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Full Length Research Paper

Identification of a restriction endonuclease (SacC1) from *Saccharomyces cerevisiae*

Mukaram Shikara

Biotechnology Division, Applied Sciences Department, University of Technology, Baghdad, Iraq.

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SacC1 is a novel restriction endonuclease from *Saccharomyces cerevisiae* that recognizes the palindromic sequence 5'CTCGAC3' cleaving both DNA strands upstream and downstream of its recognition sequence and makes a staggered cut at the distance of five bases from the recognition sequence on the upper strand and at the seventh base on the complementary strand. It shares similar characteristics with Sac I from *Streptomyces achromogenes* as well as Sst1 from *Streptomyces Stanford* and Psp124B1 from *Pseudomonas* species. It has been purified by ammonium sulphate precipitation, dialysis, and gel filtration using phosphocellulose, DEAE-cellulose and Sephadex G-100 with an optimal pH range (7.5-8.5), active at 37°C and dependent on Mg^{+2} or Mn^{2+} which increases its activity by 4- and 2-folds, respectively, while other cations decrease its activity to some extents. Cleavage on both sides of the recognition sequence is characteristic of Type IIB systems but all IIB enzymes studied so far have been found to recognize discontinuous sites and a distinctive subunit/domain organization that is not present in the SacC1 enzyme. There are similarities between SacC1 and other homing endonucleases belonging to the LAGLIDADG family such as a requirement for Mg^{2+} (or Mn^{2+}) for cleavage to take place, optimal activity at alkaline pH and stimulation of the reaction by moderate concentrations of the monovalent cation.

Key words: Purification, recognition site, restriction enzyme, *Saccharomyces, Streptomyces*, Type IIB.

INTRODUCTION

Restriction enzymes have proved to be invaluable for the physical mapping of DNA. They offer unparalleled opportunities for diagnosing DNA sequence content and are used in fields as disparate as criminal forensics and basic research. In fact, without restriction enzymes, the biotechnology industry would certainly not have flourished as it has (Roberts, 2005). Restriction enzyme systems are aim to destroy foreign DNA without destroying their own bacterial or fungal DNA (Roberts and Macelis, 1994). The key feature of restriction endonucleases is not only their ability to cleave DNA at their recognition sequences, but rather their ability to avoid cleaving DNA at any other sequence. The ability of these enzymes promoted extensive screening of bacteria and fungi by biochemical assays and by genome analyzes (Murray, 2000). They have been classified into three main groups according to their cofactor requirements and the type of DNA cleavage (Wilson, 1991). The Type II restriction enzymes recognize specific DNA sequences and cleave at constant positions at or close to that sequence to produce 5 -phosphates and 3 -hydroxyls. Usually they require Mg^{2+} ions as a cofactor, although some have more exotic requirements (Richard et al., 2003). They are also the simplest ones with respect to other properties such as subunit structure and cleavage characteristics and are composed of two separate enzymatic activities. One is a restriction endonuclease that cleaves DNA at a specific recognition sequence. The second is a DNA methyltransferase, which is able to methylate the same sequence and render it refractive to cleavage by the corresponding endonuclease (Halford, 2001).

In Iraq, several separate attempts to purify restriction endonucleses from local sources were tried with the earliest attempts made in mid 1990s (Putrus, 1995; Al-Khafagi, 1999).

*Corresponding author. mukaramshikara2010@yahoo.com.

Table 1. The oligonucleotide duplexes used in SacC1 activity stimulation experiments.

Number of duplex	Duplex	Oligonucleotides	Stimulation of SacC1 activity
1	15-mer	5'-agCACGAGCTCGCta	Yes
		3'-tcGTGCTCGAGCGat	
2	13-mer	5'-tagCACCTGCtat	No
		3'-atcGTGGACGata	
3	18-mer	5'-atagCAGAGCTCCGtata	Yes
		3'-tatcGTCTCGAGGCatat	
4	18-mer	5'-atagCACGAGCTCTtata	Yes
		3'-tatcGTGCTCGAGAatat	
5	20-mer	5'-atagGAGCTCCGGCTTtata	Yes
		3'-tatcCTCGAGGCCGAAatat	
6	20-mer	5'-atagCACCTGCtataaagt	No
7	20-mer	3-'tatcGTGGACGatatttcag	

The present attempt (with the help of Al- Azytoonah University, Amman, Jordan) aims to purify a restriction endonuclease from *Saccharomyces cerevisiae*, This yeast is a member of the largest genus that produces antibacterials, antifungal, immunosuppressants, and industrial enzymes including restriction enzymes where several restriction enzymes were purified from it. In addition, establishing a line for production of this important enzyme is very important as new restriction enzymes are still required in order to increase the range of DNA manipulation.

