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DNA cleavage based on high voltage electric pulse

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A high voltage electric pulse was applied to DNA cleavage. The DNA cleavage reaction was dependent on the voltage amplitude, pulse number and pulse width. Radical scavengers and ESR data indicated the possibility that active species such as OH radical were strongly related to DNA cleavage.

Electrochemistry; Oxygen radical; DNA cleavage; ESR; Electric pulse; Radical scavenger

1. INTRODUCTION

Various kinds of biological reactions such as cell fusion [1], DNA transformation [2] and ATP synthesis can be induced under high electric fields (kV/cm). These phenomena are considered to be mainly caused by agglutination of the cell body and an increase in the permeability in cell membrane which is primarily due to dielectric forces. Our previous work has indicated that a 10 kV/cm high voltage electric pulse was effective in transforming DNA in yeast cell [3]. This pulse amplitude was considered high enough to cause pore formation in the cell membrane based on a comparison with other results reported previously. There was less consideration about the effects of electrochemical reactions proceeded at electrodes.

Recently we found that DNA cleavage occurred during the application of high electric field pulse to cells. This discovery offers serious new and profound promise in the application to DNA transformation experiments. Therefore, in the present work, we investigated DNA cleavage phenomena under pulsed high voltage conditions.

In order to clarify factors which are strongly related to DNA cleavage, we paid special attention to electrochemical effects. According to Palecek's paper [4] on the electrochemistry of DNA, adenine (A) and cytosine (C) are reduced at an electrode. The reduction of A and C occurs due to their protonation, which may take place in the electrode region at neutral pH provided adenosine N-1 or cytosine N-3 is able to accept the proton. Furthermore DNA containing guanine offers an anodic peak in the vicinity of -0.3 V in cyclic voltammetry with mercury electrode. A reduction peak due to adenine appears in a subsequent cycle. An anodic peak due to guanine can be observed on the same voltammogram after subsequent cycles. The guanine residue in the DNA chain represents a chemically reversible redox system because guanine is reduced around -1.8 V to dihydroguanine, which is oxidized back to guanine around -0.3 V [4]. In this way, DNA can directly react with the electrode. However, no research has been performed on the cleavage of the DNA chain by electrochemical reactions.

On the other hand, reactive oxygen species are known to play an important role in DNA cleavage. For example, hydroxy radical attacks the deoxyribose sugars along the backbone of the DNA molecule and breaks the DNA chain with almost no sequence dependence [5].

A study of DNA cleavage by pulsed high voltage is presented in this paper to examine the effects of electrical parameters on DNA cleavage activity and

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to estimate how the active species formed relative to the DNA cleavage reaction.

2. MATERIALS AND METHODS

2.1. *DNA and reagents*

λ phage DNA was purchased from Takara Shuzou. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD) (bovine liver; EC 1.15.1.1). All other reagents used were of commercially analytical grade. All solutions were prepared using distilled water.

2.2. *Electric circuits for DNA cleavage*

A simple circuit for supplying an electric pulse was constructed using a capacitor, a switch controlled by a relay unit and a power generator. Initially the capacitor was charged to the terminal voltage and then the discharging circuit connecting to the electrodes and turned on. The voltage between the electrodes decreased exponentially. The time constant of the exponential decay was controlled by changing the capacitance value. In these experiments, the peak voltage was varied between 1 and 8 kV/cm. Capacitances were set at 1–3 μF with which time constants were 300–900 μs. The electrolyses were carried out using a Pt wire working electrode (diameter 0.1 cm × 1 cm), an Ag/AgCl electrode as reference and a Pt counter electrode (3 × 2 cm). Potential was set with a HA-501 potentiostat (Hokutodenko, Japan) and a HB-104 function generator (Hokutodenko, Japan).

2.3. *Assay for DNA cleavage*

The extent of cleavage of the DNA molecules was measured using electrophoresis in agarose gels. Electrophoresis was carried out in horizontal slabs of gel containing 0.5–0.8% agarose dissolved in Tris-borate buffer as described by Maniatis et al. [6]. Electrophoresis was performed either for 16 h at 0.2 V/cm or 4 h at 1.2 V/cm. Then the gels were run immersed in 0.5 μg/ml ethidium bromide solution and photographed under UV light (360 nm) using a Polaroid type film. The profiles of cleaved DNA on photographic films were recorded using a densitometer (PAN803, Jyoukou Japan).

2.4. *Effect of the addition of scavengers on DNA cleavage*

DNA was incubated with and without oxygen radical scavengers (200 μg/ml SOD or 100 mM mannitol) for 5 min at 37°C. After incubation, treated DNA was visualized by gel electrophoresis.

2.5. *S_1 nuclease reaction*

The procedure was described by Maniatis et al. [7].

2.6. *ESR measurements*

ESR spectra were taken with a Varian spectrometer (9.5 GHz) with a field modulation frequency of 100 kHz, using microwave power of 10 mW. The hfsc values from the spectra were measured by Mn^{2+} in MgO as reference.

