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Comparative Analysis of Biochemical Properties of Site Specific Methyl-directed DNA Endonucleases BisI, BlsI and EcoBLI Recognizing 5’-G(5mC)NGC-3’/3’-CGN(5mC)G-5’.

VA Chernukhin*, DA Gonchar, MA Abdurashitov, EV Kileva, OA Belichenko, VS Dedkov, NA Mikhnenkova, SKh Degtyarev

SibEnzyme Ltd., Russia.

ABSTRACT

A comparative analysis of biochemical properties of three similar site specific methyl-directed DNA endonucleases BisI, BlsI and EcoBLI recognizing 5’-G(5mC)NGC-3’/3’-CGN(5mC)G-5’ has been done. Optimal concentrations of MgCl2, NaCl or KCl, pH of reaction buffers as well as the optimal reaction temperatures for these enzymes were determined. It was shown that BisI has the maximal activity in the buffer 10 mM Tris-HCl (pH 9.0 at 25^{0}C), 15 mM MgCl2, 150 mM KCl, 1 mM DTT. An optimal buffer for MD-endonuclease BlsI is 10 mM Tris-HCl (pH 8.0 at 25^{0}C), 15 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT. The enzyme EcoBLI displays a maximal activity in the buffer 10 mM Tris-HCl (pH 8.5 at 25^{0}C), 10 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT. Optimal temperature for all enzymes is 37^{0}C.

Keywords: methyl-directed DNA endonuclease, MD-endonuclease, C5-methylated DNA, endonuclease activity assay.

Abbreviations: 5mC – 5-methylcytosine, ENase – restriction endonuclease, MD-endonuclease – site specific methyl-directed DNA endonuclease, MTase – DNA Methyltransferase, ORF – open reading frame, u.a. – units of activity.

**Corresponding author*

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INTRODUCTION

Site specific 5mC-directed DNA endonucleases (MD-endonucleases) recognize and cleave DNA sequences with 5-methylcytosines, and don't cleave unmethylated DNA. About 15 prototypes of these enzymes with different recognition sites have been described during last ten years. Like restriction endonucleases MD-endonucleases are characterized both recognition sequence and a cleavage position. But unlike restriction enzymes the methylation pattern of recognition sites is very essential for activity of MD-endonucleases. This is well illustrated by the examples of endonucleases that recognize methylated sequence 5'-GCNGC-3'. Thus, among the enzymes with this recognition site and the cleavage position after the central nucleotide "N" BlsI effectively cleaves it in the presence therein of at least two 5-methylcytosines [1], while PkrI – only in the presence of three 5-methylcytosines [2]. Among the enzymes with cleavage position before the central nucleotide "N" MD-endonuclease BisI effectively cleaves the sequence 5'-GCNGC-3' when it contains at least two 5-methylcytosines [3] as well as a new MD-endonuclease EcoBLI [4], whereas GluI – in the presence of only four ones [5].

BisI was discovered the first among this group of enzymes [3]. A preliminary results on BisI digestion of methylated plasmids and synthetic deoxyoligonucleotide duplexes have shown this enzyme to cut DNA sequence 5'-G(5mC)^NGC-3'/3'-CGN^(5mC)G-5' as indicated by arrows. This site is the product of Fsp4HI MTase action resulting in the first cytosine occurs to be methylated in all sequences 5'-GCNGC-3' on both strands [6]. Recently a genome of Bacillus subtilis T30 – producer of BisI, has been determined and a gene coding BisI has been identified [7]. Last year a search of bisI gene homologues among the sequenced genomes of enterobacteria has revealed a highly homologous ORF from well-known strain Escherichia coli BL21 (DE3) (ACT 43858). It was shown this ORF (WP 001276099.1) to code MD-endonuclease named EcoBLI, an isoschizomer of BisI. The ecoBLI gene was amplified and cloned in E.coli ER2267, and recombinant enzyme preparation was obtained [4].
MD-endonuclease BlsI is a neoschizomer of BisI. It recognizes the same nucleotide sequence 5'-G(5mC)NGC-3'/3'-CGN(5mC)G-5', but unlike BisI, BlsI is the first site specific methyl-directed endonuclease that cleaves DNA with the formation of 3'-protruding ends. [1].

This article describes the results of experiments on comparative analysis of biochemical properties of three similar MD-endonucleases – BisI, BlsI and EcoBLI.