MATERIALS AND METHODS

Sources of media and analytical chemicals

All analytical chemicals, Phage Lambda, plasmid DNAs, all enzymes, DNA markers and dialysis tubing cellulose membrane (diameter 6 mm and width 10 mm) were purchased from Sigma Aldrich. [α-^{33}P]dATP is from Amersham. Oligonucleotides (Table 1) and the DNA sequencing kit are from InterScience. Kd ladder is from Genomics, Agilent Technologie. pMXBIO control plasmid containing the endonuclease target sequence served as a double-strand template using the forward and reverse primer 1212 and 1233 are obtained from New England Biolabs.

Isolation of yeast

S. cerevisiae strain R-Z128 was used throughout the study. Yeast is grown in YPD medium (1% yeast extract, 2% Bacto-peptone and 2% dextrose) at 30ºC with constant shaking for 3 days and then the cells were lysed by sonication in ultrasonic bath (Sonicator Branson 5210) for 20 x 10s and broken cells were removed by centrifugation (2 min, 15,600 ×*g*). The supernatant was centrifuged at 3000 x g for 30 min at 4°C and the supernatant has been used as the "crude extract" and the source of the enzyme.

Purification of the restriction endonuclease

Solid ammonium sulphate was added to the "crude extract" to form 0 - 30%, 30 - 50% and 50 - 80% saturation fractions, respectively. After centrifugation at 4000 g for 15 min, the pellet (of each fraction) was suspended in 40 mM potassium phosphate (pH 7.5) containing 5 mM 2-mercaptoethanol, and 10% glycerol (buffer A) was then dialyzed with two changes against 4 L of the above buffer for 24 h and measured for endonuclease activity.

The 50 - 80% saturation fraction was found to have a high endonuclease activity, so it has been purified further by layered onto a 1.5 x 40 cm phosohocellulose column that was previously equilibrated with 4 L of buffer A. The endonuclease was eluted from the column with a linear gradient of 0 - 0.6N NaCl in buffer A. The peaked fractions (Fractions 45 - 50) were pooled together and dialyzed for 5 h against 4 L of 40 mM Tris-HCl, pH 7.5 containing 5 mM 2-mercaptoethanl and 10% glycerol (buffer B) and then loaded onto a DEAE-cellulose column (1.5 x 1m) that was previously equilibrated with buffer B. Two peaks were obtained, the first peak with a very small activity was eluted in the washing region (fractions 36 - 50), while the second peak with most endonuclease activity was eluted at 0.76 - 0.84 N NaCl (Fractions 113 - 121) (Figure 1). The peaked fractions were pooled, dialyzed for 5 h and loaded into Sephadex G-100 column (Figure 2) and eluted with buffer B (see Methods). The top fractions (82 - 85) were used as the purified restriction enzyme. The homogeneity of the enzyme was determined by 10% SDS–PAGE. The total yield of SacC1 protein was 2.5 mg.L^{-1} of induced culture. All steps were carried out at 4°C. Endonuclease activity, protein and carbohydrate concentrations were determined for all fractions.

Determination of endonuclease activity

Endonuclease activity was assayed according to Brown and Smith method (1980) by incubation of 0.3 pmol lambda DNA with 3.0 pmol of the purified endonuclease enzyme in a final volume of 20 µL containing 40 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 0.1 mg.mL^{-1} bovine serum albumin for 1 h at 37°C. The reaction mixture was stopped by cooling at 0ºC and with the addition of 20 mM EDTA. The cleavage products electrophoresed on a 0.8% agarose gel and DNA was visualized by staining with ethidium bromide. One unit of the enzyme was defined as the amount of the enzyme that can digest 1 µg of Lamba DNA for 1 h at 37°C under standard conditions.

Determination of the recognition sequence and the cleavage site

The recognition sequence of SacC1 was determined by mapping of the recognition sites on phage λDNA. The fragments predicted by

