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A spectroscopic method to determine the activity of the restriction endonuclease EcoRV that involves a single reaction

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Abstract

A one-step protocol is presented to determine the activity of EcoRV as a model of restriction enzymes. The protocol involved a molecular beacon as DNA substrate, with the target sequence recognized by EcoRV in the stem. EcoRV cleaved the stem forming two fragments one of which contained the fluorophore and quencher, initially bound by three base-pairs. This shorter fragment rapidly dissociated at 37 °C causing an increase of fluorescence intensity, which was used to gauge the reaction kinetics. The reaction can be described using the Michaelis-Menten mechanism and the kinetic parameters obtained were compared to literature values involving other protocols.

Keywords

Molecular beacon; restriction endonuclease; fluorescence; protein-DNA interaction

The mechanism whereby endonuclease EcoRV cleaves double stranded DNA involves non-specific binding of the enzyme to any sequence of DNA followed by one-dimensional diffusion along the DNA molecule until the GAT[^]ATC target sequence is found [1]. When EcoRV encounters its cognate sequence it binds Mg²⁺ with high affinity and this is accompanied with a large DNA distortion, followed by the hydrolysis of the phosphodiester bond [1-4]. The activity of the enzyme decreases several orders of magnitudes when other divalent metal ions (e.g., Co²⁺, Mn²⁺, and Cd²⁺) are employed as cofactors [4] or when there is at least one base mismatch in the cognate sequence [5]. In vitro experiments have shown that the cutting rate by restriction enzyme increases in proportion with the length of the oligonucleotides that contain the target sequence [6]. However, this antenna effect may not be that important in live processes because numerous proteins involved in DNA metabolism may impede the diffusion of the restriction enzymes [7].

In spite that the most widely used assays to determine the activity of restriction enzyme are gel electrophoresis, filter binding, high-performance liquid chromatography (HPLC) [8], and enzyme-linked immunosorbent assay (ELISA) [9, 10], such essays have the drawback that are time consuming, expensive and discontinuous. Therefore, spectroscopic methods have

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emerged as an alternative to circumvent some of the limitations mentioned. For example, Ma et al. [11] developed an elegant protocol to determine the activity of *RsaI* endonuclease with high efficiency and sensitivity (up to 1.0 U/ml). Their method involved the preparation of an oligonucleotide that served as a substrate for *RsaI*. Then, one strand of one of the fragments produced during the reaction catalyzed by *RsaI* was hybridized to the loop sequence of a molecular beacon (MB), which served as primer for a DNA polymerase. The basic concept was to form a double stranded region in the loop region of the MB to favors the open form of the MB, which was accompanied with an increase in the fluorescence intensity.

In this work, we present a straightforward spectroscopic method to evaluate the activity of EcoRV as model of restriction enzymes. The reaction of interest is [12]

where E denotes the restriction endonuclease EcoRV, and P₁ stands for the product that contains the loop portion and half of the original stem and P₂ the product that has the fluorophore and the quencher bound to it. The rate constants k_2 and k_3 describe the phosphodiester hydrolysis and product release, respectively. Reaction 1 is schematically represented in Scheme 1. The DNA substrate is a molecular beacon that contains the palindromic sequence recognized by EcoRV in the stem portion. The MB is cut in two portions upon adding EcoRV in the presence of Mg²⁺. P₂ consists of two fragments, held together by three base pairs of the original stem, one of which has the fluorophore bound to it and the other the quencher. Owing to the low stability associated to the interaction of three base pairs at 37 °C, P₂ dissociates into the strand that contains the fluorophore and the strand that contains the quencher. Because the fluorophore is no longer forced to interact with the quencher, the dissociation process is accompanied with an increase in the fluorescence Intensity.

In our analysis, we employed the steady-state initial reaction conditions, and made the following assumptions: (i) under the reaction conditions that we employed the MB substrate exists mainly in the closed form. (ii) The three-bp product (P₂) that contains the fluorophore-quencher pair dissociates immediately after its formation producing the free acceptor and the quencher.

According to reaction (1), the Michaelis constant and turnover number are

$$K_m = \frac{k_3(k_2 + k_{-1})}{k_1(k_2 + k_3)} \quad (2)$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3}$$

Below we present the experimental details. Molecular beacon oligo #1: 5'(HEX)(C6-NH) GAT ATC GTC GTTTT TTT TTT TTC GAC GAT ATC (DabcyI) 3' phosphodiester DNA, oligo #2: 5'(HEX)(C6-NH) GAT ATC GTT TTT TTT TTT TTC GAT ATC (DabcyI) 3' phosphodiester DNA, PAGE/HPLC purified, were synthesized by Trilink Biotechnologies, Inc. (San Diego, CA). Molecular beacon 5'(HEXTM) CCAA (T)₁₆

TTGGG (Dabcyl) 3' were purchased from Integrated DNA Technologies (Coralville, IA). Enzymes EcoRV (20,000U/ml, supplied in 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 200 ug/ml BSA and 50% glycerol) and EcoRI (20,000U/ml, supplied in 300 mM NaCl, 10 mM KPO₄ (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.15% Triton X-100, 200 ug/ml BSA and 50% glycerol) were purchased from New England Biolabs (Ipswich, MA). Buffer conditions. For EcoRV: 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9 at 25°C, 100 ug/ml BSA; for EcoRI: 50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100, pH 7.5 at 25°C. All chemicals were analytical reagents and were used without further purification. Deionized-distilled water was used to prepare all solutions. All the kinetic studies were done at 37°C in a volume of 400 µl. The fluorescence studies were carried out in a Varian Cary 50 fluorometer. The fluorescence recording normally started with the addition of enzymes. The fluorophore HEX was excited at 535 nm and the emission was observed at 552 nm. We measured the MB's melting temperature to be 67°C for oligo #1 and 53°C for oligo #2 at the applied buffer condition by monitoring fluorescence intensity at 552 nm (data not shown).

