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A novel restriction endonuclease *Bsu121* I from *Bacillus subtilis*

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Restriction endonucleases are molecular scissors that bind to specific sequences in DNA and cut within or adjacent to these sequences. The ability of type II restriction endonucleases to cleave DNA *in vitro* at their recognition sites has led to many applications in rDNA technology and genetic engineering. We have established the growth curve for *Bacillus subtilis* strain 121. Our data suggest that the bacterial growth saturation was observed at 16-20 h (0.62 O.D.) which is known late logarithmic phase. Cells were harvested at this phase for isolation of restriction endonuclease. A novel type II restriction endonuclease which we designated as *Bsu121* I has been isolated from grampositive *Bacillus subtilis* strain 121 and partially purified. The restriction endonuclease was isolated from cell extracts using step-wise purification through ammonium sulphate precipitation, followed by phosphocellulose column chromatography. SDS-PAGE profile showed denatured molecular weights (23 and 67 kDa) of the endonuclease. The partially purified enzyme restricted pBR322 DNA into two fragments of 3,200 and 1,700 bp. The endonuclease activity required Mg^{+2} as cofactor.

Key words : *Bacillus subtilis*, *Bsu121* I, pBR322 DNA, Type II restriction endonuclease

INTRODUCTION

Restriction endonucleases provide a range of site-specific tools for analysis, rearrangement, cloning and sequencing of DNA in molecular biology and genetic engineering. Type II restriction endonucleases recognize specific DNA sequences and cleave both strands of the DNA at fixed locations or near their recognition sites. Many restriction enzymes are dimeric proteins that recognize palindromic DNA sequences in symmetric fashion (Chandrasegaran, 2001; Halford, 2001). The sequences usually comprise 4 or 8 defined nucleotides, which can be continuous or interrupted, symmetric or asymmetric, unique or degenerate. They require Mg^{2+} as cofactor for cleavage activity and cleavage generally occurs on the 5' side of the phosphate, leaving DNA fragments with 5' phosphate and 3' hydroxyl termini (Pingoud and Jeltsch, 2001). The ability of the type II restriction endonucleases to cleave DNA *in vitro* at their recognition site(s) has lead to their use in many applications in analysis and manipulation of DNA. This has prompted extensive screening of bacteria for these enzymes by biochemical and by genome analysis (Kong *et al.*, 2000). During the last three decades since the discovery of restriction endonucleasea, several bacteria have been screened for site-specific endonucleolytic enzymes. Some bacteria contain more than 20 different restriction-modification systems. Currently 3514 type II enzymes have been identified, encompassing 238 different specificities according to available data, REBASE (http://rebase.neb.com). In many instances, the enzymes from different species cleave DNA at same sites (Roberts and Halford, 1993). Type II restriction-modification systems comprise pairs of enzymes with matching DNA sequence specificity. The modification enzyme is a DNA methyltransferase that specifically methylates either adenosyl or cytosyl residues within the recognition sequence, thus making DNA resistant to the restriction activity. The restriction enzyme is an endodeoxyribonuclease that cleaves unmethylated DNA at a precise location within or around the recognition sequence. As a consequence, foreign

double stranded DNA, unmethylated at the restriction sites recignized by the cell's restriction-modification systems, is quickly degraded (Rocha *et al.*, 2001). We describe here the isolation and partial purification of a novel restriction endonuclease *Bsu121*I from *Bacillus subtilis* strain 121 and its activity on pBR322 DNA.

MATERIALS AND METHODS

Biological materials

Bacillus subtilis strain 121 was obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India. pBR322 DNA, standard molecular markers were products of *Bangalore Genei*, India and 1 kb DNA ladder was from MBI Fermentas. The cells were grown aeronbically with shaking (130 cycles/sec) in incubator (orbitek-LE, Scigenics Biotech, India) at 30°C in a medium (pH 7.0) containing 16g/1 tryptone, 10g/1 yeast extract, 8g/1 beef exteact and 5g/1 Nacl, up to late logarithmic phase (16 h). The cells were collected by centrifugation and stored at –20°C until use.

Standard growth curve

250 µl of *Bacillus subtilis* culture was added to 50 ml of YBT medium and was incubated at 30°C for a period of 24h. Absorbance was measured at 600 nm for 24 h at an interval of two hours.