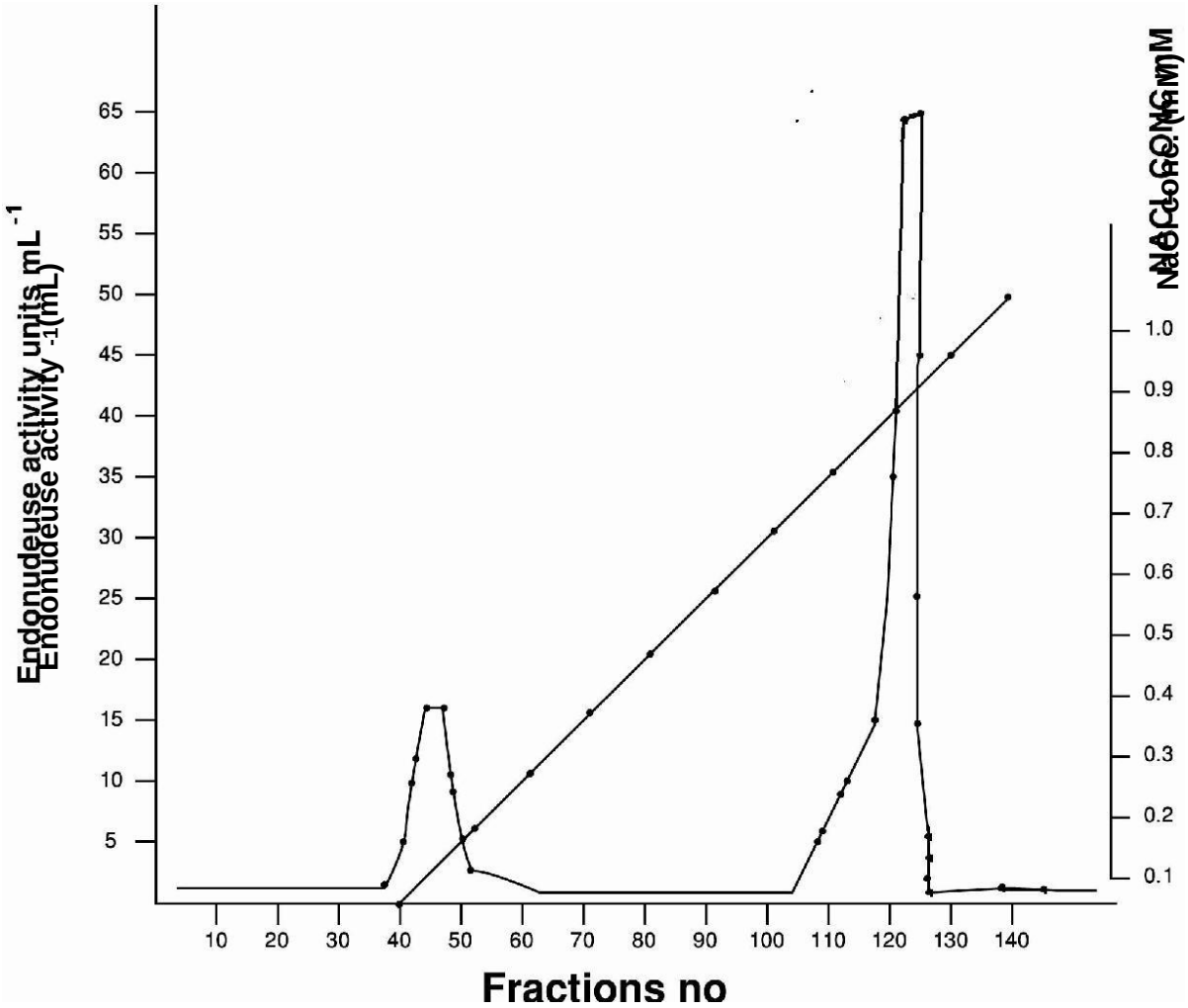

Figure 1. DEAE-cellulose chromatography of SacC1 endonuclease activity. The pooled active fractions from phosphocellulose column were eluted through the DEAE-cellulose column with 300 NaCl gradients (0-1M) as described in "Methods".

cleavage of the inferred recognition sites were compared with the observed fragments from SacC1 cleavage of different DNAs, while λ DNA was used as a template to characterize the cleavage site of SacC1. A 20 mer oligodeoxyribonucleotide complementary to λDNA was used in direct sequencing through the SacC1 recognition site. Four dideoxy sequencing reactions using [α^{33}P] dATP and a DNA sequencing kit were carried out. The same primer and template were used in an extension reaction, which also included T7 DNA polymerase, dNTP and [α^{33}P] dATP. The extension reaction was heat inactivated, treated with SacC1 in the presence of 0.5 µM oligonucleotide duplex and the reaction mixture was divided into two. The aliquot was treated with T4 DNA polymerase in the presence of dNTP. All samples were diluted with sequencing dye solution and loaded on a standard sequencing gel along with the dideoxy sequencing reaction.

Determination of optimal pH, temperature, stability and metal ions

The influence of pH and the temperature of endonuclease activity were examined. The optimal pH was determined at 37°C in buffer B, while the enzyme activity at various temperatures (20 - 80°C) were determined in buffer B, pH 8.0. The pH stability was studied by measuring the endonuclease activity at pH 8.0 after the enzyme was incubated at 37°C for 24 h. The effects of different metal ions were also determined.

Estimation of purity and the molecular weight

Sephadex G-100 was used according to the method of Andrews (1964). SacC1 samples (250 µg) were loaded onto a Sephadex G-100 column (1 x 50 cm) which was equilibrated with a buffer B. Separation was carried out at a flow rate of 0.2 mL.min^{-1}. The column was calibrated with dextran blue (>100 kDa), bovine serum albumin (66.2 kDa), Egg albumin (45 KDa), chymotrypsinogen A (25 kDa), lysozyme (14.4 kDa) and cytochrome C (12.4 kDa), Elution profiles were monitored by measuring absorbance at 280 nm and the specific Lamba DNA digestion profile by Sac I and SacC1. For the interpolation of unknown molecular mass, a linear dependence of the logarithm of the molecular mass on the elution time was assumed. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 12%