3. RESULTS

The degradation of phage DNA which occurred after pulse application could be visualized by agarose gel electrophoresis. DNA bands became smaller with an increasing number of pulses (fig.1). This indicates that the DNA cleavage reaction is more severe with an increase in number of pulses. The effect of pulse amplitude on DNA cleavage was also investigated. As shown in fig.2, the rate of lower molecular size DNA production increased with pulse amplitude. The effect of molecular size is shown in fig.3. When pulsed voltage was applied to a mixture of fragments digested by a restriction enzyme, each fragment peak was reduced and the relative position of each fragment shifted to a lower molecular size. The pulse width was controlled by changing the time constant of exponential decay. DNA cleavage increased with an increase of pulse width.

Radical scavengers were used for estimating

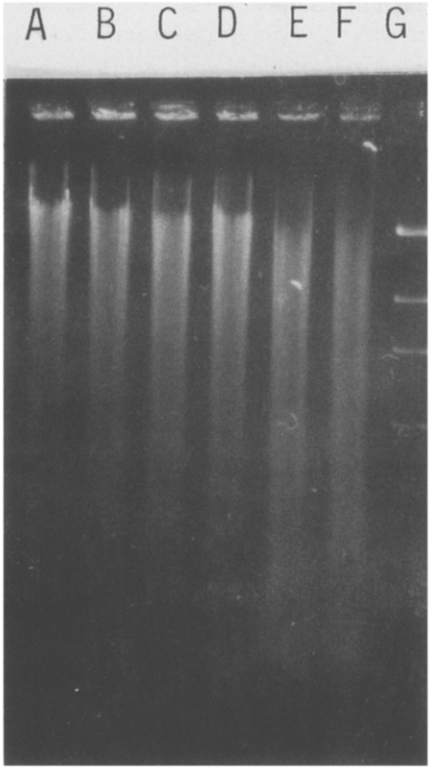

Fig.1. Effect of the number of pulses on DNA cleavage. A pulse of 8 kV/cm was applied to the λ phage DNA solution. Electrophoretic conditions are described in the text. Lanes: A, initial DNA without pulse; B,C, 10 pulsed DNA; D, 20 pulsed DNA; E, 30 pulsed DNA; F, 40 pulsed DNA; G, *Hin*dIII digests of A.

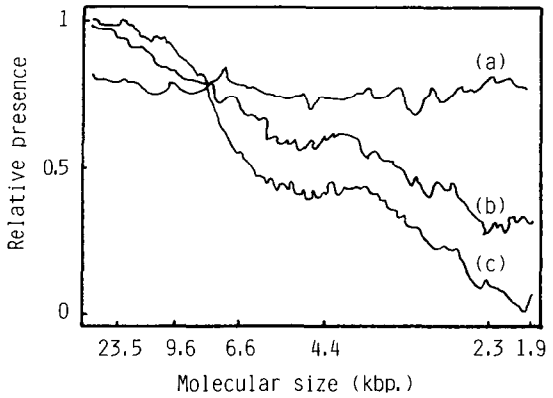

Fig.2. Densitometer scan of electrophoretic patterns with various pulse amplitudes. Forty pulses with various amplitudes were applied to the λ phage DNA solution. Pulse amplitudes were used as follows: (a) 8 kV/cm; (b) 3 kV/cm; (c) none.

what chemical species were involved in DNA cleavage [8,9]. DNA cleavage by an electric pulse could occur in the presence of SOD and mannitol suggesting that both $O_2^{\cdot -}$ and $OH^{\cdot}$ are essential for the cleavage effect (table 1). The spin-trapping method was used to confirm formation of oxygen radicals. This method consists of using a spin trap such as DMPO, which forms a relatively stable nitroxide radical by reacting covalently with unstable free radicals such as $O_2^{\cdot -}$ and $OH^{\cdot}$. The hyperfine splitting of these adducts provides the information about the identification of original

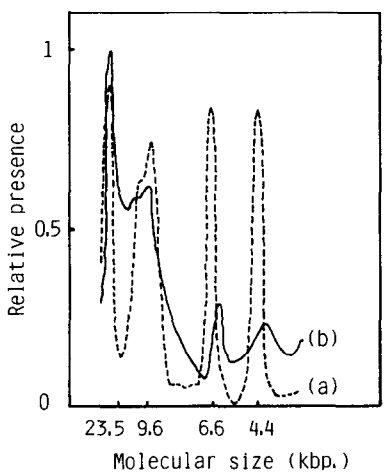

Fig.3. Effect of molecular size on DNA cleavage. Twenty pulses of 8 kV/cm were applied to *Hin*dIII digests of λ phage DNA. (a) With pulse; (b) without pulse.

