycerol, and 0,02 % bromphenol blue.

Samples were submitted to electrophoresis on slab gels consisting of 1,4 % agarose in Tris acetate and 0,5 μg/mL ethidium bromide. The resulting DNA-banding pattern was visualized by ethidium bromide fluorescence under short-wave ultraviolet light.

Thus, one unit of the enzyme was defined as the amount required to completely digest 1 μg of λ-DNA in 1 h, at 37 °C.

Assays for the presence of contaminating nucleases
In addition to the assay to measure the activity of the restriction enzymes,the following assays are performed throughout the purification process to determine in which chromatographic fractions exonucleases and nonspecific endonucleases are located. The careful assessment at each step of the ratios of the activities enables pooling of the maximum restriction endonuclease activity and the minimum of contaminating activities.

Assay for Nonspecific Endonuclease
A sample, usually of 5 μL, is incubated with 1 μg of pCB121 plasmid for 6 h, in the standard restriction endonuclease assay buffer, in a volume of 0,02 mL. Therefore the cleavage of a single phosphodiester bond in the DNA by a contaminating endonuclease is detected by converting the DNA form I to the DNA form II.

Assay for Exonuclease Contaminations
Different amounts of Sac I (35, 25 and 15 units) were incubated (usually 3 h) with 100 fmoles of an oligodeoxyribonucleotide (oligo 40 mer), labeled at the 5' end with [γ^{32}P] ATP, in the standard assay buffer in a volume of 100 μL at 37 °C. The reactions were then analyzed by proteins electrophoresis.

Assay for phosphatase
The test for phosphatase activity was carried out as follows:

Different amounts of Sac I (20 to 100 units) were incubated for 20 min at 37 °C with 5 pmoles of [γ^{32}P] ATP. After the incubation period, 1 μL of the reaction

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mixture was analyzed by thin layer chromatography in a polyethylenimine cellulose paper, which was developed with K_2HPO_4 750 mM pH 3,5. Under these conditions, it is possible to detect 10^{-6} units of phosphatase activity by one unit of Sac I enzyme.

Accelerated stability study of the restriction enzyme Sac I
This assay was carried out to predict a quantitative decrease of enzymatic activity in time. The test involves the incubation of the enzyme at different temperatures (4 °C, 15 °C, 25 °C and 37 °C). The measurement of the relative rates of degradation (decrease of enzymatic activity in time) was done essentially as described in Materials and Methods (Assay for Restriction Enzyme Activity), and the fitting of the Arrhenius equation was made in order to relate the degradation rate at high temperatures to the slow degradation rate at low temperatures (4).

Other methods
To estimate the molecular weight of the Sac I, 20 µg of the pure enzyme were analyzed onto 12,5 % SDS-PAGE (10). After PAGE, the gels were stained with Coomassie blue. The protein concentration was quantified by the Coomassie blue method (11) using BSA as a standard.

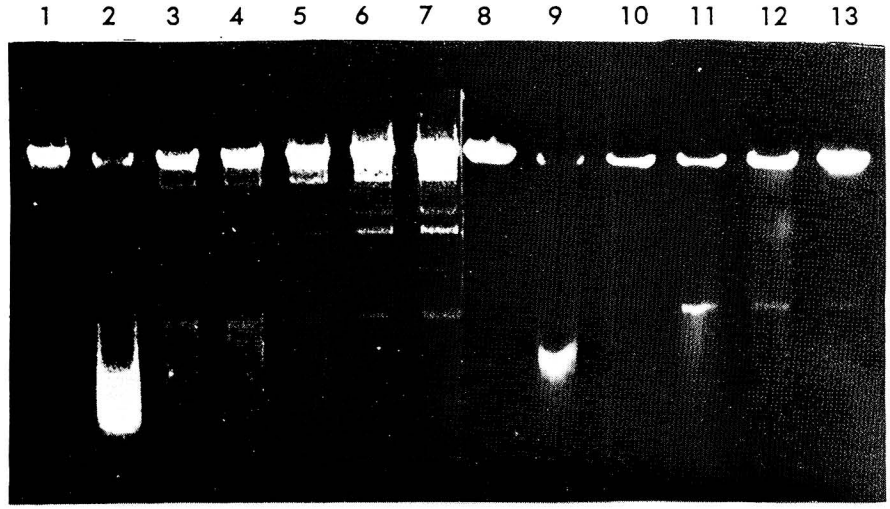

Figure 1. *Isolation of Sac I after precipitation with ammonium sulfate. Sac I, II and III activities are present in the crude extract. The bulk of Sac II and Sac III remained in the supernatant while Sac I activity was found in the precipitate when the crude extract was treated with ammonium sulfate. Left to right: Lane 1: λ-DNA as a negative control. Lane 2-7: λ-DNA digested with different enzyme units (1 u, 3 u, 5 u, 10 u, 15 u and 20 u per µL) of the crude extract. Lane 8: λ-DNA as a negative control. Lane 9-13: λ-DNA digested with different enzyme units (1 u, 3 u, 5 u, 10 u and 20 u per µL) of 40 % (w/v) ammonium sulfate precipitate.*