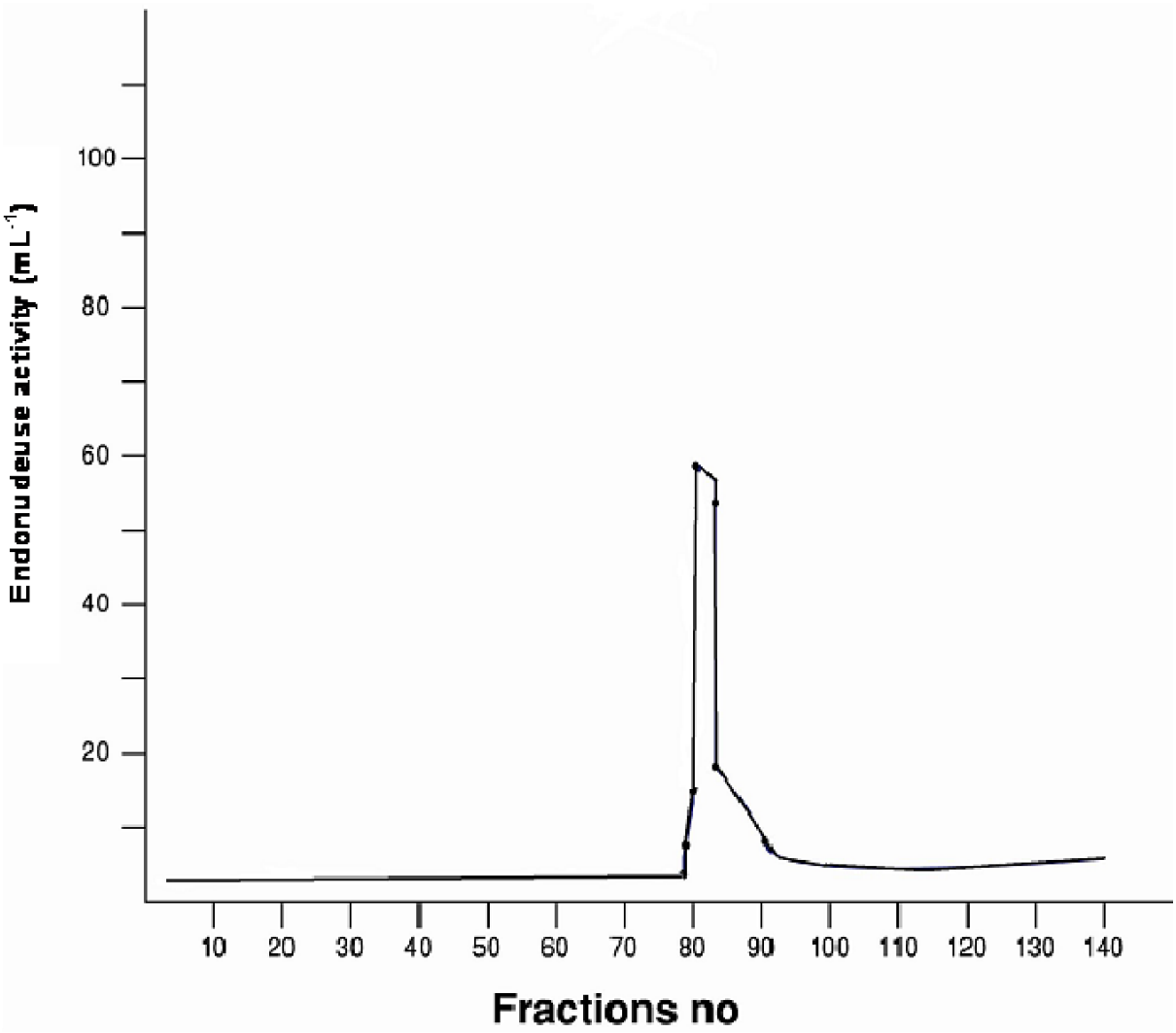

Figure 2. Sehadex G- 100 Chromatography of SacC1 endonuclease activity. The pooled active fractions from DEAE-cellulose column were eluted through the Sephadex G-100 column as described in "Methods".

polyacrylamide gels as described by Laemmli (1970) and as modified by Maizel (1971). Molecular weight markers were obtained from Boehringer, Mannheim, Germany.

Estimation of protein and carbohydrates

Protein contents were determined by the method of Lowry et al. (1951) using Bovine serum albumin as a standard. Carbohydrates contents were determined by the method of Dubois et al. (1956) using glucose as a standard. In the case of electrophoresis, an endonuclease activity unit is defined as the ability of one volume of restriction enzyme to completely cut 1 ug of lambda DNA at 37°C for 1 h under standard conditions.

Determination of the optimal conditions for the *in vitro* endonucleolytic activity of the purified restriction enzyme

To determine the optimal conditions for the *in vitro* endonucleolytic activity of SacC1, different buffers were tested, taking into account ionic strength, pH, Mg^{2+} and ion concentrations.