This work consists of two main experiments. Figure (1A) presents the time courses of reaction 1 changing the substrate concentration from 0.02 to 2.0 µM, while keeping fixed concentration of EcoRV. The concentration of the enzyme was 100 U/ml (about 1 nM), which was much lower than the concentration of substrate MB. It was observed that the increase in the MB concentration is accompanied with an increase in fluorescence intensity. Figure (1B) displays the observed reaction rate versus substrate MB concentration. In reaction 1, it is assumed that the product released is the rate-limiting step, which may be related to k_3 and thus is proportional to the fluorescence intensity change. Using a simple Michaelis-Menten mechanism, we have $K_m \approx 0.5 \mu\text{M}$ for both MB oligos, and $k_{cat} \approx 20 \text{ min}^{-1}$ for oligo#1 and $k_{cat} \approx 1.5 \text{ min}^{-1}$ for oligo#2, respectively, assuming $k_2 \approx k_3$ [12, 13].

The second experiment explored the dependence of reaction 1 on the EcoRV concentrations. This is illustrated in Figure (2A) in which the enzyme concentration was increased from 5 U/ml to 250 U/mL, keeping fixed the MB concentration ($[\text{MB}] \approx 0.1 \mu\text{M}$). It is apparent from Figure 2B that the increase in $[\text{EcoRV}]$ is accompanied with an increase in the fluorescence intensity. To establish a detection limit we tested that we could still observe the fluorescence change for EcoRV at 1 U/ml (data not shown).

We carried out the following control experiments: (i) observed fluorescence from the MB substrate in solution without adding the enzyme for a time period equivalent to that used to study reaction 1, (ii) carried out reaction 1 adding EcoRI (target sequence GAT[^]ATC) instead of EcoRV, and (iii) added EcoRV to a MB substrate that does not have the sequence recognized by EcoRV, but with the same loop sequence. In the three cases no reaction was observed confirming the high specificity of EcoRV toward the target sequence located in the stem of MB. Furthermore, we performed HPLC experiment that confirm that MB is cleaved by EcoRV (data not shown).

It is interesting to note that the value of K_m (0.5 µM) reported here using a 7 bp-stem MB and 10-bp stem MB is similar to the literature value (0.6 µM) obtained employing a 12 bp oligonucleotide, using the same buffer conditions ($[\text{MgCl}_2]=10 \text{ mM}$, $[\text{NaCl}]=100 \text{ mM}$)[12].

This indicates that EcoRV can take substrates of 7 bp (the length of the stem of the MB), a number of bp that coincides with the length of the cognate sequence of the enzyme, but is shorter than 10, which is the number of bp required to complete a helix turn. Therefore, we can argue that this 7 bp-stem acquires a secondary structure that the enzyme binds to and cleaves.

In the literature there are reports that involved dsDNA substrates of 10-12 bp or longer [1, 12, 14]. In part, this is because it is known that pieces of DNA of this length form well-defined secondary structures. Another important piece of information is that the crystallographic diameter of EcoRV is about the length of a fragment of DNA of about 15 bp [15]. Considering these two facts together, it is then difficult to conceive that EcoRV can take a substrate with a length of 7 bp. The magnitude of catalytic constant is another source of information. Consider the following information. The value that we obtained for k_{cat} (1.5 min^{-1}) for the 7-bp stem MB is much smaller than k_{cat} (20 min^{-1}) for the 10-bp stem MB, which in turn is smaller than that reported in the literature for an oligonucleotide (33 min^{-1}) of 12 bp [14]. This means that the cleavage rates increase as the double stranded DNA region increases, which may be attributed to the larger cross-section for non-specific binding of the longer DNA substrates. In contrast, it is not evident why the magnitude of k_{cat} determined for 7-bp stem MB in the present work is similar to that reported for a DNA plasmid (0.9 min^{-1}) [5, 7]. A plausible explanation would be that proteins bound to the DNA molecule in the plasmid environment restrict the one-dimensional diffusion of EcoRV, which means that the enzyme has to bind directly to the target sequence, as it is the case for the MB substrate.

In summary, we present a straightforward fluorescence assay to evaluate the EcoRV activity that involves only one reaction. To the best of our knowledge, this is the first report of a reaction catalyzed by restriction enzymes in which the length of dsDNA substrate is equal to the target sequence recognized by the enzyme. While the experiments here reported suggest that the method may be directly extended to develop activity assays for other restriction enzymes, this must be confirmed experimentally.

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Abbreviations used

MB	molecular beacon
HPLC	high-performance liquid chromatography
ELISA	enzyme-linked immunosorbent assay
EDTA	diaminoethanetetraacetic acid
dsDNA	double-stranded DNA
bp	base-pair

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Scheme 1.

Schematic representation of process designed to measure the activity of EcoRV using designed MB. After the cleavage of the stem the fluorophore and quencher separate, resulting in an increase on the fluorescence intensity.

Figure 1. (A) Real-time monitoring of the EcoRV endonuclease activity using MB oligo #1 and #2, keeping fixed [EcoRV] at 100U/mL, but varied substrate concentration. (B) Plot of the observed reaction rate versus substrate MB concentration, showing the typical Michaelis-Menten kinetics for both MB oligomers.

Figure 2.

(A) Real-time monitoring of the EcoRV endonuclease activity using both MB oligo #1 and #2, maintaining fixed [MB] = 0.5 μ M, but varied enzyme concentration. (B) Plot of the observed reaction rate versus endonuclease EcoRV concentration, showing a good linear correlation for both MB oligomers.