BceII, a new type IIS restriction endonuclease

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ABSTRACT

A new Type IIS restriction endonuclease was identified, partially purified and characterized from a *Bacillus cereus* subsp. *fluorescens* strain. The enzyme recognizes the nonpalindromic sequence ACGGC and cleaves at a distance from it. The cleavage appears to occur with a +1 basepair uncertainty. Thus the cleavage and recognition site is as shown below:

ACGGC(N) 11-13
TGCCG(N) 12-14

INTRODUCTION

Type II restriction endonucleases are important tools in recombinant DNA technology. As every enzyme with new specificity extends the possibilities of restructuring DNA at will, the search for new enzymes has been intensively pursued in the past decade and the list of these enzymes is permanently growing (1,2). One interesting subgroup of these enzymes has recently been named Type IIS (1). These enzymes have nonpalindromic recognition sequences with a well defined cleavage site outside the recognition sequence. In this paper we report the isolation and characterization of a Type IIS restriction endonuclease with a novel recognition and cleavage specificity.

MATERIALS AND METHODS

Cells were routinely grown in yeast-tryptone broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) at 37°C.

FokI was from New England Biolabs, HinfI was a gift of Dr. M. Hartmann (Jena), all other restriction endonucleases and T4 DNA ligase were prepared in this laboratory.

Sequencing was done by the Maxam-Gilbert technique (3).

alpha-³²P-dATP and gamma-³²P-ATP were from IZINTA (Budapest), Klenow-fragment and T4 polynucleotide kinase were purchased from New England

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Biolabs. End-labelling of DNA fragments was carried out as described in the handbook of Maniatis et al. (4).

Exonuclease contamination was tested by two methods. DNA was end-labeled either at the 5'-end or at the 3'-end with ^{32}P . It was then incubated with excess BceI enzyme under optimal conditions for 4 hours. The radioactivity of the DNA was then compared before and after incubation: 1. By precipitating the DNA with trichloroacetic acid and collecting and counting the insoluble radioactive material on glass-fiber filters. 2. By spotting an aliquot of the samples on PEI cellulose thin layer plates. The plates were chromatographed in 0.75 M KH_2PO_4 , and autoradiographed.

RESULTS

The bacterial strain producing the enzyme was identified as Bacillus cereus subsp. fluorescens by Dr.M.Konkoly-Thege (Budapest). It grows well at 37°C with vigorous shaking in yeast-tryptone broth with a doubling time of 25 min.

The BceI enzyme was purified as follows. Cells (50 g wet weight) were harvested in early stationary phase by centrifugation and disrupted by sonication (20 x 30 sec) after suspension in one volume of pH 7.5 Tris-HCl with 0.01 M 2-mercaptoethanol. The sonicated extract was clarified by 60 min ultracentrifugation with 35000 rpm in a Beckmann Ti60 rotor. The supernatant as adjusted to 1 M NaCl, and it was chromatographed on a 90 x 4 cm Bio-Gel A-0.5 m column equilibrated and eluted with 1 M NaCl, pH 8 0.02 M Tris-HCl, 0.01 M 2-mercaptoethanol. Active fractions were pooled, dialyzed against PC buffer (0.01 M pH 7.4 Na-phosphate, 0.1 mM EDTA, 0.01 M 2-mercaptoethanol, 5% glycerol) and applied to a 3 x 25 cm DEAE-cellulose column equilibrated with PC buffer. Proteins were eluted with a 2 x 0.5 liter gradient from 0 to 0.5 M NaCl in PC buffer. Active fractions, eluted between 0.15-0.20 M NaCl were pooled, dialyzed against PC buffer and applied to a 1.5 x 15 cm phosphocellulose column. This was developed with a 0 to 1 M NaCl gradient in PC buffer (2 x 100 ml). Active fractions, eluted between 0.5-0.7 M NaCl were pooled and after the addition of bovine serum albumin (final concentration 200 ug/ml) dialyzed against storage buffer (50 mM KCl, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol, 50 % glycerol). The enzyme at this stage was sufficiently pure for practical purposes. It gave a clear banding pattern after 10-fold overdigestion, and more than 90% of the resulting fragments could be ligated and recut with the enzyme. Further work to purify the enzyme to homogeneity is in progress in this laboratory. By this procedure

the approximate yield of the enzyme is 50 units/gram wet weight of cells.

