Double-strand break of giant DNA: protection by glucosyl-hesperidin as evidenced through direct observation on individual DNA molecules

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Abstract **We have performed a real-time observation of photo-induced breakage on individual long DNA molecules stained by a cyanine dye, YOYO, under the intense illumination ($\lambda = 450$–490 nm) in solution. It was shown that the double-strand breakage is accelerated by Fenton's reagent (Fe^{2+}/H_2O_2). In addition, it was found that the breakage reaction is protected in the presence of a water-soluble flavonoid, glucosyl-hesperidin. To interpret the kinetic process of the double-strand breakage reaction, a two-step mechanism is proposed: under light illumination on giant DNA molecules, single-strand breaks are generated to induce nicks in a stochastic manner, and then reactive oxygen attacks these nicks causing the double-strand break.**

Keywords: Long duplex DNA; Double-strand break; Glucosyl-hesperidin; Fluorescence microscopy

1. Introduction

There is increasing evidence that DNA damage, including single- and double-strand breaks, and modification of the bases, induces mutagenic and carcinogenic processes [1–3]. Among these changes, DNA double-strand breaks are considered to be the most biologically significant lesions in living cells [4]. However, most in vitro studies on double-strand breaks have been performed at the level of DNA oligomers [5,6]. There is little information available on the double-strand breaks of giant genome DNA molecules. This may be partly due to the lack of a suitable methodology to monitor the breakage of giant DNA molecule.

Recently, we made direct observations of single giant DNA molecules in solution using fluorescence microscopy and found that giant DNA molecules undergo a large discrete transition from an elongated state to a compact state upon the addition of various condensing agents [7,8]. We have also shown that an anticancer drug, daunomycin, is able to loosen the packing of DNA and induce double-strand breaks [9,10].

In the present study, we applied the experimental technique of single-molecule observation to evaluate the kinetic process of photo-induced double-strand breaks in DNA molecules. We performed fluorescence microscopic observation of DNAs stained by a fluorescent cyanine dye, YOYO, under intense illumination ($\lambda = 450$–490 nm) in solution. YOYO has been widely used for different fluorescence applications, including the detection of DNA on electrophoresis gels and the visualization of individual DNA molecules by fluorescence microscopy [11–13]. It is also known that YOYO has the ability to photo-cleave probed DNAs by generating hydroxyl radicals [11,12]. We measured the breakage time of individual DNA molecules in solution and analyzed the kinetics of the breakage reaction in a quantitative manner. In addition, we found that glucosyl-hesperidin (GHSP) is able to prevent double-strand breakage and reduce the breakage rate by more than one order of magnitude. GHSP is a water-soluble analogue of hesperidin, which is a flavonoid found in citrus fruit peel [14]. The chemical structure of GHSP is shown in Fig. 1.

2. Materials and methods

2.1. Materials

T4 phage DNA (166 kbp, contour length 57 μm) was purchased from Nippon Gene (Toyama, Japan). The fluorescent cyanine dye YOYO (trade name YOYO-1) was purchased from Molecular Probes, Inc. (Oregon, USA). An antioxidant, 2-mercaptoethanol (2-ME), was purchased from Wako Pure Chemical Industries (Osaka, Japan). GHSP was a kind gift from Toyo Sugar Refining Co. Ltd (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries.

2.2. Fluorescence microscopic observations

For fluorescence microscopic measurements, T4 phage DNA was dissolved in 10 mM Tris–HCl buffer solution with 0.8 μM YOYO and 10% (v/v) glycerol at pH 7.4. Observations were carried out at room temperature at around 20 °C under air atmosphere. To minimize intermolecular DNA aggregation, measurements were conducted at a low DNA concentration: 0.3 μM in nucleotide units. Pipetting of the DNA solution has been performed with special care to avoid the mechanical stress on the giant DNA molecules. To investigate acceleration by Fenton's reagent, 2.5 μM $FeCl_2$ was first added to the DNA solution. The reaction was started by adding 12 mM (0.04%) H_2O_2. The possible effect of GHSP on the reduction of breakage was evaluated by adding 1 mg/ml GHSP to the DNA solution. Illumination with 450–490 nm light was performed with an optical excitation filter, using a 100 W mercury lamp and a 50% transmission filter (ND filter). The fluorescence of YOYO was observed at 510 nm. In order to reduce photocleavage to the level suitable for the real-time observation, 4% (v/v) 2-ME was mixed with samples before the addition of Fe^{2+} and/or H_2O_2. Fluorescent DNA images were obtained using a microscope

