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Journal of Alloys and Compounds 408–412 (2006) 391–395

Journal of ALLOYS AND COMPOUNDS

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Application of cerium(IV)/EDTA complex for future biotechnology

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Received 31 July 2004; received in revised form 14 December 2004; accepted 14 December 2004
Available online 17 June 2005

Abstract

A new artificial system for site-selective hydrolysis of single-stranded DNAs was prepared. By using two oligonucleotide additives that bear a monophosphate group at the termini, gap structures were formed at predetermined positions in substrate DNA. The phosphodiester linkages in the gap were efficiently and selectively hydrolyzed by Ce(IV)/EDTA complex (EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate) at pH 7.0 and 37 °C. Furthermore, the fragments formed by the site-selective scission were connected with various oligonucleotides by using T4 DNA ligase, producing desired recombinant DNAs. A new tool for manipulation of single-stranded DNA in biotechnology has been successfully obtained.

Keywords: DNA; Cerium; Artificial restriction enzyme; Hydrolysis

1. Introduction

Site-selective hydrolysis of DNA has been one of the most attractive and challenging themes for chemists and biochemists [1–4]. Versatile applications to biotechnology and molecular biology have been proposed. In current biotechnology, the DNAs of bacteria and viruses are manipulated by use of natural restriction enzymes. For the manipulation of huge DNA of higher animals and plants, however, the sequence selectivity of them is insufficient (most of them recognize a specific sequence of four or six bases). Therefore, artificial restriction enzymes, which show far higher sequence-specificity than naturally occurring ones, are required.

Theses artificial enzymes are generally prepared by hybridizing catalytic residue for scission of DNA with sequence recognizing residue (Fig. 1). Their site-selectivity originates primarily from favorable activation-entropy term for the hydrolysis of target phosphodiester linkage. Various natural and synthetic compounds, which selectively bind to specific sequence in DNA, are now available. However, phosphodiester linkages are extremely resistant to hydrolysis (the half-life of the linkage is estimated to be 200 million years under physiological conditions) [5], and the molecular design of the catalytic site is quite difficult.

About 10 years ago, non-enzymatic DNA hydrolysis was for the first time accomplished by use of Ce(IV) [6–8]. The DNA scission proceeds totally via hydrolytic pathway without the contribution of oxidative scission. The acceleration by Ce(IV) is as large as 10^{11}-fold, and this large catalytic activity is specific to Ce(IV) [9,10]. At pH 7.0 and 50 °C, the half-life of the phosphdiester linkage in DNA is reduced to a few hours. The hydrolytic scission fragments of DNA could be transformed into substrate for natural enzymes so that the matching between Ce(IV) and the current biotechnology is straightforward [11].

However, the Ce(IV) ions readily form metal hydroxide gel at physiological pH, and this imposes a significant limitation to the scope of application of Ce(IV). Recently, we found that the complex of Ce(IV) with EDTA is homogeneous at pH 7 and active for DNA hydrolysis [12]. Here we report that the

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doi:10.1016/j.jallcom.2004.12.075

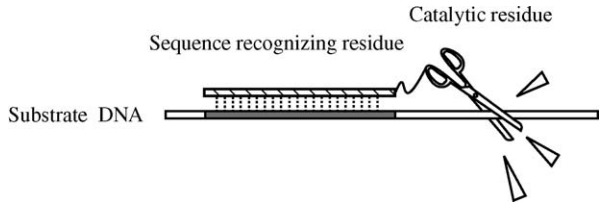
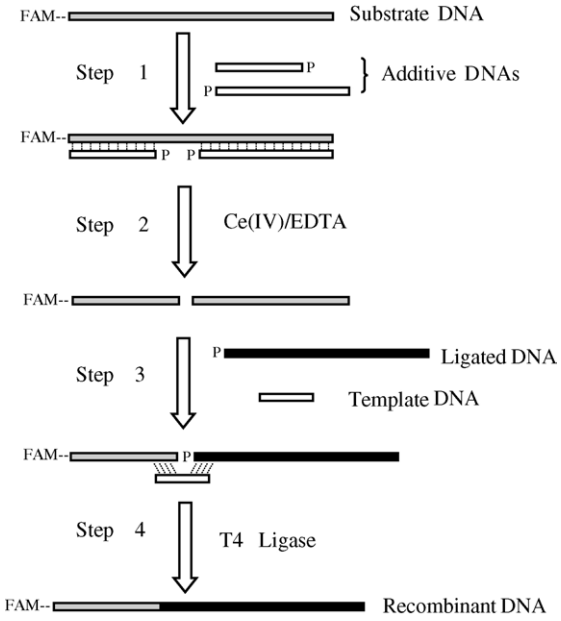