RESULTS

Purification of the SacC1

Table 2 summarizes the purification steps.

Homogeneity

The homogeneity of the enzyme was determined by 10% SDS–PAGE. The total yield of purified enzyme's protein was 2.5 $mg.L^{-1}$ of induced culture.

Determination of the recognition site

To determine the substrate specificity of the enzyme, cleavage sites of SacC1 on λ DNA were mapped by double digestion with SacI and SacC1 (Figure 3). A

Table 2. Purification steps of SacC1.

Step	Total volume (mL)	Total activity (units)	Total protein (mg)	Specific activity (unit.mg^{-1})	Purification	(%)
Crude extract	500	2345	7.61	308.2	1	100
50-80% $(NH_4)_2SO_4$ fraction	28	1495.2	3.58	417.7	1.4	63.7
Phosphocellulose	50	1296	0.61	2124.6	6.9	42.7
DEAE-Cellulose	30	827.6	0.18	4597.7	14.9	35.3
Sephadex G-100	16	464.6	0.007	66371.4	215.4	19.8

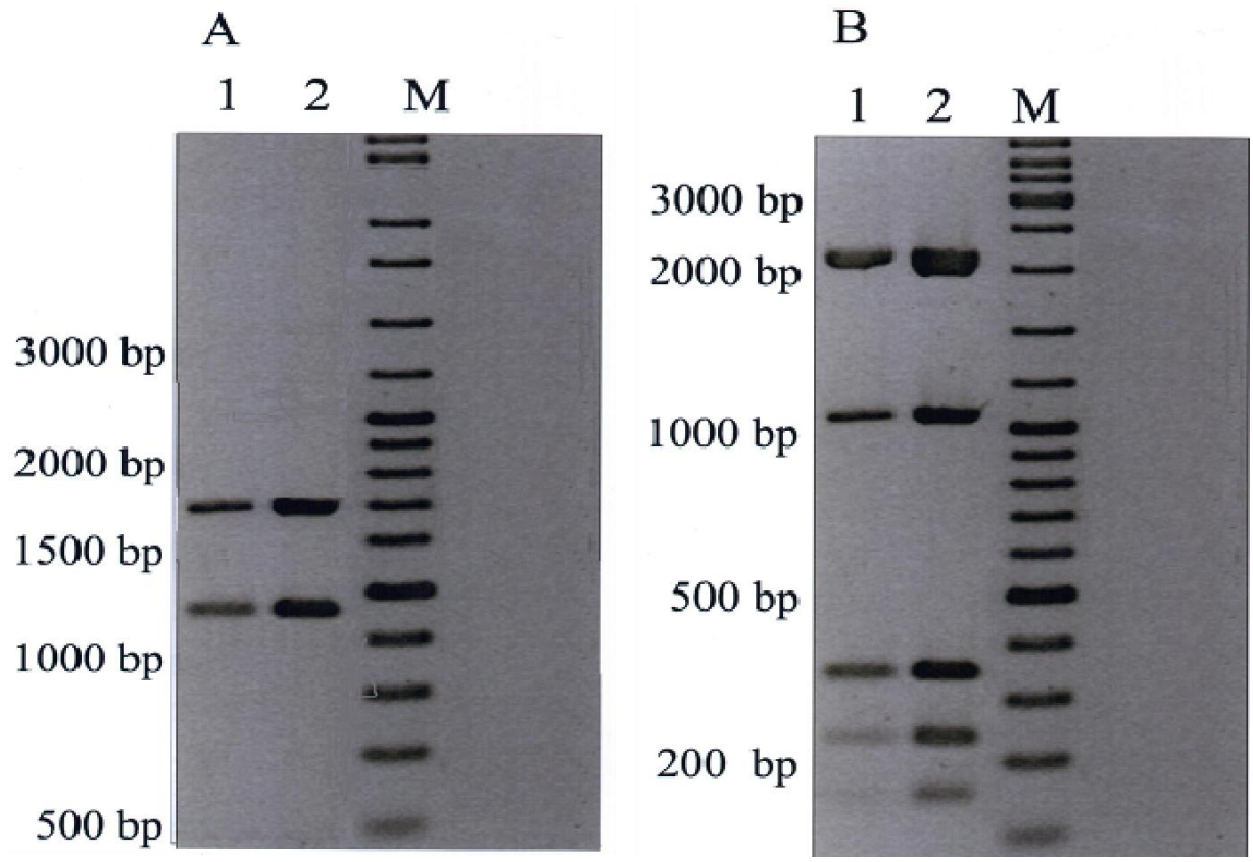

Figure 3. Determination of the recognition sequence of SacC1 a) Comparison of the plasmid DNA cleavage by SacC1 (lane 1) and SacI (lane 2).B) double digestion of the plasmid DNA with SacC1 and SacI. M) DNA molecular weight marker (100-10000).

computer-aided search of homologous nucleotide sequences revealed only one common sequence, 5'GAGCTC3', for the mapped SacC1 sites. The numbers of DNA fragments generated by SacC1 and SacI cleavage of λDNA and ϕX174 DNAs were 2 and 1 sites, respectively. pBR322, pUC19, pTZ19R, and M13mp18 were not cleaved since they do not contain 5'GAGCTC3' sequences.