MATERIALS AND METHODS

Isolation of MD-endonuclease preparations:

BlsI and BisI MD-endonuclease preparations were isolated by the methods described before [1, 3]. EcoBLI enzyme preparation was purified from the recombinant E.coli strain producer, transformed with the plasmid carrying the cloned ecoBLI gene. One chromatographic step on Heparin-agarose ("Sigma", USA) was performed. All purification procedures were carried out at 0-4^{0}C. Frozen cell paste was thawed with 50 ml of Buffer A (10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 7 mM 2-mercaptoethanol) with addition of 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL Lysozyme. Then the cells were disrupted by sonication on the ultrasound desintegrator Soniprep 150 (MSE, UK) using five 1 min impulses with 1 min interval. Cell debris was removed by centrifugation at 15,000 rpm for 30 min in J2-21 centrifuge ("Beckman", USA). The supernatant was loaded onto Heparin-agarose column (20 ml) equilibrated with Buffer A with 0.2 M NaCl, and washed with two volumes of Buffer A containing 0.2 M NaCl. Adsorbed material was eluted with 500 mL of linear gradient (0.2-1 M NaCl) in Buffer A. The fractions containing a peak of EcoBLI activity were collected and dialyzed against 300 mL of Buffer A with 550 g/L Glycerol for 16 hours. The purified enzyme preparation was stored at -20^{0}C. EcoBLI activity assay in the chromatographic fractions was performed by addition of 2 µL aliquots from the fractions to 20 µL of reaction mixture containing 1 µg of pFsp4HI3 plasmid DNA [5] previously linearized with DriI restriction enzyme (pFsp4HI3/DriI) in SE-buffer «B» (10 mM Tris-HCl, (pH 7.6 at 25^{0}C), 10 mM $MgCl_2$, 1 mM DTT) for 1 hour at 37^{0}C. The reaction was stopped by addition 10 µL of 0.25 M Na-EDTA, pH 8.5, 500 g/L sucrose and 5 g/L bromphenol blue. The products of digestion were determined by electrophoresis in 1% agarose gel. The electrophoresis was carried out in 8 g/L of LE agarose ("Segenetic", Germany) in TAE buffer at 5 V/cm for 2.5 h as described [8]. The fluorescence of DNA was revealed on the Herolab GmbH device.

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Enzyme activities assay:

The analysis of enzymatic activity with BisI, BlsI and EcoBLI was performed by adding the aliquots of enzymes into 20 µL of reaction mixture containing 0.5 µg of pFsp4HI3/DriI plasmid DNA in different buffers and incubating the reaction mixture for 1 hour at different temperatures. The products of digestion were determined by electrophoresis in 1% agarose gel. In order to find out how effectively these three endonucleases can digest 5'-GCNGC-3' sequence with different number of 5-methylcytosine residues the pFsp4HI3/DriI cleavage pattern was analyzed after incubation with 4 separate aliquots of enzymes differing by 2 times (from 1 up to 1/8 µL). The enzyme preparations was diluted with the Buffer "B100" (10 mM Tris-HCl, (pH 7.6 at 25^{0}C), 50 mM KCl, 0.1 mM EDTA, 200 µg/mL BSA, 1 mM DTT , 500 g/L Glycerol).

All enzyme preparations, DNA, buffers, as well as molecular weight marker "1 kb DNA Ladder" used in the work, were from "SibEnzyme" (Russia).