Fig. 1. Traditional molecular design of artificial restriction enzymes.

catalytic activity of Ce(IV)/EDTA complex is strongly dependent on the substrate DNA structure (Fig. 2). Single-stranded portions of the substrate DNA are selectively hydrolyzed by the complex [13,14]. Both the scission efficiency and the selectivity are increased by monophosphate groups located near the single-stranded portion [15,16]. Furthermore, the application of the selective scission to manipulation of single-stranded DNA is also reported (Scheme 1).



Scheme 1. Strategy for manipulation of single-stranded DNA.

2. Experimental methods

2.1. Materials

All the phosphoramidite monomers were purchased from Glen Research Co. The oligonucleotides were prepared on an automated synthesizer, purified by usual methods, and completely characterized by MALDI-TOF MS. Water was deionized by MILLIPORE WATER PURIFICATION SYSTEM and sterilized by an autoclave immediately before use. Commercially obtainable $Ce(NH_4)_2(NO_3)_6$ (from NACALAI TESQUE) and EDTA·4Na (from TOKYO KASEI KOGYO) were used without further purification. Homogeneous Ce(IV)/EDTA complex was prepared immediately before use by mixing equimolar amounts of $Ce(NH_4)_2(NO_3)_6$ and EDTA (4Na salt) in Hepes buffer.

2.2. DNA hydrolysis

The hydrolysis of DNA substrate (^{32}P-labelled at the 5′-end using T4 polynucleotide kinase) was initiated by adding the solution of Ce(IV)/EDTA complex to the reaction mixtures, and carried out at pH 7.0 (Hepes buffer) and 37 °C. After a predetermined time, the reactions were

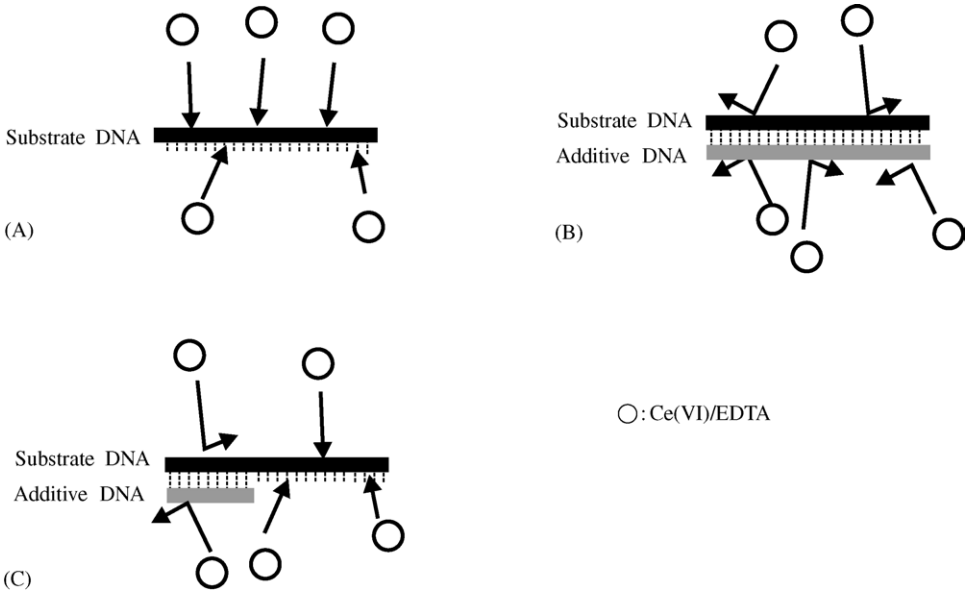


Fig. 2. Hydrolysis of single-stranded DNA and double-stranded DNA by homogeneous solution of Ce(IV)/EDTA complex.

stopped, and the mixtures were then analyzed by denaturing 20% polyacrylamide gel electrophoresis. The scission fragments were quantified with a Fuji Film FLA-3000G imaging analyzer.