In order to identify the recognition sequence, the BceFI enzyme was first tested on pBR322 plasmid DNA and it was found that this DNA was cleaved at three sites (Fig.1.). As the sizes of the resulting fragments did not correspond to the DNA fragments obtained by cleavage with any of the known restriction endonucleases (or with any possible combinations of them), it was recognized that BceFI represents a new specificity. The three cleavage sites were approximately localized by double digestions with BceFI, and BspI, HpaII, AluI, MboII, AvaII, SalI, HindIII, BamHI, BalI, PstI, BglI or FokI enzymes respectively, to between positions 600-620, 1150-1170 and 2950-2980 of the pBR322 sequence. The known sequences at and around these regions were then searched by computer for possible homologies. No common palindromic sequence was found but two possible 5 basepair homologous regions, CCTAA and ACGGC were identified as candidates for the recognition sequence. The entire sequence of bacteriophage lambda was then screened for the occurrence of these

Fig.1. Digestion patterns with BceFI

Electrophoresis on 2% agarose.

Lane 1: lambda DNA digested with BceFI

Lane 2: pBR322 " " " BceFI (predicted fragment-sizes: 1996, 1810, 557 bp)

Lane 3: pKLT1 DNA digested with BceFI (predicted fragment-sizes: 2091, 936, 740, 573, 208, 136, 129, 52 bp)

Lane 4: lambda DNA digested with BglI (molecular mass marker)

Fig.2. Determination of the BceI cleavage site: The relevant region of the pKLT1 DNA sequence.

two sequences. CTTAA occurs in lambda DNA only 18 times, and as the digestion of lambda with BflI results in many more fragments, this possibility was eliminated. The sequence ACGGC occurs in lambda DNA 115 times and the predicted fragment pattern seemed to be compatible with the experimental observation. This pattern however was obviously too complex for positive identification. Therefore two other plasmids, constructed in this laboratory, with known sequence, were also screened for the presence of the ACGGC sequence, and these two plasmids were also digested with BceI and the abovementioned other enzymes. pKLT1 was predicted to have 8, pTIL1 4 ACGGC sites. The results of the single and double digestion experiments showed perfect agreement with these predictions. Thus, on the basis of digestion patterns of four different DNA molecules, it could be concluded with confidence that the recognition sequence of BceI is ACGGC (or GCCGT), a new specificity. Fig. 1 illustrates some of these simple digestions with BceI).

From all the double digestion experiments described in the previous section, it could also be concluded that the cleavage site of BcefI, similarly to all restriction endonucleases with asymmetric recognition sequence, lies outside the recognition region, thus BcefI is a Type IIS enzyme. Moreover this cleavage site, similarly to that of the FokI or Tth111I cleavage sites, seemed to lie at a considerable distance from the recognition sequence. The accurate location of the cleavage site was determined as illustrated on Fig.2. Briefly: the 5 kb large plasmid pKLT1 was cleaved by HindIII (it cleaves at two sites at positions 504 and 1074) and the resulting larger fragment was labelled either at the 3'-ends by "filling in" with Klenow fragment and alpha-³²P-dATP, or at the 5'-ends with polynucleotide kinase and gamma-³²P-ATP. The two labelled DNA preparations were then digested with BcefI, the resulting small (less than 30 bp) HindIII-Bcef I fragments were isolated and run on a sequencing gel alongside a sequencing ladder of similarly labelled (i.e.: labelled only at the HindIII end, either 5', or 3') 270 bp long HindIII-HinfI fragments from the same plasmid DNA. As

Fig.3. Autoradiogram after Maxam-Gilbert sequencing of the HindIII-HinfI fragment

Lane 1: 3'-labelled HindIII-Bcefi fragment

Lanes 2-5: 3'-labelled HindIII-HinfI fragment. C+T, C, G+A, G

Lane 6: 5'-labelled HindIII-Bcefi "

Lanes 7-10: 5'-labelled HindIII-HinfI " C+T, C, G+A, G

the autoradiogram on Fig. 3. shows, contrary to expectations the cleavage with Bcefi produced several bands with either labelling.

