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Electrochemical assay for deoxyribonuclease I activity

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ABSTRACT

A thiolated oligonucleotide having three ferrocenes was immobilized on a gold electrode through the sulfur–gold linkage. This electrode showed a current response based on the redox reaction of the ferrocene moieties and this response was decreased after treatment with deoxyribonuclease I (DNase I), suggesting the disappearance of the ferrocene moieties on the electrode by the DNase I digestion. A linear correlation between i_0 and i, which are current peaks before and after DNase I treatment, respectively, was observed and this slope was decreased with increase in the amount of DNase I. No current decrease was observed in the presence of EDTA or RNase A instead of DNase I. These results suggested that the current decrease responded specifically to the amount of DNase I and this electrode could be used for an electrochemical DNase I assay. Under the optimum conditions of DNase I digestion at 37 °C for 30 min, a quantitative analysis could be achieved in the range of 10^{-4}–10^{-2} units/μl of DNase I.

Deoxyribonuclease I (DNase I,[1] EC 3.1.21.1) is a nonspecific endonuclease that cleaves phosphodiester linkages of single- or double-stranded DNA to give di- and oligonucleotides with a 5′-phosphate and a 3′-hydroxyl group [1–3]. However, it has some preference for the cleavage site of purine and pyrimidine bonds such as adenine–cytosine sequences [4,5]. DNase I plays an important role in biological events such as DNA metabolism [6] and has been suggested to be involved in the autoimmune disease systemic lupus erythematosus [7,8] or apoptosis [9,10]. Recently, Pulmozyme recombinant human DNase I was cloned, expressed, and produced for the treatment of patients with cystic fibrosis [11]. Diagnostic use of serum DNase I activity as a novel early-phase marker in acute myocardial infarction has also been reported [12]. Due to the biological importance of DNase I and related DNases and their clinical and diagnostic uses, it is essential to measure DNase activity. A variety of methods have been developed to determine the DNase I or DNase activity, but few simple and sensitive detection methods are known including a single radial enzyme diffusion (SRED) method [13] or fluorometric DNase detection methods using PicoGreen dye [14] or the DNaseAlert QC System [15] with an oligonucleotide substrate having fluorescein and rhodamine dyes at both termini. These methods are useful, but still have certain drawbacks such as the difficulty in automatic assay readout and the relatively expensive cost of the fluorogenic oligonucleotide required.

The aim of this work was to develop an electrochemical DNase assay method as a rapid, simple, and sensitive one with an inexpensive and compact instrument using a ferrocenyloligonucleotide-immobilized electrode. Especially, the ferrocenyloligonucleotide-immobilized electrode has been developed from the viewpoint of electrochemical DNA detecting technology [16–20]. However, an electrochemical DNase assay using the ferrocenyloligonucleotide-immobilized electrode has not been reported yet except for our preliminary report [21], although an electrochemical detection of DNA cleavage by DNase I pioneered by Palecek and co-workers [22–26] or an electrochemical detection of DNA-related enzymes [27–30] has been reported over the years. Our idea of the electrochemical DNase I assay is illustrated in Fig. 1. A thiolated oligonucleotide carrying ferrocene (Fc) moieties (Fc-oligo-SH) was prepared by the reaction of an oligonucleotide disulfide derivative with ferrocenylcarbodiimide (FCDI) followed by treatment with dithiothreitol (DTT) which was developed as a simple ferrocenylation reagent for DNA research by our group [31–33]. Different means of DNA labeling especially with electrochemically active reagents have been described in detail in Refs. [31,34]. When the electrode is treated with an aqueous solution containing DNase I, the Fc parts would be removed from the electrode after cleavage by DNase I. This should cause a decreased electrochemical response depending on the amount of DNase I. This system will be the first report, but photosignal change after DNase I cleavage was reported in a homogeneous medium by using the DNA duplex formed from oligonucleotides having fluorescence and Au nanoparticles [35].

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[1] *Abbreviations used:* DNase I, deoxyribonuclease I; DTT, dithiothreitol; Fc, ferrocene; FCDI, ferrocenylcarbodiimide; 3-HPA, 3-hydroxypicolinic acid; QCM, quartz crystal microbalance; TEAA, triethylammonium acetate.

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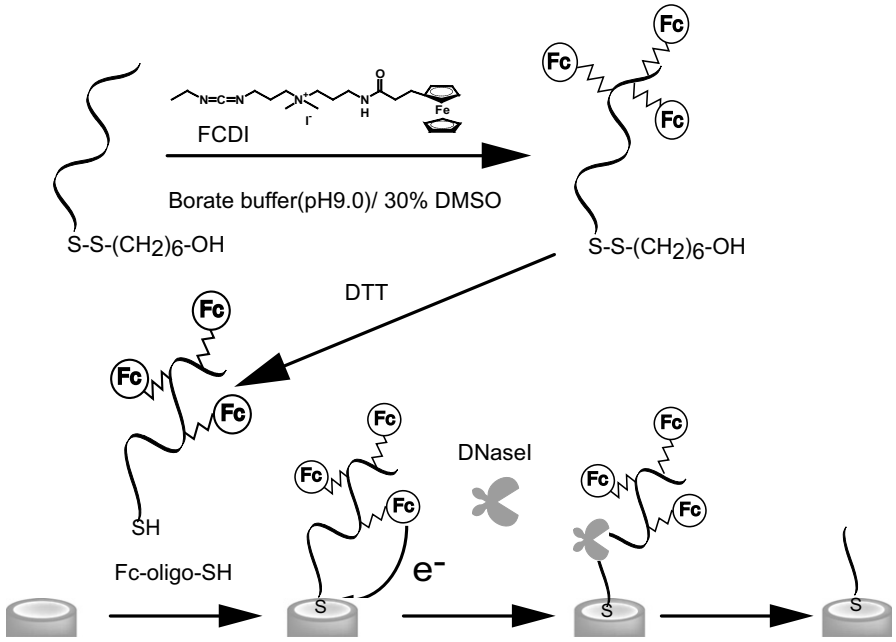

Fig. 1. Principle of electrochemical DNase I assay based on Fc-oligo-SH-immobilized electrode and chemical structure of FCDI.

