OPTIMIZATION OF THE *Eco*RI*-ACTIVITY OF *Eco*RI ENDONUCLEASE

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Received 4 April 1978

1. Introduction

Type II restriction endonucleases, defined as those restriction endonucleases which cleave in their specific recognition sites, are widely used in analysis and manipulation of DNA molecules [1]. The *Eco*RI endonuclease recognizes the symmetrical sequence:

5′–G↓AATT–C–3′

3′–C–TTAA↑G–5′

and cleaves it a high ionic strength, and pH ~7.3 [2,3]. When ionic strength is lowered and pH adjusted to 8.5 the recognition specificity of the *Eco*RI endonuclease is reduced to the tetranucleotide sequence:

5′–N↓AATT–N–3′

3′–N–TTAA↑N–5′

The enzyme activity responsible for this substrate recognition is referred to as *Eco*RI*-activity [4]. Cleavage of DNA under the *Eco*RI* conditions described usually results in a number of partial digest products which reflect differential efficiencies of cleavage at different sites.

In analogy to *Eco*RI* it was also observed that *Bsu*I, which cleaves in the middle of the tetranucleotide sequence:

5′–GG↓CC–3′

3′–CC↑GG–5′

decreases its substrate specificity at high pH, low ionic strength and high glycerol concentration, predominantly to the:

5′–G↓C–3′

3′–C↑G–5′

sequences [5]. I have attempted to optimise the *Eco*R*-activity through lowering of the ionic strength of the medium and by the addition of organic solvents. This study is of interest in elucidating the mechanism of interaction between a nucleic acid sequence and a restriction endonuclease and furthermore for the evaluation of the useful range of incubation conditions for the practical application of restriction nucleases.

2. Materials and methods

2.1. *Chemicals*

All reagents were of certified grade and purchased from Merck, Darmstadt. Hydroxyapatite was prepared as in [6]. Lubrol (ethylenoxid condensate of fatty alcohols) was purchased from Sigma. DNA: *Col*E1-DNA and λ-DNA were prepared as in [7,8]. λ-dv1-DNA fragments digested with *Bsu*I were a gift from H. G. Zachau and M. Steinmetz.

2.2. *Restriction nucleases*

*Eco*RI was prepared by a modification of the procedure in [9]. Cells, 200 g wet wt, harvested in the late log phase of a 20 l fermenter were suspended in 600 ml buffer A (0.01 M K_2HPO_4–KH_2PO_4) pH 7, 7 mM 2-mercaptoethanol, 0.2 M NaCl, 0.1% Triton N101 and sonicated by a Branson sonifier to 1/4 of the starting A_{458}. Cellular debris was removed through

addition of 10% (w/v) solution of *N*-cetyl-*N,N,N*-trimethylammoniumbromide to final conc. 1% (v/v) and stirred 20 min at 4°C. After centrifugation at 25 000 × *g* at 2°C for 1 h the enzyme activity of the supernatant was purified according to [9] including the hydroxyapatite step. The endonuclease was stored with 50% glycerol at −20°C, without noticable loss of activity for a year. 1 μl contains 300 U normal *Eco*RI activity. 1 U is defined as a complete digestion of 1 μg λ-DNA in 1 h at 37°C. The yield is approx. 4×10^7 U / 200 g cells.

To test for unspecific nuclease activity [^{3}H]thymidine-labelled *Col*E1-DNA (8×10^3 cpm) prepared in absence of chloramphenicol *Eco*RI was incubated by an excess 3000 U for 3 h at 37°C. In an alkaline sucrose gradient the DNA sedimented as a sharp peak in the position of linear DNA and no smaller fragments were visible. In addition the enzyme activity was free of exonuclease activity as judged by the following criteria:

1. 1000 U *Eco*RI endonuclease were incubated with sheared ^{32}P-labelled chromosomal DNA (1×10^6 cpm/ug) 3 h at 37°C under *Eco*RI conditions and no radioactivity over the background could be detected as trichloroacetic acid-soluble material.

2. Linear *Col*E1-DNA obtained by treating with a 100-fold excess of *Eco*RI was converted to covalently-closed circles upon treatment with T4-ligase and DNA-ligase under the conditions in [10,11] (not shown).

*Bam*HI was prepared according to [12], *Hin*dIII according to [13] and *Sma*I according to our conditions. *Bsu*I was a gift from T. Trautner.