We attributed this heterogeneity at first to exonuclease contamination. This possibility was ruled out however when we were unable to detect even traces of exonuclease by two different exonuclease tests in our Bcefi preparations (the results of one such test are shown on Fig. 4.). The

Fig.4. Exonuclease test

Autoradiogram showing the result of the exonuclease test described in Methods (2.)

Lane 1. 5'-end labelled DNA without incubation.

Lane 2. 5'-end labelled DNA incubated for 4 hours with excess BcefI

Lane 3. 3'-end labelled DNA without incubation.

Lane 4. 3'-end labelled DNA incubated for 4 hours with excess BcefI

appearance of 2-3 neighbouring bands was also independent from the amount of the enzyme and the duration of digestion in these experiments. Of course the ambiguity was not restricted to this single cleavage site. Fig 5. shows that cleavage of a different DNA also results in the generation of three fragments (52±1 bp) detectable even without end-labeling. Thus we had to conclude that the ambiguity of the cleavage site was an inherent property of our enzyme and its recognition and cleavage site should be characterized as:

ACGGC(N)₁₁₋₁₃

TGCCG(N)₁₂₋₁₄

The requirements of the enzyme were analyzed only so far as needed for the practical application. Thus it was established that it does not require Na or K ions for activity, the pH optimum is around 7.8, the enzyme requires Mg²⁺ ions (optimum 3 mM), and its temperature optimum is around 37°C.

DISCUSSION

The BcefI enzyme, described here, represents an entirely new recognition specificity. Although several restriction endonucleases have been described from various Bacillus cereus strains (5), only some of these have been characterized, and none of them are commercially available. In order to avoid confusion, we decided to name the new enzyme after the subspecies name (fluorescens) BcefI. The strain producing the new enzyme can be easily grown on conventional medium, the purification procedure is simple and straightforward, thus BcefI will probably be a useful new research tool in recombinant DNA technology.

An interesting property of the BcefI enzyme is the slight ambiguity of

Fig.5. Demonstration of the ambiguous cleavage with unlabelled DNA. pBR322 DNA was cleaved with HindII and BceII. The digest was run on a nondenaturing 12% polyacrylamide gel. The small fragment running at the position of the xylene cyanol dye is 52 ± 1 bp long.

the cleavage site. It seems that in this case the recognition is perfectly specific, but the cleavage site is only approximately determined, with a ± 1 basepair tolerance or "wobble". A partly similar behaviour of the HphI enzyme has been reported by Kleid et al. (6). The difference is that in the case of HphI the distance of the cutting site from the recognition site seems to depend on the particular sequence (but within one sequence it is definite). On the other hand, BceII seems to cut ambiguously within any particular context. As Fig.3. indicates, it seems likely (but not proven) that each cleaved molecule has 1 nucleotide long 5'-overhanging ends. Nevertheless, the

inaccuracy of the cleavage does not impair the possibility of ligation because all fragments could have only 4 different sticky ends and in any complex reaction mixture all possible ends are present with sufficiently large probability.

It is tempting to speculate that the BceFI enzyme represents an evolutionary "missing link" between the TypeII and TypeIII enzymes. It might be an interesting model to study the enzymology of sequence- specific DNA cleavage.

It must also be noted that recently new techniques have been published (7,8,9,10) that exploit the special features of the TypeIIS enzymes. Probably BfII can also be used for these purposes. It remains to be seen whether the observed ambiguity of cleavage is an advantage or a drawback for such applications.

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