In this paper, we describe the construction of a sensor electrode carrying Fc-oligo-SH to achieve the electrochemical DNase I assay.

Experimental

Materials

Ferrocenylcarbodiimide (Fig. 1) as a ferrocenylation reagent for DNA was synthesized according to the route described previously [31]. An oligonucleotide disulfide derivative, 5′-HO$(CH_2)_6$-SS-$(CH_2)_6$-dA_{10} ACA AAT AAC AAA TAT-3′, was custom-synthesized by Genenet Co. (Fukuoka, Japan). BioPak water was purified by a Milli-Q system Gradient A10 coupled with an Elixs3 kit (Millipore, Billerica, MA). DNase I (RNase free) was purchased from TaKaRa Bio Inc. (Shiga, Japan). A 10X DNase I buffer (400 mM Tris-HCl (pH 7.5), 80 mM $MgCl_2$, and 50 mM DTT) from TaKaRa Bio Inc. was used to dilute DNase I.

HPLC

The HPLC system used in this experiment was composed of the following components: Hitachi C-7300 column oven, L-7450H diode array detector, L-7100 pump, and D-7000 interface chromatograph (Hitachi High-Technologies Co., Tokyo, Japan). Reversed-phase HPLC was run using a Mightysil RP-18 column (0.5 × 25 cm) (Kanto Chemicals Co. Inc., Tokyo, Japan) with the gradient conditions, where the acetonitrile (CH_3CN) content in 100 mM triethylammonium acetate (TEAA) buffer (pH 7.0) was linearly changed from 10 to 95% over 30 min at a flow rate of 1.0 ml/min with detection at 260 nm.

MALDI-TOF MS

Oligonucleotides modified with FCDI were characterized by MALDI-TOF MS (Voyager Linear-SA, PerSeptive Biosystems Inc., Foster City, CA) measurement of the products separated by HPLC. They were dissolved in a solution of 50 mg/ml 3-hydroxypicolinic acid (3-HPA) in 0.1% trifluoroacetic acid (TFA)/50% CH_3CN and dried. Mass spectra were measured by the negative mode.

Preparation of Fc-oligo-SH

Thirty microliters of a solution of 5′-HO$(CH_2)_6$-SS-$(CH_2)_6$-dA_{10} ACA AAT AAC AAA TAT-3′ (30 nmol) in 50 mM borate buffer (pH 9.0) was mixed with 30 μl of a solution of 100 mM FCDI in 50 mM borate buffer (pH 9.0) containing 60% DMSO. After overnight shaking at 37 °C, the reaction mixture was justified to 1 ml by 1 ml with 0.1 M TEAA buffer (pH 7.0) and then this solution was seeped into the top of the NAP-10 column (Pharmacia Sephadex G-25, Amersham Biosciences Co., Uppsala, Sweden) to remove unreacted FCDI. The NAP-10 column was replaced by 0.1 M TEAA buffer (pH 7.0) by passage of 1.5 ml of its solution. One milliliter of the first fraction was collected and freeze-dried. The lyophilized product was dissolved in 60 μl of autoclaved BioPak water and purified by reversed-phase HPLC. The fraction eluted at 20 min was collected, dried, and dissolved in 50 μl sterilized water. The oligonucleotide disulfide modified by three ferrocenes thus obtained, HO$(CH_2)_6$-SS-$(CH_2)_6$-dA_{10} ACA AAT^{Fc} AAC AAA $T^{Fc}AT^{Fc}$-3′ (Fc: FCDI modified part), was identified by MALDI-TOF MS, and reduced by treatment with 0.04 M DTT in water to afford the desired thiolated ferrocenyloligonucleotide (Fc-oligo-SH).

DNase I digestion of Fc-oligo-SH in homogeneous solution

The varied amounts of DNase I were mixed with a solution of 10 μM Fc-oligo-SH in 1X DNase I buffer. The reaction mixture was incubated at room temperature for 30 min and the enzymatic hydrolysis was stopped by heating at 80 °C for 10 min. Fc-oligo-SH did not show any decomposition under such conditions of heating in the absence of the enzyme. The reactivity of DNase I to Fc-oligo-SH was assessed by reversed-phase HPLC and MALDI- TOF MS.

Preparation of Fc-oligo-SH-immobilized electrode

A gold electrode of 2.0 mm^2 in area (commercially available electrode, Bioanalytical Systems (BAS) Inc., Tokyo, Japan) was polished with 6 and 1 μm of diamond slurry and with 0.05 μm of alumina slurry in this order, and sonicated in Milli-Q water for 15 min (3 times). This electrode was electrochemically polished by scan-