2.3. Manipulation of single-stranded DNA

The ligated DNA having 5′-terminal monophosphate and the template DNA were synthesized on an automated synthesizer. The substrate single-stranded DNA was labeled with fluorescein at the 5′-end and treated with Ce(IV)/EDTA in the presence of additive DNAs. The reaction mixtures were subjected to polyacrylamide gel electrophoresis, and the desired fragments were collected. The products were purified by ethanol precipitation, and dissolved in water. To this solution, T4 DNA ligase and the ligation buffer (both are from DNA Ligation Kit of Takara), as well as the ligated DNA and the template DNA, were added. The enzymatic reaction was accomplished at 16 °C for 30 min, and the products were directly analyzed by polyacrylamide gel electrophoresis. The sequences of ligation products were determined on an ABI PRISM 310 Genetic Analyzer using dye terminator labeling method. Prior to the analysis, the products were amplified by PCR, inserted into pGEM(R)*-T Easy vector (Promega, Tokyo), and screened.

3. Results

3.1. Hydrolysis of single-stranded DNA and double-stranded DNA by homogeneous solution of Ce(IV)/EDTA complex

As summarized in Fig. 2, there exists remarkable substrate-specificity in the DNA hydrolysis by these homogeneous solutions of Ce(IV)/EDTA complex. Homogeneous solution of Ce(IV)/EDTA complex cleaved the substrate single-stranded DNA almost randomly without any specific base-preference (Fig. 2A). Hydrolytic character of the scission has been definitely confirmed by the HPLC analysis. In the presence of DNA which is complementary with the whole part of substrate DNA, however, the DNA was hardly cleaved (Fig. 2B). When a predetermined portion of the substrate DNA formed a duplex with the additive, the single-stranded portion in the substrate was hydrolyzed far more efficiently than was the double-stranded portion (Fig. 2C). In the scission of single-stranded portion, no specific base-preference or sequence-preference was observed. These facts, therefore, indicate the possibility of new strategy for site-selective DNA scission (Fig. 3).

For the purpose of comparison, similar DNA scission experiments without EDTA were carried out. When $Ce(NH_4)_2(NO_3)_6$ was dissolved in HEPES buffer and the pH was raised to 7, gel of Ce(IV) hydroxide was formed. Significantly, this hydroxide gel hydrolyzed both single-stranded DNA and double-stranded DNA at almost the same rates. This result is highly in contrast with the preferential scission of single-stranded DNA by homogeneous Ce(IV)/EDTA complex.

3.2. Gap-selective DNA hydrolysis by homogeneous solution of Ce(IV)/EDTA complex

Based on the strategy in Fig. 3A, only the target scission site is kept single-stranded, and the other portions are protected from the scission by forming a duplex with DNA additives. As expected, the scission by homogeneous solution of the Ce(IV) complex selectively occurred at unpaired nucleotides. The double-stranded region was not hydrolyzed to a measurable extent, except for the minor scission near the gap-edges. In addition to these gap-strategies, site-selective DNA hydrolysis by the Ce(IV)/EDTA complex was successfully accomplished by forming a bulge-structure at the target site (Fig. 3B). Only the single-stranded site in the bulges was selectively hydrolyzed. The scission efficiency increased with increasing bulge-length and, in all the cases, was the largest at around the center of bulge.

3.3. Improvement of efficiency of gap-selective DNA hydrolysis by use of oligonucleotide additives bearing monophosphate group at the terminal position

In order to improve the efficiency of gap-selective hydrolysis by Ce(IV)/EDTA, the strategy illustrated in Fig. 1

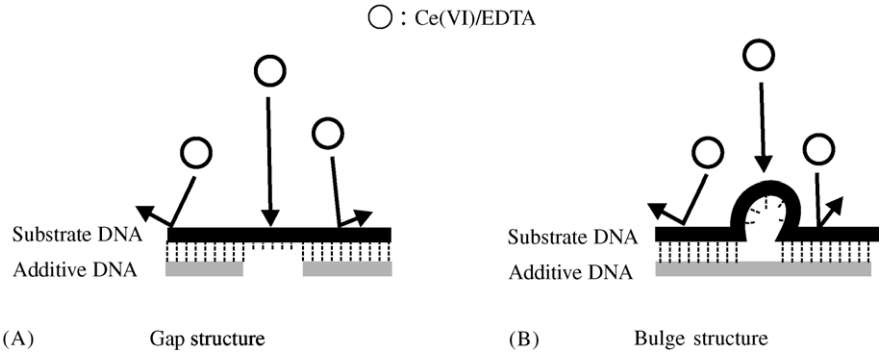


Fig. 3. New strategy for site-selective DNA hydrolysis which requires no covalent fixation of molecular scissors.