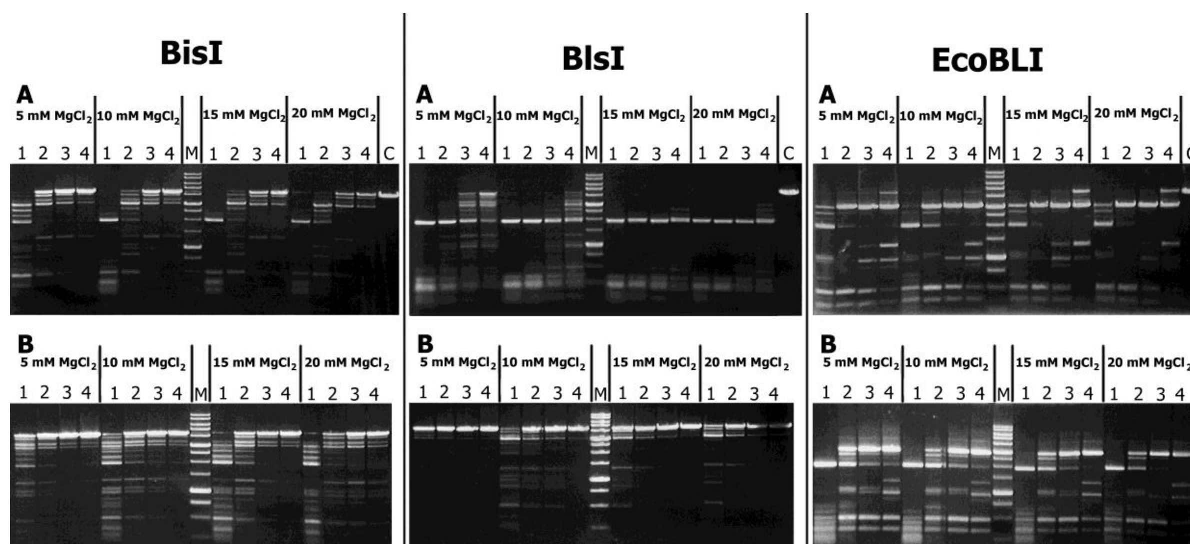
RESULTS AND DISCUSSION

To comprise a biochemical properties of three enzymes we have made the treatment of pFsp4HI3/DriI DNA with BisI, BlsI or EcoBLI enzyme preparations (1 u.a./µL of each). The pFsp4HI3 plasmid DNA comprises the gene coding Fsp4HI MTase resulting in the first cytosine occur to be methylated in all sequences 5'-GCNGC-3' on both strands. This plasmid also contains three hypermethylated DNA regions due to the presence of two or three overlapped Fsp4HI MTase recognition sites [5]. All reactions for BisI, EcoBLI and BlsI activity comparison were performed in 20 µL of reaction mixture, containing 0.5 µg pFsp4HI3/DriI DNA for 1 hour. The endonuclease activities were assayed at 4 different enzyme volumes: 1, 1/2, 1/4 and 1/8 µL. Each enzyme preparation was diluted with the Buffer "B100".

Determination of optimal $MgCl_2$ concentration

At the first optimization step the enzyme activities were analyzed at different $MgCl_2$ concentrations in the reaction mixture containing 10 mM Tris-HCl (pH 8.5 at 25^{0}C) at 37^{0}C. Mg^{2+} ion is the essential cofactor for the enzymatic activity and at the same time $MgCl_2$ is a reaction mixture component affecting on ionic strength of the solution. Therefore its optimum concentration was determined at two alternative NaCl concentrations (0 and 100 mM). The results are presented in Figure 1.

Figure 1: Dependence of BisI, BlsI and EcoBLI activities on MgCl2 concentration at two different NaCl concentrations. A – optimization at 100 mM NaCl; B – optimization without NaCl. Lanes: 1-4 – serial dilutions of the enzyme preparations, ranging from 1 µL (1 u.a.) to 1/8 µL; M – 1 kb DNA molecular weight marker; C – control DNA pFsp4HI3/DriI.



As can be seen from this figure the maximal activity of BisI and BlsI was shown at 15-20 mM $MgCl_2$ at high ionic strength while EcoBLI showed maximal activity at 10 mM $MgCl_2$ at low ionic strength. Further optimization experiments were carried out at 15 mM $MgCl_2$ for BisI and BlsI or at 10 mM $MgCl_2$ for EcoBLI.

Determination of optimal NaCl or KCl concentration

The analysis of the ionic strength influence was performed using two salts – NaCl and KCl in the reaction mixture containing 10 mM Tris-HCl (pH 8.5 at 25^0C), definite concentration of $MgCl_2$ at 37^0C. The results of three enzyme activities assay at different concentrations of NaCl and KCl are shown in Figure 2.

Figure 2: Dependence of BisI, BlsI and EcoBLI activities on NaCl and KCl concentrations. Lanes: 1-4 – serial dilutions of the enzyme preparations, ranging from 1 µL (1 u.a.) to 1/8 µL; M – 1 kb DNA molecular weight marker; C – control DNA pFsp4HI3/DriI.