Results and Discussion

Purification of the Restriction Endonuclease Sac I.
The restriction endonuclease Sac I was purified 143-fold with an overall yield of 16 % and a specific activity of 26 666 U/mg (Table 1). This already large-scale purification scheme could easily be further scaled up.

A major problem found with the purification of the Sac I enzyme was the presence of Sac II and Sac III in the final preparation. After $(NH_4)_2SO_4$ precipitation, the Sac II and Sac III activities were removed, with only the Sac I activity remaining in the precipitate (Figure 1).

The Q-Sepharose column was used in order to increase the flow rate. Elution of the enzyme from Q-Sepharose required about 400-650 mM NaCl. After this step, pure Sac I was obtained, although, traces of nonspecific nucleases still remained .

The best purification was achieved on phosphocellulose. In this case, the enzyme was eluted from this column at about 450 mM NaCL, and a removal of most of the nonspecific nucleases after this step was also obtained. The Sac I enzyme appeared as a narrow peak of activity.

The results on Figure 2 show no evidence of nonspecific endonuclease activity, even with an 800-fold overdigestion of the pUC18 plasmid.

The integrity of the oligonucleotide demonstrates that there was no nuclease activity present in the

Table 1. *Purification of the Sac I restriction endonuclease. A summary.*

Purification step	Total U (U)	Protein (mg/mL)	Total P. (mg)	Spec. act. (U/mg)	Yield (%)	Purif. fact.
Crude extract	500 000	53,7	2 685	186,0	100	1,00
Ammon. sulfate	400 000	26,0	1 040	385,0	80	2,00
Q-seph	150 000	3,2	96	1562,2	30	1,83
P-11	80 000	1,5	3	26 666	16	143,36

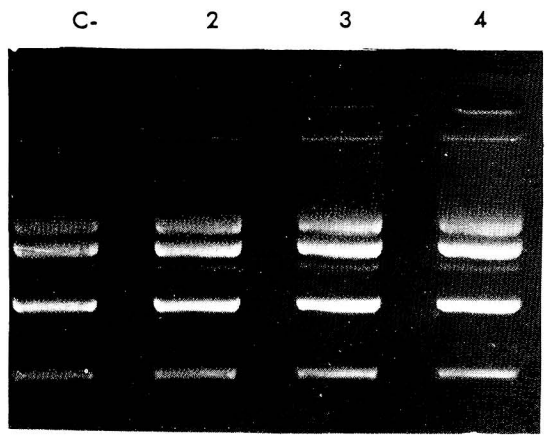

Figure 2. *Electrophoresis of pCB121 in a 1 % agarose gel at different overdigestions with Sac I. Lane 1: pCB121 as a negative control. Lane 2, 3, 4: pCB121 overdigested with 100,400, and 800 units of Sac I respectively.*