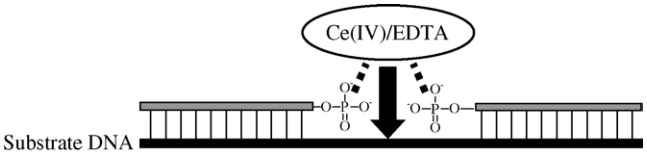


Fig. 4. Hydrolysis of DNA at gap by combining Ce(IV)/EDTA complex with additive DNAs bearing monophosphate group.

was combined with the gap-strategy. The monophosphate group is widely known as a ligand of Ce(IV) so that a monophosphate group was introduced at the end of the gap-forming additive DNAs (Fig. 4). Fig. 5 shows the gel electrophoresis patterns for the DNA hydrolysis by homogeneous solution of Ce(IV)/EDTA complex at pH 7.0 and 37 °C. In lane 6, $DNA^{(L)}$-P and $DNA^{(R)}$ were combined, and a monophosphate group was placed at the 5′-side edge of the 10-base gap in substrate DNA. The scission by the Ce(IV)/EDTA complex was strictly restricted to the gap-region. Similarly, gap-selective scission was successfully achieved by combining $DNA^{(L)}$ with P-$DNA^{(R)}$ and placing a monophosphate at another edge of the gap (lane 7). When two DNAs without the terminal monophosphate ($DNA^{(L)}$ and $DNA^{(R)}$) were combined, the scission was almost marginal under these conditions (see lane 5). Enormous promotion of gap-selective hydrolysis by the monophosphate groups has been conclusively confirmed. When only either $DNA^{(R)}$-P or P-$DNA^{(L)}$ was used alone (without the coexistence of another additive DNA), the single-stranded portion was hydrolyzed almost randomly (lane 4). The gap structure is necessary for these site selective scissions. When $DNA^{(L)}$-P and P-$DNA^{(R)}$ were combined and two monophosphate groups were placed at both edges of the gap, the gap-selective DNA scission was still more efficient