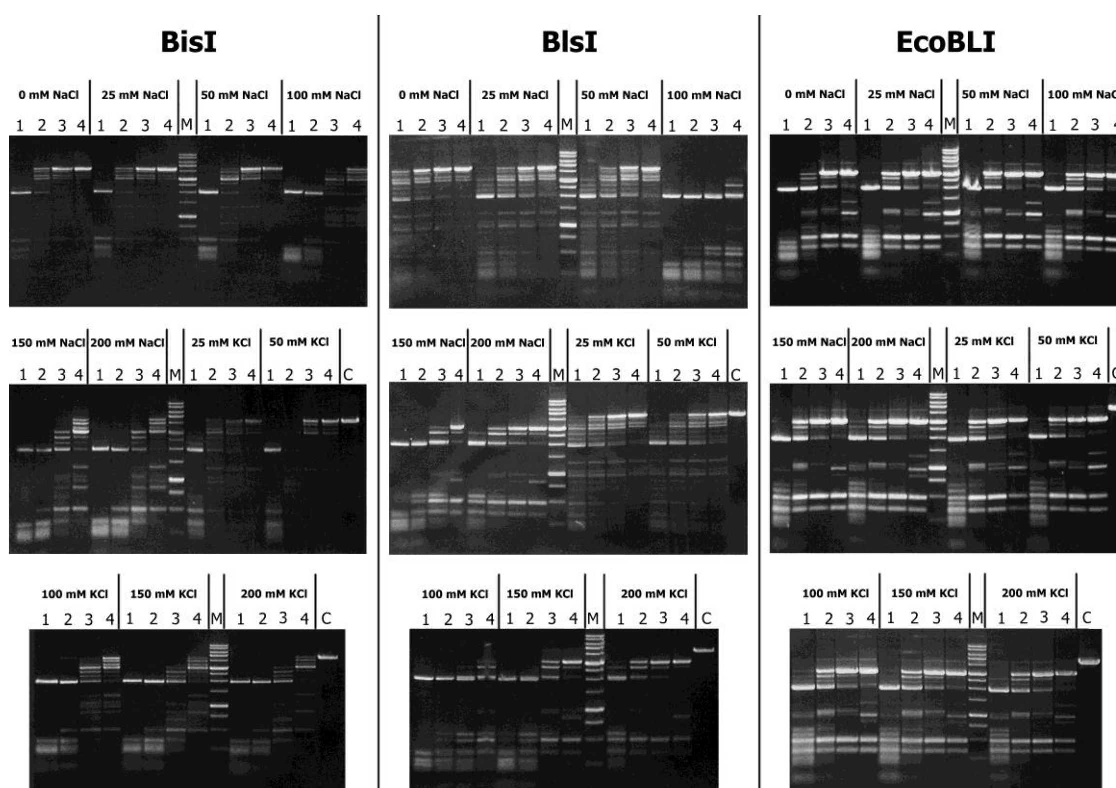


Figure 2 shows that the optimal NaCl or KCl concentration for BisI is 100-200 mM, for BlsI – 100-150 mM. Surprisingly, EcoBLI has revealed the maximal activity at 0-25 mM NaCl or KCl. Further optimization was carried out at 150 mM KCl for BisI, 100 mM NaCl for BlsI, and 25 mM NaCl for EcoBLI.

Determination of optimal pH value

The experiments on BisI, BlsI and EcoBLI activities dependence from pH value were performed at $MgCl_2$ and NaCl or KCl concentrations that were chosen in preliminary experiments. Four variants of Tris-HCl buffer with pH 7.5, 8.0, 8.5 or 9.0 and the incubation of the reaction mixtures at 37^0C for 1 hour were used. The results of pH value optimization are presented in Figure 3.

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Figure 3: Dependence of BisI, BlsI and EcoBLI activities on pH value. Lanes: 1-4 – serial dilutions of the enzyme preparations, ranging from 1 µL (1 u.a.) to 1/8 µL; M – 1 kb DNA molecular weight marker; C – control DNA pFsp4HI3/DriI.

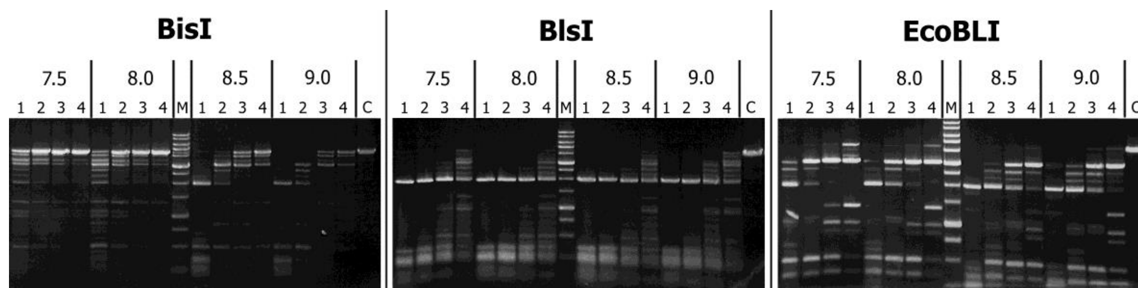
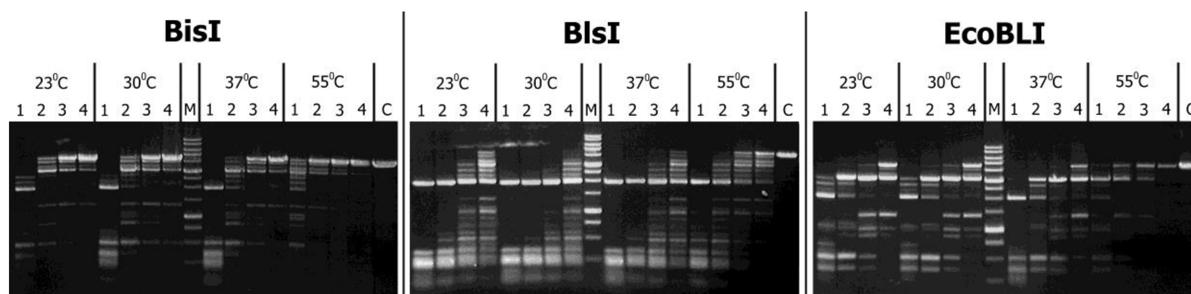