The cleavage position was determined by the method of primer extension (Sanger et al., 1977) through comparison of the dideoxy sequence ladders with fragments generated by SacC1 cleavage and T4 DNA polymerase action on the digestion product (Figure 4). The fragment generated by SacC1 digestion co-migrates with the T band of the sequence ladder indicating that the cutting point is 5 nt away from the recognition sequence 5'-GAGCT/C3'. The single band obtained after treatment with T4 polymerase co-migrates with the C band (lane 1) of the sequence ladder, confirming that the cleavage point on the complementary DNA strand is 7 nt away from the recognition sequence. In summary, SacC1 cleaves to double-stranded DNA, generating fragments five and seven bases upperstream and downstream of its recognition sequence, respectively.

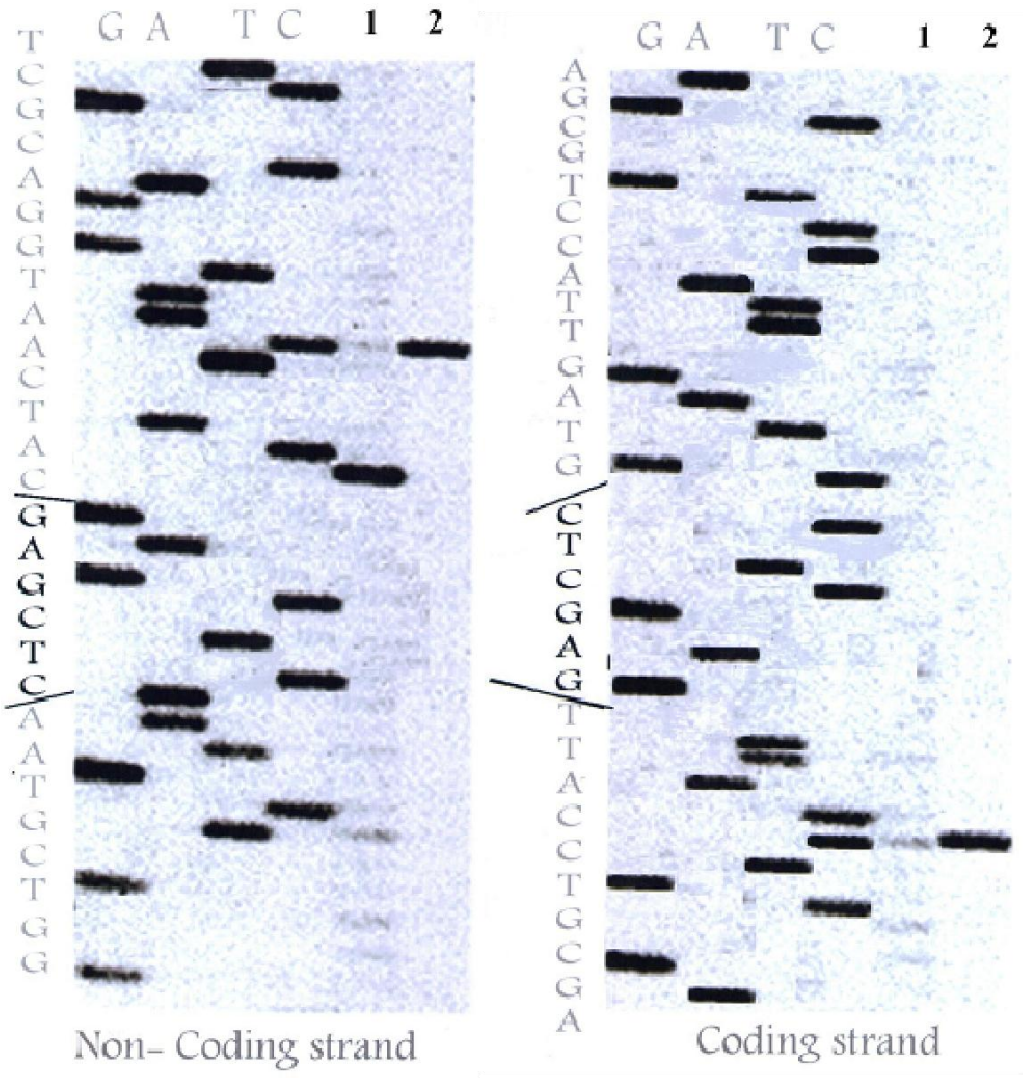

Figure 4. Determination of the cleavage site. Autoradiogram is showing the extension polymerization products digested or not with SacC1 and the corresponding sequence ladder. The cleavage sites are indicated in bold letters. Lane 1. T4 polymerase action on the SacC1digest, Lane 2. The product of the primed synthesis reaction cleaved with SacC1.