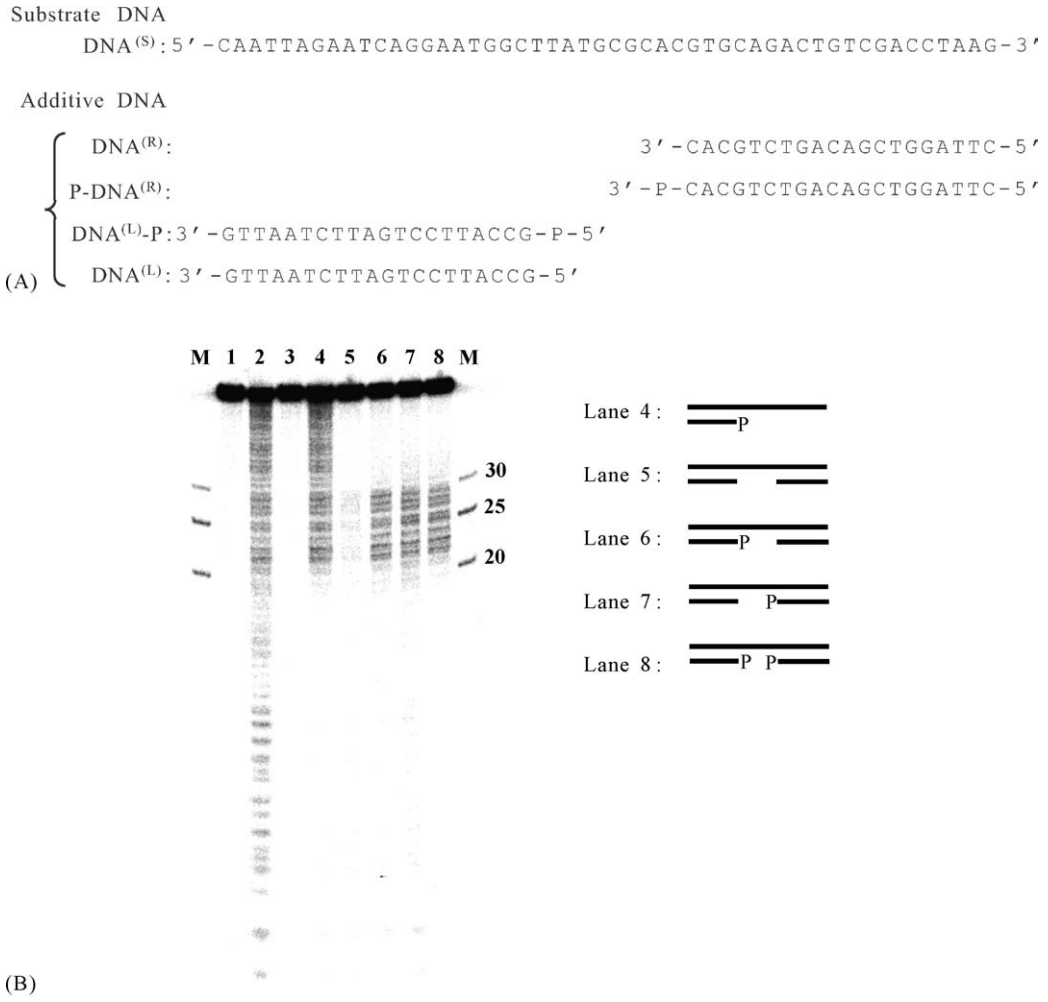


Fig. 5. (A) DNA substrates and oligonucleotide additives used. (B) Gel electrophoresis patterns for the hydrolysis of DNA by combining Ce(IV)/EDTA complex with various additive DNAs. Lane 1, control; lane 2, Ce(IV)/EDTA complex only; lane 3, $DNA^{(L)}$-P/P-$DNA^{(R)}$ (without Ce(IV)/EDTA complex); lane 4, $DNA^{(L)}$-P alone (with Ce(IV)/EDTA complex but without the second additive DNA); lane 5, $DNA^{(L)}$/$DNA^{(R)}$; lane 6, $DNA^{(L)}$-P/$DNA^{(R)}$; lane 7, $DNA^{(L)}$/P-$DNA^{(R)}$; lane 8, $DNA^{(L)}$-P/P-$DNA^{(R)}$. The 10-base gap is formed between the two markers M20 and M30. The structures in lanes 4–8 are schematically depicted in the right-hand side. Reaction conditions: $[DNA^{(S)}]_0 = 1.0\ \mu M$, [each of the additive DNAs]$_0 = 1.5\ \mu M$, $[NaCl]_0 = 100$ mM, and [Ce(IV)/EDTA complex] = 0.5 mM at pH 7.0 (10 mM Hepes buffer) and 37 °C for 15.5 h (M = mol dm^{-3}).

(lane 8). For the selective scission of shorter gap length, the combination of Ce(IV)/EDTA and oligonucleotide additives bearing monophosphate group was also effective.

3.4. Application of the present site-selective scission to single-strand DNA manipulation

The strategy for manipulation of single-stranded DNA is shown in Scheme 1. Single-stranded substrate DNA was first hybridized to gap-forming additive DNAs (Step 1), and was selectively hydrolyzed by adding Ce(IV)/EDTA (step 2). Then, the scission fragments were incubated in the presence of both a template DNA and a ligated DNA having a phosphate at the 5′-end (step 3). The 3′-side portion of this template is complementary with 3′-side of hydrolyzed DNA and the remainder of the template is complementary with the 5′-side of ligated DNA. Finally, the mixtures were treated with T4 DNA ligase (step 4). After the enzymatic treatment, a longer DNA was obtained. According to the sequencing experiment, this DNA corresponded to the desired recombinant DNA (the ligation product between the scission fragment and the ligated DNA).

4. Summary

When gap structures are formed at predetermined site in substrate DNA by using monophosphate-bearing oligonucleotides as cofactors and these conjugates are treated with Ce(IV)/EDTA complex, the phosphodiester linkages at the gap site are selectively and efficiently hydrolyzed. Furthermore, the resultant DNA fragments can be successfully connected with various oligonucleotides by using T4 DNA ligase. These results confirm hydrolytic character of the present site-selective scission, and also indicate strong potential of this method for molecular biology.

Acknowledgments

This work was partially supported by PROBRAIN. The support by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology, Japan is also acknowledged.

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