Figure 3 shows that the optimal pH value for BisI is 9.0, for BlsI – 8.0-8.5, for EcoBLI – 8.5. Further optimization was carried out at optimal pH value for each enzyme.

Determination of optimal temperature

To determine an optimal temperature for the reaction with BisI, BlsI and EcoBLI an incubation of the mixtures for 1 hour at 23, 30, 37 and 55^{0}C was performed. BisI preparation was analyzed in the buffer containing 10 mM Tris-HCl (pH 9.0 at 25^{0}C), 15 mM $MgCl_2$, 150 mM KCl, 1 mM DTT. BlsI was added to the reaction containing the buffer with 10 mM Tris-HCl (pH 8.0 at 25^{0}C), 15 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT. New enzyme EcoBLI was analyzed in the buffer containing 10 mM Tris-HCl (pH 8.5 at 25^{0}C), 10 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT. The results of temperature optimization are presented in Figure 4.

Figure 4: Dependence of BisI, BlsI and EcoBLI on temperature.
Lanes: 1-4 – serial dilutions of the enzyme preparations, ranging from 1 µL (1 u.a.) to 1/8 µL; M – 1 kb DNA molecular weight marker; C – control DNA pFsp4HI3/DriI.



As can be seen from this figure all studied enzymes have the maximal activity at 37^{0}C.

Thereby, the optimal physico-chemical parameters for maximal activities of BisI, BlsI and EcoBLI MD-endonucleases may be summarized in Table 1.

Table 1: Optimal conditions for the reaction with BisI, BlsI or EcoBLI

MD-endonuclease	$MgCl_2$ molar concentration, mM	NaCl or KCl molar concentration, mM	pH value	Temperature, ^{0}C
BisI	10-20	100-200	8.5-9.0	37
BlsI	15-20	100	8.0-8.5	37
EcoBLI	10	0-25	8.5-9.0	37

CONCLUSION

A comparative analysis of biochemical properties of three similar MD-endonucleases BisI, BlsI and EcoBLI has been performed. Optimal concentrations of $MgCl_2$, NaCl or KCl, pH values of reaction buffers as well

as the optimal reaction temperatures for these enzymes were chosen. BisI has revealed a peak of its activity in the buffer containing 10 mM Tris-HCl (pH 9.0 at 25^{0}C), 15 mM $MgCl_2$, 150 mM KCl, 1 mM DTT at the temperature 37^{0}C. These reaction conditions are almost identical to ones determined earlier [3]. MD-endonuclease BlsI has revealed a maximal activity in the buffer comprising 10 mM Tris-HCl (pH 8.0 at 25^{0}C), 15 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT at the temperature 37^{0}C. These conditions are sufficiently closed to ones determined earlier too [1], the differences were: 10 mM $MgCl_2$, pH 8.0 and temperature 30^{0}C. The optimal conditions for MD-endonuclease EcoBLI were defined first time: 10 mM Tris-HCl (pH 8.5 at 25^{0}C), 10 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT. Despite of its isoschizomers BisI and BlsI the new enzyme displays an optimum activity at low NaCl (or KCl) concentration. Remarkably newly found MD-endonuclease ElmI from enterobacteria with the same recognition site has similar to EcoBL optimal conditions [9]. The newly acquired information may be useful to scientists conducting experiments in the field of epigenetics, e.g. determination of DNA methylation pattern or study of mammalian DNA.

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