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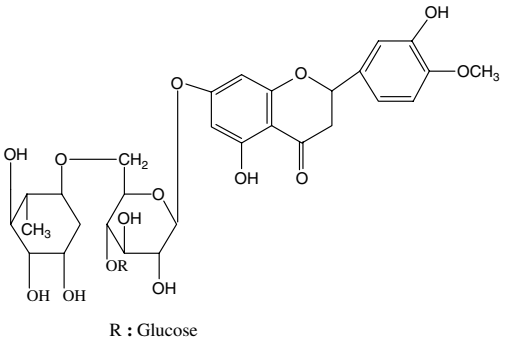


Fig. 1. Chemical formula of GHSP.

(Axiovert 135 TV, Carl Zeiss, Germany) equipped with a 100× oil-immersion objective lens and a highly sensitive Hamamatsu SIT TV camera, which allowed the recording of images on video tapes. The video images were analyzed with an image processor (Argus 20, Hamamatsu Photonics, Hamamatsu, Japan).

3. Results and discussion

3.1. Double-strand breakage visualized by fluorescence microscopy

We measured the breakage time, τ of individual DNA molecules in solution under focused illumination by fluorescence microscopy. Fig. 2 exemplifies the real-time observation of the breakage of a single T4 DNA molecule in the presence of Fenton's reagent (Fe^{2+}/H_2O_2), indicating that the breakage reaction is actually observed at the level of individual DNA molecules.

Fig. 3 shows a histogram of the breakage time distribution, where ca. 100 molecules were observed in each condition. In buffer solution, the average breakage time was $\langle\tau\rangle = 37$ s (see also Table 1). With the addition of hydrogen peroxide together with Fe^{2+} to the buffer solution, the breakage time of DNA became much shorter; $\langle\tau\rangle = 12$ s. On the other hand, the addition of GHSP increased the breakage time ($\langle\tau\rangle = 52$ s), indicating that GHSP significantly protects against DNA breakage. Moreover, GHSP was able to increase the breakage time from 12 to 36 s, even in the presence of Fenton's reagent (Fe^{2+}/H_2O_2). The protective effect of GHSP observed here corresponds well to the experimental trends in previous studies on flavonoids as potential antioxidants [14,15].

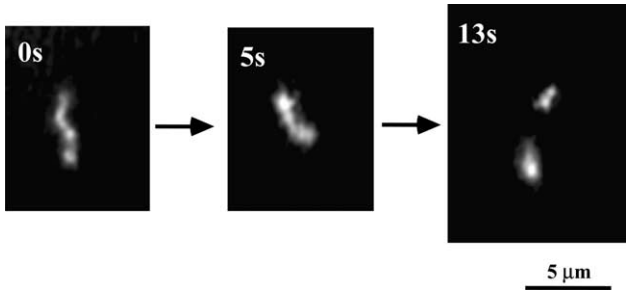


Fig. 2. Fluorescence microscopic observation of a T4 DNA molecule, showing double-strand breakage in the presence of active oxygen with 12 mM H_2O_2 and 2.5 μM Fe^{2+}.