Purification of restriction endonuclease

All steps were carried at 4°C. Frozen cells (16 g wet weight) of a log phase culture as described above, were thawed in 100 ml buffer A (8 mM NaH_2PO_4, 1 mM EDTA, 15 mM Tris. C1, 10% glycerol, 5 mM M β-mercaptoethanol) containing 0.1 M NaCI and disrupted by sanitation (Bronson Sonifier II, USA) : 5 cycles of 2 min each with an interval of 5 min. After sonication, insoluble material was removed from crude extract by centrifugation (Hitachi CR26H, Japan) at 1200 g for 2 h.

The crude extract was precipitated using solid ammonium sulfate with constant stirring. Enzyme activity was found at 50%-80% saturation. The precipitate was suspended in 10 ml of buffer A and applied on to a phosphocellulose column (1.2 × 8 cm), pre-eqilibrated with buffer A, containing 0.1 to 1.0 M NaCI. Fractions of 5 ml were collected and assayed for endonuclease activity. The endonuclease was eluted at 0.1—0.2 M NaCI. Active fractions were pooled and was stored in 50% glycerol at –20 °C. The fractions were analyzed by 10% SDS-PAGE. Restriction endonuclease protein content was estimated by Bradford method (Bradford, 1976).

SDS-PAGE analysis

SDS-PAGE on 10% (m/v) polyacrylamide gels was conducted according to Laemmli (1970). Samples from crude extract, ammonium sulfate precipitation and phosphocellulose were solubilized in 2x-SDS sample buffer containing 62.5 mM Tris-HCI (pH 6.8), 5%(m/v) SDS, 1 mM PMSF, 2% (V/V) 2-mercaptoethanol, 10% sucrose and 0.01% bromophenol blue and heated at 70°C for 3 min. The apparent molecular masses of proteins were estimated by comparison with the mobility of standard proteins (*Bangalore Genei*, Bangalore, India) on the gel after SDS-PAGE. Proteins on the gel were visualized by coomassie Beilliant Blue staining following standard protocols (Sambrook *et al.*, 1989).

Endonuclease activity

The restriction eneonuclease activity was assayed in 20 µl reaction mixture containing 2 µl of pBR322 DNA (250 ug/ml) with final concentration of 5 µg/ml in reaction mixture and 2 µl of restriction buffer (10 mM Tris. HCI pH 7.5, 10 mM $MgCl_2$, 1mM Dithiotheritol, 0.1 mg/ml bovine serum albumin, 0.1 M NaCl). column samples (4 µl) were added to reaction mixture and incubated for 90 min at 37°C. The reaction was stopped by adding 2 µl of 10X loading dye, centrifuged for 30 sec and an aliquot (10 µl) from each solution was then electrophoresed in 0.7% agarose gel.

RESULTS AND DISCUSSION

Type II restriction endonucleases are indispensable tools in creating recombinant DNA molecules. The isolation and characterization of restriction enzymes have rapidly advanced during the past 30 years. Molecular biologists have isolated and

characterized novel restriction-modidication systems from different genera of bacteria. The search for endonucleases with hitherto unknown specificity and for analogs of already known enzymes has gained a lot of importance in recent years. Initially, we have investigated the growth curve of *Bacillus subtilis* (Fig. 1) under standard conditions as described in materials and methods section. Our data suggest that the growth saturation was observed at 0.62 O.D., which is known as stationary phase in the growth curve. The lag phase or initial growth was observed up to 6 h. The log phase or exponential phase started after 6 h of incubation. We observed a drastic increase in the growth rate up to 20 h. After 16 to 20 h of incubation, the stationary phase started in which, the growth rate was almost steady. In this phase the growth rate will stimulate the production of secondary metabolites, such as antibiotics.After prolonged stationary phase, death phase starts, when there is nutrient deficiency (Allen, 2000).

Table 1 : Partial Purification of *Bsu121*I (16g wet paste)

S. No.	Fraction	Total Protein (mg)
1.	Crude extract	1200
2.	Ammonium sulphate	576
3.	Phosphocellulose	192

Our main objective was to isolate novel restriction enzyme from *Bacillus subtilis* and determine its sites of restriction on pBR322 DNA. We have now standarsized a protocol for this purpose. According to suggested nomenclature (Nathans and Smith, 1973), we tentatively named this enzyme as *Bsu121*I. The *Bsu121*I enzyme was stable at 4°C. The *Bsu 121*I endonuclease at this partially level was found to be suitable for producing restriction digests. There is a rapid decrease in protein content with purification, which indicates that the purification fold of the enzyme has been increased (Table 1). Our data showed 1200 mg, 576 mg and 192 mg of protein content in 3 fractions of crude, ammonium sulphate and phosphocellulose fractions respectively (Table 1). Our study suggests that partial purification of the enzyme is better done with phosphocellulose. Our data also indicate the subunit molecular weights of partially purified enzyme are around 67 kDa and 23 kDa (Fig. 2).