Table 1

Effect of radical scavengers

Radical scavengers (concentration)	Relative DNA cleavage activity[a]
None	100
200 μg/ml SOD[b]	45
100 mM mannitol[b]	32

[a] This activity was estimated from relative amount of cleaved DNA below 4.4 kbp based on densitometric data

[b] The reaction conditions are described in section 2

40 pulses of 8 kV/cm were applied to λ phage DNA

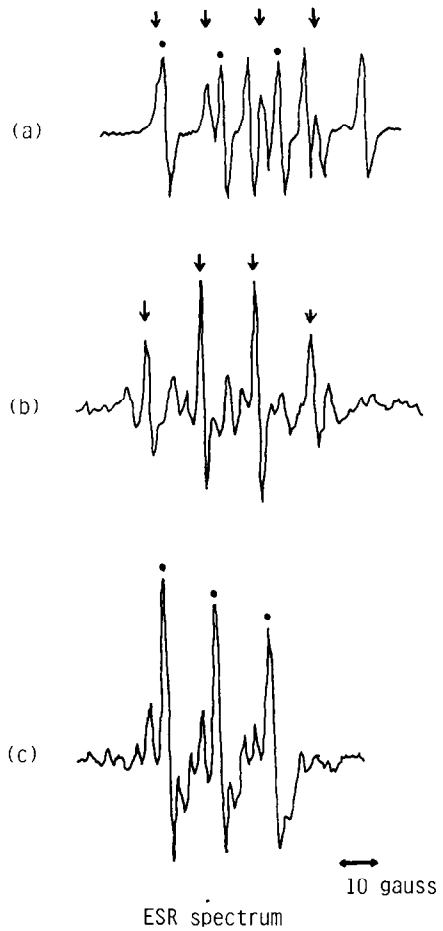

Fig.4. ESR spectra of 0.1 M DMPO in Tris-EDTA buffer after application of voltage. Voltage conditions were indicated as follows: (a) 20 pulses of 8 kV/cm; (b) −0.4 V (vs Ag/AgCl) for 20 min; (c) 0.8 V (vs Ag/AgCl) for 20 min. Arrows indicate the 1:2:2:1 quartet due to DMPO-OH.

radicals. Fig.4a shows the ESR spectrum of an 8 kV/cm pulse applied to a DNA sample. This spectrum consists of more than 10 lines. Fig.4b shows the spectrum when −0.4 V was applied to the Pt electrode versus Ag/AgCl in the presence of DMPO. The four specific lines indicated the generation of hydroxy radicals because the ratio of each of the line strengths was 1:2:2:1 which indicated a typical spectrum of the DMPO-OH spin adduct [10,11]. Unidentified spin adducts were obtained at positive potentials. From a comparison of the spectrum (fig.4a) at 8 kV/cm, it is supposed that this spectrum consisted partially of both parts shown in fig.4b and c. This is because both oxidation and reduction products mix in the electrode cell.

4. DISCUSSIONS

The cleavage of DNA is strongly dependent on the voltage amplitude, pulse number and pulse width. Clements et al. [12] investigated the electric breakdown of water induced by a high voltage pulse. They estimated the production of active oxygen species produced under a high electric pulse from an emission spectrum. Hydroxy radical has been considered to attack the deoxyribose sugars arrayed along the surface of DNA [13]. Secondary reactions of the resulting deoxyribose-centered radicals cause the backbone to break essentially by disintegration of the sugars themselves [14–16]. Our radical scavengers and ESR experiments indicate the possibility that oxygen radicals are strongly related to the DNA cleavage reaction. When a high electric pulse was applied to DNA with DMPO, more complicated ESR spectra were obtained than those in fig.4a (not shown). Hydroxy radicals attacked carbon atoms of the sugar group in DNA, and this reaction was followed by generation of carbon radicals. Therefore these complicated spectra might be based on the participation of DMPO-carbon-centered radical adducts. λ phage DNA became more sensitive to S_1 nuclease digestion after pulse application. In particular, the increase in S_1 nuclease sensitivity was observed even after less than 10 pulses. This fact indicated that single-stranded scission occurred more frequently than double-stranded scission. A hydroxy radical has already been applied to the 'foot-printing' method because this radical reacts with each deoxyribose resulting in nonspecific DNA cleavage [5]. Cleaved DNAs obtained by our pulse method gave a broad band pattern in gel electrophoresis. This result coincided with nonspecific cleavage of the hydroxy radical.

As the lifetime of a hydroxy radical is very short (~1 ns) [17], only DNA adsorbed on the surface of electrode will be cleaved. Repetition of pulses was thought to be effective for both desorption of cleaved DNA and adsorption of intact DNA. This hypothesis is supported by the observation that DNA cleavage activity was strongly dependent on the pulse number as shown in fig.1.

The electric pulse method can be applied to DNA-mediated transformation with 3 pulse repetition [3], in which DNA cleavage cannot be observed. Therefore it is not considered to be a serious problem in DNA transformation experiments, if electric pulse is used under the correct conditions. Our methods can also be used for in vivo DNA cleavage, if an implantable microelectrode is inserted into an intact cell nucleus. Furthermore combined with a DNA selective binding protein, preferential cleavage of DNA may be accomplished.

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