2.3. *Digestion of DNA, enzyme assays, gel electrophoresis*

Digestion with *Eco*RI* was carried out by the following procedure: to 20 μl *Col*E1-DNA (1 μg) were added 20 μl 250 mM Tris–HCl pH 8.5, 20 mM $MgCl_2$ buffer, 2 μl 10% (w/v) Lubrol and different volumes of organic solvents and H_2O to final vol. 100 μl and 3 μl *Eco*RI and incubated at 37°C. Standard conditions of DNA digestion with *Bam*HI, *Hin*dIII, *Sma*I, *Bsu*I have been described [14]. Electrophoresis of DNA fragments was performed in 5% (w/v) acrylamide–0.25% (w/v) bisacrylamide gel in a vertical electrophoresis apparatus using 0.09 mM Tris, 2.5 mM EDTA, 90 mM H_3BO_3, pH 8.3, buffer with 150 mA, 105 min at 4°C. Gels were soaked in ethidium bromide (1 μg/ml) and photographed under ultraviolet light.

3. Results

3.1. *Optimization of EcoRI* activity*

The *Eco*RI activity itself cleaves *Col*E1-DNA once to give linear DNA and the smaller fragments are the result of the *Eco*RI* activity. After addition of ethylene glycol from 38–58% (v/v) the reaction can be optimized. The optimum pH for the *Eco*RI* activity after addition of ethylene glycol was determined from pH 7.5–9.5. The activity was maximal at pH 8.0–8.5. The *Eco*RI* endonucleolytic digestion of *Col*E1-DNA results in a complex fragment pattern. To examine the influence of ethylene glycol concentration, different amounts of ethylene glycol were added to the samples and incubated 16 h at 37°C. With the lowest concentration of 38% (v/v) ethylene glycol following fragments of *Col*E1-DNA are obtained, as shown in fig.1f–k: With the highest ethylene glycol concentration of 58% (v/v) fragment B with 8.9×10^5

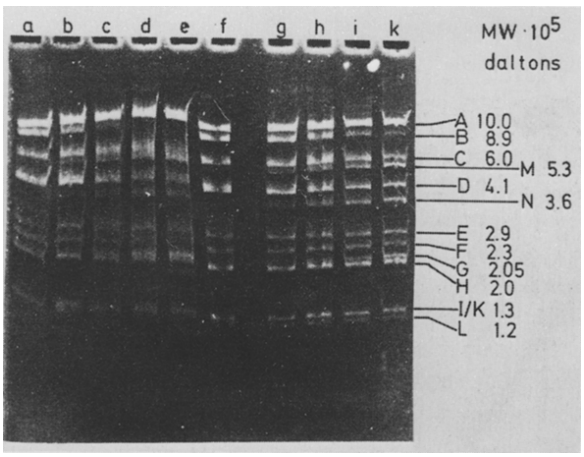

Fig.1. Electrophoresis of *Eco*RI* cut 1 μg *Col*E1-DNA for 16 h at 37°C in a 5% acrylamide slab gel under various concentrations of glycerol: (a) 40 μl; (b) 50 μl; (c) 60 μl; (d) 70 μl; (e) 100 μl; and of ethylene glycol: (f) 40 μl; (g) 50 μl; (h) 60 μl; (i) 70 μl; (k) 100 μl.

disappears and new fragments M with 5.3×10^5 and N with 3.6×10^5 appear as shown in fig.1f–k. The molecular weights of the fragments are calculated with λ-dvl-DNA digested with *Bsu*I as marker ($\times 10^6$): 11.19; 8.76; 5.88; 3.46; 3.0; 2.31; 1.73; 1.43; 1.37; 1.15; 0.95; 0.86; 0.53.

The sum of the molecular weights of the fragments of *Col*E1-DNA, obtained after 16 h incubation, is approx. 4.2×10^6, which is in correspondence with an estimated size of *Col*E1 of 4.2×10^6 [15].

To examine the time course of *Eco*RI* digestion of *Col*E1-DNA under the optimal ethylene glycol concentration 58% (v/v) samples were incubated for different times with a fixed enzyme concentration. After incubation for 1 h no linear form was detectable, and all fragments present in the limit digest are visible as well as some partial digest fragments. After 16 h incubation a limit digestion of *Col*E1-DNA was obtained in which the fragments are identical in number and size to those shown in fig.1f–k (fig.2).

The kinetics and the concentration dependency of ethylene glycol show, that a limit digest is obtained after 16 h. It seems that some cleavage sites are much more resistant to cleave than others, as also found [4].

In addition to the effect of ethylene glycol the influence of other organic compounds, which lower the ionic strength of the medium, was examined.