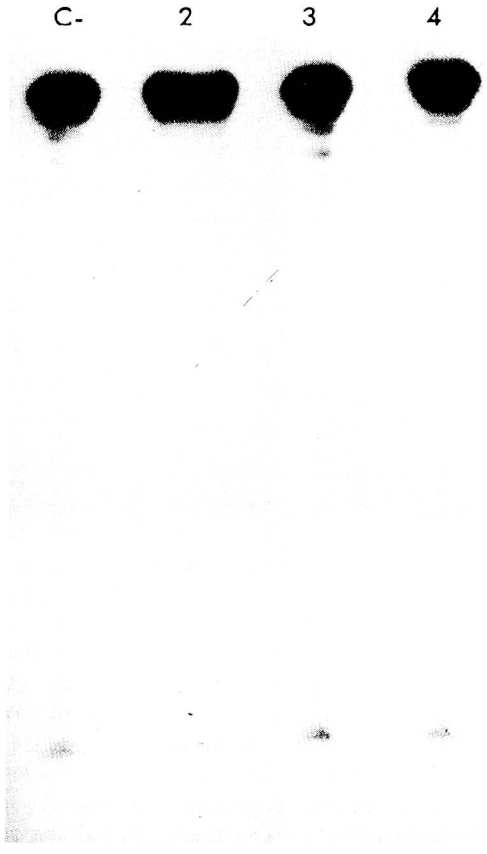

Figure 3. *Nuclease test of Sac I preparation using [γ^{32}P] ATP labeled oligonucleotide λ-DNA (1μg). Lane 1: Oligonucleotide as a negative control. Lane 2: Oligonucleotide with 15 units of Sac I. Lane 3: Oligonucleotide with 25 units of Sac I. Lane 4: Oligonucleotide with 35 units of Sac I.*

preparation (Figure 3). The preparation was also free from phosphatases (data not shown). Our final product, with a specific activity of about 26 666 U/mg of protein, was free from nonspecific nucleases.

Comparison of the detection levels by SDS-PAGE with the quantity of protein sample applied, indicates that the final preparation was highly pure. Batches of Sac I purified according to this procedure have been used successfully in our laboratory for cloning purposes (data not shown).

Size of the Sac I protein

The molecular weight of the Sac I by using SDS-PAGE and High Performance Liquid Chromatography was determined. It was of about 50 kDa (Figure 4). The structure of the enzyme under native conditions was estimated by gel filtration on a TSK 3 000 SW column. Sac I eluted from the TSK 3 000 column as a single activity peak that corresponded to the monomer with a size of 50 kDa (as confirmed by SDS-PAGE).

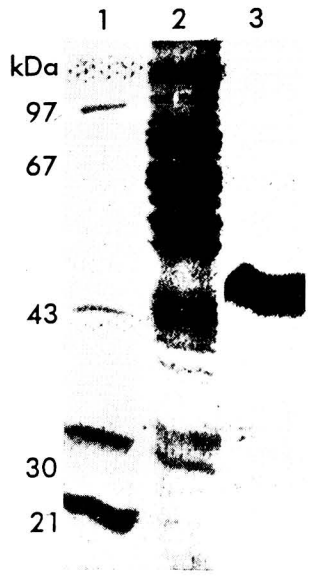

Figure 4. *12.5 % SDS-PAGE was made according to Laemmli. (1970). Lane 1: Protein standards (20 μg of each protein): phosphorylase B (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa). Lane 2: 20 μg of the crude extract. Lane 3: 20 μg of the final preparation of Sac I.*

Stability study

K was calculated and the specific rate of the enzymatic activity decrease was plotted, using log K against the

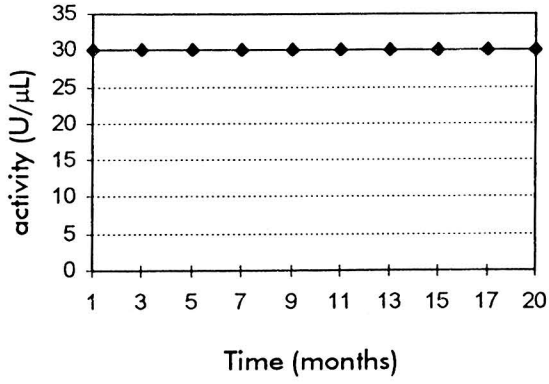

Figure 5. *Plot of Sac I residual activity versus time in order to predict the enzyme stability at -20 °C. The abscissa shows the decrease of the enzymatic activity expressed in U/μL of the enzyme using λ-DNA as a substrate. The ordinate shows the time in months. This graph shows the slow inactivation rate of the enzyme under storage condition (-20 °C) during 20 months.*

absolute reciprocal temperature. As a result, the enzyme Sac I was stable at -20 °C for at least 20 months (Figure 5). The activity of enzyme was rechecked, and the data were conformed.

Conclusions

1. After two steps of purification, a homogeneous preparation of Sac I free of nonspecific nucleases, and free of Sac II and III enzymes was obtained.
2. The molecular weight of the Sac I enzyme was estimated, being of approximately 50 kDa.
3. Sac I was stable at -20 °C for at least 20 months.

Acknowledgments

The authors would like to thank G. Reyes and J. Madrazo for the technical assistance. They also wish to thank, A. Suárez for the helpful computer program and Diana Cruz-Bustillo and Ricardo Silva for revising the English version of the article.

Recibido en marzo de 1995. Aprobado en septiembre de 1995