A type II enzyme will cleave a supercoiled (SC) plasmid with one site first in one strand to give the open circle (OC) form of the DNA and then in the

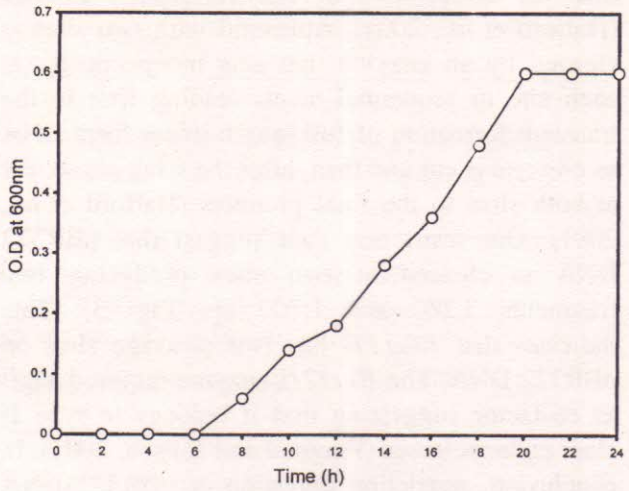

Fig. 1 : Growth curve for *Bacillus subtilis* strain 121.

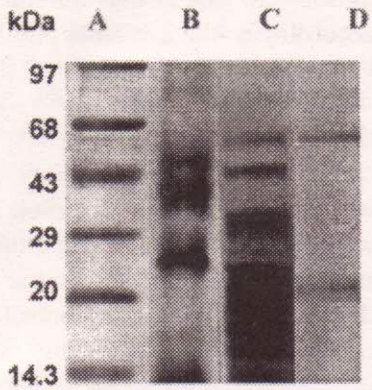

Fig. 2 : Protein profile (kDa) of restriction endonuclease from *Bacillis subtilis*. Protein was analyzed using 10% SDS-PAGE as described in materials and methods. Lane A (molecular marker); Lane B (Crude Extract); lane C (Ammonium sulfate fraction); Lane D (Phosphocellulise fraction).

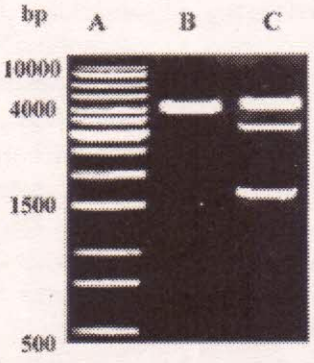

Fig. 3 : Profile of digested fragments of pBR322 DNA by restriction endonuclease isolated from *Bacillius subtilis* strain 121 on 0.7% agarose gel. Lane A (1kb molecular ladder); lsnr B (Unrestricted pBR322 DNA); Lane C (Restricted pBR322 DNA).

other strand, to give the full-length linear form (FLL). The cutting of both strands is often faster than the dissociation of enzyme from the DNA (Halford *et al.*, 2001). Aplasmid with two sites is cleaved by an enzyme that acts independently at each site in sequential steps, leading first to the transient formation of full length linear form DNA as one site is cut and then, after the a lag phase, cut at both sites to the final products (Halford *et al.*, 2001). Our restriction data suggest that pBR322 DNA is cleaced at two sites producing two fragments 3,200 and 1,700 bp (Fig. 3). This indicates that *Bsu121*I has two cleavage sites on pBR322 DNA. The *Bsu121*I enzyme required Mg^{2+} as co-factor suggesting that it belongs to type II class endonucleases (Pingoud and Jeltsch, 2001). In conclusion, restriction digestion of pBR322 DNA by partially purified enzyme suggested that it is a novel restriction enzyme from *Bacillus subtilis* strain 121 according to the available published data in REBASE (http://rebase.neb.com/).

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