Glycerol also shows an activating effect. The number of DNA fragments, which were obtained in tests

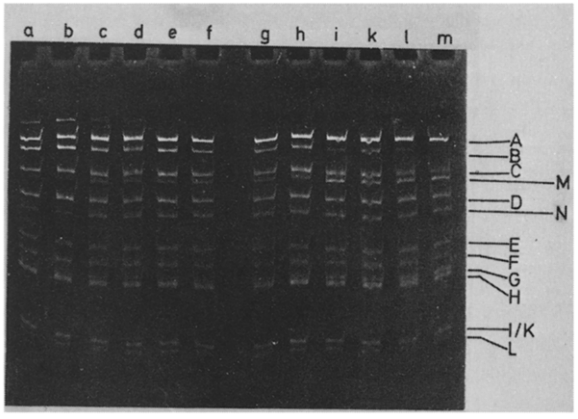

Fig.2. Electrophoresis of *Eco*RI* cut of 1 μg *Col*E1-DNA under optimal ethylene glycol concentration (100 μl) for different times at 37°C in 5% acrylamide slab gel: (a,b) 30 min; (c,d) 1 h; (e,f) 2 h; (g,h) 3 h; (i,k) 5 h; (l,m) 16 h.

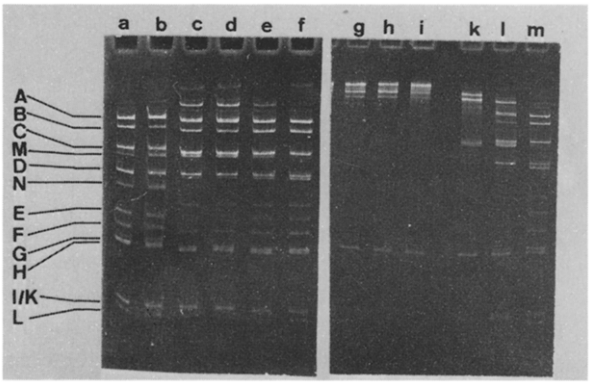

Fig.3. Electrophoresis of *Eco*RI* cut of 1 μg *Col*E1-DNA with various concentrations of different organic solvents for 16 h at 37°C in a 5% acrylamide slab gel: (a) 70 μl; (b) 100 μl ethylene glycol; (c,d) 20 μl; (e,f) 100 μl thriethylene glycol; (g) 1 μl; (h) 5 μl; (i) 10 μl; (k) 20 μl; (l) 50 μl; (m) 100 μl dimethylsulfoxide.

of the kinetic and concentration dependency is the same as that obtained with ethylene glycol. The 16 h digest with the highest glycerol concentration also lead to the digestion of the B fragment and the production of the two fragments D and F (fig.1a–e).

With triethylene glycol, dimethylsulfoxide a complete digest of *Col*E1 could not obtained, as shown in fig.3. By addition of formamide, *N*-methylformamide, pyrrolidone-(2), 1-methylpyrrolidone-(2) the same uncomplete digestion patterns as found with DMSO can be observed.

To test ethylene glycol and glycerol as a general effector of reduced specificity for restriction endonucleases these organic solvents were added to incubations with the enzymes *Bam*HI, *Hin*dIII and *Sma*I, at pH 7.5 and pH 8.5. Using λ-DNA, no alteration in the specificity was found to be effected.

4. Discussion

The data presented demonstrate that *Eco*RI* activity can be optimized by lowering the ionic strength of the solution. One can speculate that the splitting of the DNA by the 'star' activity is a property of *Eco*RI itself. Since it has been demonstrated in a number of systems that polar organic compounds can exert an effect on protein–DNA interactions, polar organic

compounds, including DMSO, increase RNA synthesis in vivo [16] and in vitro [17]. An optimization effect has been found with *Bsu*I, which can be induced to recognise the dinucleotide instead of the normal tetranucleotide sequence at the cleavage site [5]. From the observation, that other restriction nucleases, including the isoschizomer *Hae*III of *Bsu*I do not show any activating effect on the presence of polar organic compounds, one can conclude that the effect on *Bsu*I is specific for the enzyme molecule. *Eco*RI is known to be a tetramer molecule [18,19], which is in equilibrium with a dimer form. The interaction forces of the subunits could be weakened by organic compounds and influence the specificity.

The catalytic sites of depolymerizing enzymes have been suggested [20] to be constructed of subunits, recognising a series of adjoining residues on the polymer chain. The finding of altered kinetics at the recognition sites on the DNA and especially for reduced specificity of *Eco*RI and *Bsu*I would favour this suggestion.

The findings here demonstrate that, by optimization of incubation conditions, reproducible limit digests can be obtained for *Eco*RI* activity. This finding is essential for the further direct application of this activity to mapping DNA sequences and for gene cloning. Previous studies have implied that only partial digests could be produced [4] or that the *Eco*RI' activity was only an intermediate loss of specificity in a chain ending finally with recognition of PuPuA$\downarrow$TPyPy sequences [21]. Recently it was shown that Mn^{2+} in place of Mg^{2+} also stimulates the *Eco*RI activity but apparently not to such a great extent as ethylene glycol [22].

Acknowledgements

I thank M. Melzer and W. Westphal for expert technical assistance and J. Collins for helpful discussions and critical reading of the manuscript. I thank H. Schütte for his help in purifying the *Eco*RI enzyme to a very high degree.

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