Estimation of molecular weight

The molecular mass of SacC1 was estimated by gel filtration on a Sephadex G- 100 column to be 58,000 and by SDS-gel electrophoresis to be 64,000 (Figure 5).

Effects of pH and temperature

The optimum pH for the enzyme was determined by estimating the percent activity at different pH values. The pH optimum of the enzyme has a range of (7.5 - 8.5), while the optimum temperature was determined by examining the enzyme activity at different temperatures. Preliminary experiments showed its stability at room temperature for 30 min, but for 6 h at 4°C and more than three months at -20°C (Table 3).

Metal ion requirements

The enzyme appears to be dependent on Mg^{+2} and Mn^{2+} 4- which increases its activity by 4- and 2-folds, respectively, while other cations decrease its activity to some extents. Salts such as sodium or potassium chlorides have no effect at all, while the presence of B-mercaptoethanol increases the activity slightly. ATP has no effect on it (Table 4). The maximum activity of the enzyme was observed at 37°C in the presence of 40 mm Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 0.1 $mg.mL^{-1}$ BSA in

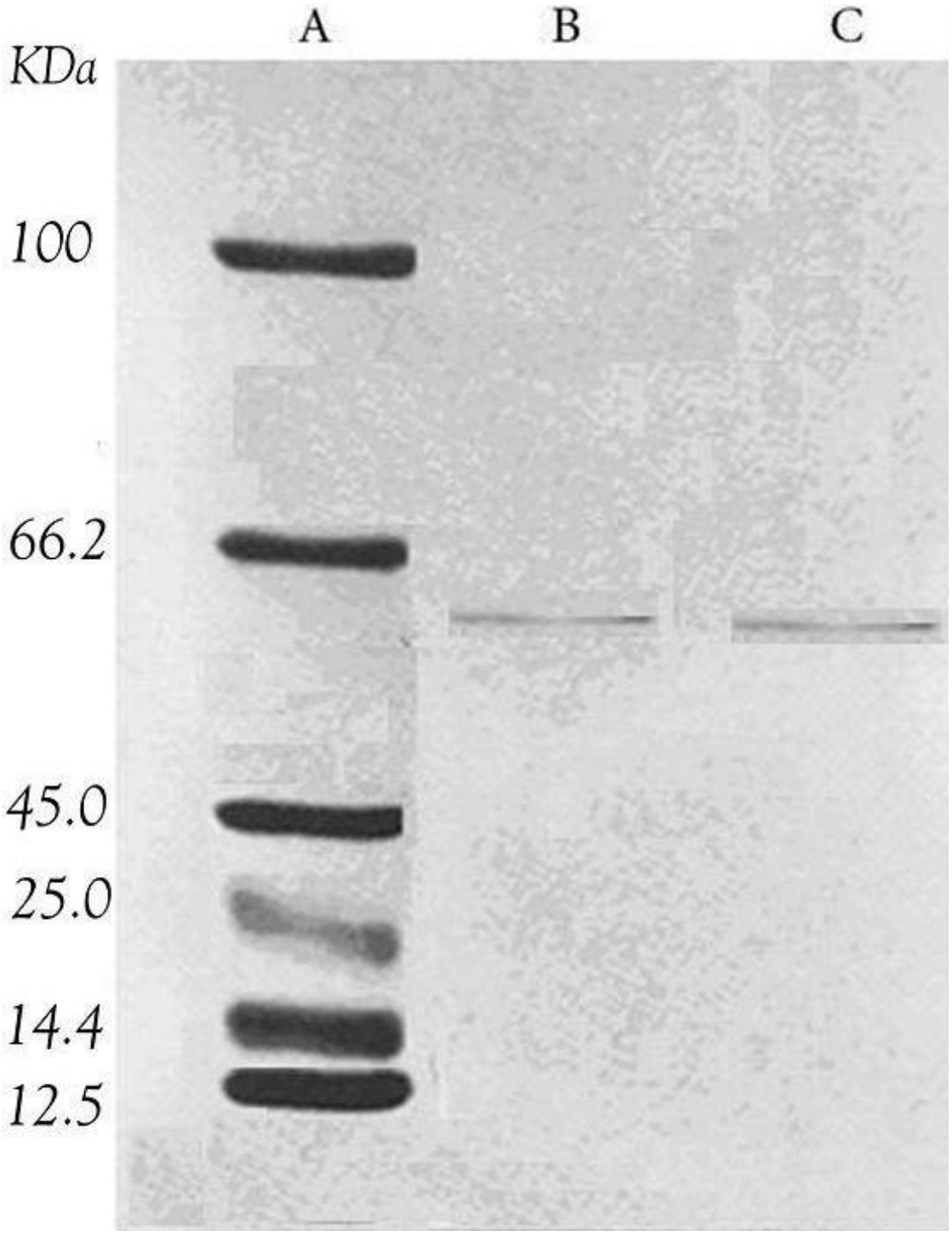

Figure 5. Determination of the molecular weight of SacC1 (B and C) while (A) is the protein ladder (10-100 kDa) was used as the protein molecular weight marker.

the absence of any salt or cofactors (Table 4).

DISCUSSION

SacC1 is a novel restriction endonuclease from *Saccha-romyces cerevisiae* that recognizes the palindromic sequence 5'CTCGAC3' cleaving both DNA strands upstream and downstream of its recognition sequence and makes a staggered cut at a distance of five bases from the recognition sequence on the upper strand and at the seventh base on the complementary strand.

Table 3. Effect of the temperatures on SacC1.

pH	Relative activity (%)	Temperature (°C)	Relative activity (%)
6.0	14	15	15
6.5	32	20	17
7.0	80	30	60
7.5	100	37	100
8.0	100	45	55
8.5	100	50	35
9.0	16	55	30
9.5	4	60	3

Table 4. Influence of different metal ions and reagents on SacC1.

Addition	Concentration (mM)	Relative activity (%)
No addition	None	100
Mg^{2+} ($MgCl_2$)	10.0	420
Ca^{2+} ($CaCl_2$)	10.0	98
Mn^{2+} ($MnCl_2$)	10.0	240
Zn^{2+} ($ZnCl_2$)	5.0	30
Fe^{3+} ($FeCl_3$)	5.0	120
Hg^{2+} ($HgCl_2$)	10.0	40
Ni^{2+} ($NiCl_2$)	10.0	51
Cu^{2+} ($CuCl_2$)	10.0	50
Cd^{2+} ($CdCl_2$)	1.0	78
EDTA	5.0	24
Na^{+} (NaCl)	1.0	110
K^{+} (KCl)	1.0	100
2-mercaptoethanol	0.5	112
Dithiothreitol	0.5	60
Sodium dodecyl sulfate	1.0	20
ATP	1.0-10.0	0
Urea	1.0	0
H_2O_2	1.0	0

SacC1 was purified from *S. cerevisiae* 215 fold with 20% recovery by ammonium sulphate precipitation, dialysis, and gel filtration using phosphocellulose, DEAE-cellulose and Sephadex G-100. The technique used for purification of SacC1 was very effective for removal of all interacting proteins. The purified fractions obtained from Sephadex G- 100 were able to break lambda DNA into smaller sizes, making this enzyme a restriction enzyme.

The enzyme has an optimal pH range (7.5-8.5) and is active at 37°C. Preliminary experiments showed its stability at room temperature for 30 min, but for 6 h at 4°C and more than three months at - 20°C. The enzyme is dependent on cations and Mg^{2+} (or Mn^{2+}) which increases its activity by 4- and 2- folds, respectively.

SacC1 shares similar characteristics with Sac I from *Streptomyces achromogenes* (Zhuravleva et al. 1987) as well as Sst1 from *Streptomyces Stanford* and Psp124B1 from *Pseudomonas* species. They all recognize 5'CTCGAC3' and have optimal temperatures of 37°C and optimal pH around 8.0. Sac I has a molecular weight of 50,000 while SacC1 has a molecular weight of 64,000. EcoRI has completely different characteristics from the above two enzymes (Tamerler et al., 2001; Dai, 2007).

Cleavage on both sides of the recognition sequence is a characteristic of Type IIB systems but all IIB enzymes studied so far have been found to recognize discontinuous (usually asymmetric) sites and a distinctive subunit/domain organization that is not present in the SacC1 enzyme. On the other hand, excision of small fragments by SacC1 bears some similarities to the action of *BcgI* (Kong et al., 1993) and *HaeIV* (Piekarowicz et al., 1999) belonging to Type IIB system. However, conversely to these enzymes, SacC1 recognizes non-interrupted sequences and ATP do not have any influence on its activity.

Type IIB enzymes excise DNA fragments by cutting

either side of their sites and some of them, especially *R. BplI* and *R. AloI* recognizes palindromic sequences (Roberts, 2005). Some of the Type IIB systems need no cofactor other than Mg^{2+} to cut DNA, like SacC1.

The characteristics of the cleavage reaction catalyzed by SacC1 are reminiscent of those already reported for other homing endonucleases belonging to the LAGLIDADG family (Belfort and Roberts, 1997; Wende et al., 1996). The similarities are a requirement of Mg^{2+} (or Mn^{2+}) for cleavage to take place, optimal activity at alkaline pH and stimulation of the reaction by moderate concentrations of the monovalent cation. SacC1 catalyzes, also, a double-strand cleavage in the vicinity of the insertion site, creating 3'-overhangs of 5 nt, a feature common to all the LAGLIDADG homing endonucleases studied so far (Jurica and Stoddard, 1999). The cleavage occurs 7 nt downstream from the insertion site for the coding and non- coding strands, respectively, a pattern also observed for *I-PorI* by Andersen et al. (1994).

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