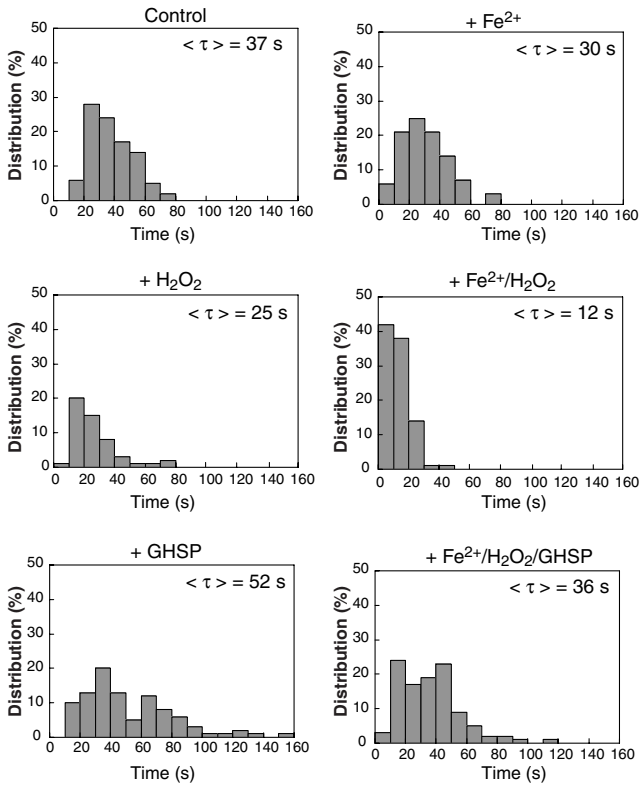


Fig. 3. Histograms of the probability of double-strand breakage of T4 DNA molecules, as monitored by single DNA observation with fluorescence microscopy, where the concentrations of Fe^{2+}, H_2O_2 and GHSP were 2.5 μM, 12 mM, and 1 mg/ml, respectively. The bar in the histograms shows the percentage of the breaks during each 10 min of observation. The breakage in the control experiment is due to the photobreakage reaction with 450–490 nm light in the presence of YOYO.

Table 1
Mean breakage time $\langle\tau\rangle$ until double-strand breakage and the effective constant A of the breakage reaction, where A is normalized to be unity ($A_0 = 1$) in the control experiment

Sample	Mean breakage time $\langle\tau\rangle$ (s)	Effective kinetic constant[a] A/A_0
Control (DNA alone)	37	1.0
$+Fe^{2+}$	30	1.3
$+H_2O_2$	25	1.7
$+Fe^{2+} + H_2O_2$	12	12.2
+GHSP	52	0.4
$+Fe^{2+} + H_2O_2$ + GHSP	36	0.9

[a] Experimental error is estimated to be ±0.2.

Fig. 4 shows the time course for the increase of damaged DNA molecules under various conditions, which has been deduced from the direct measurement on the double-strand breakage of individual DNAs. The breakage reaction in the presence of the Fenton's reagent is largely inhibited by the addition of GHSP. It is to be noted that there is a delay in the double-strand breakage reaction except for breakage in Fenton's reagent without GHSP. Ackerman and Tuite [11,12] reported that in the presence of YOYO, the number of double-strand breaks increases quadratically with time, except for the existence of time-lag in the early stage. In the next section, we

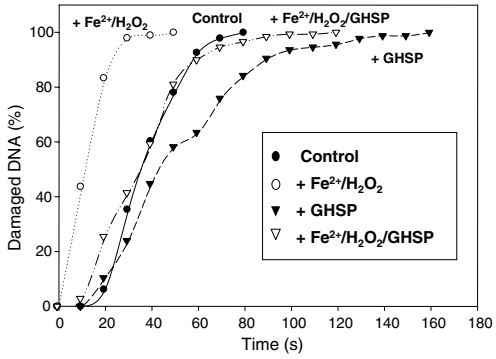


Fig. 4. Time-dependence of the percentage of damaged DNA molecules, deduced from the summation of probability in the histograms of Fig. 3.

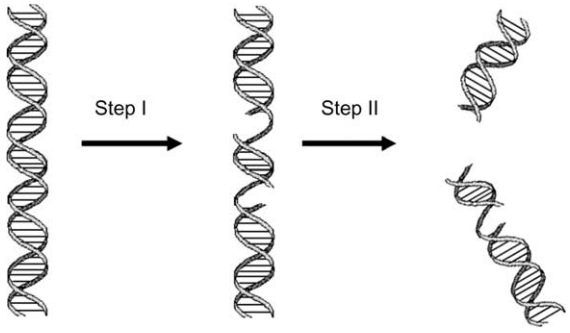
will discuss the characteristic profile of the kinetics of the breakage reaction including the aspect of the time-delay.

3.2. Analysis of the kinetics of double-strand breakage

We analyzed the breakage time distribution to clarify the physico-chemical mechanism of the breakage reaction. To observe individual DNA molecules stained with a fluorescence dye, YOYO, a rather intense light is illuminated on a narrow region on the order of tens of micrometer in the sample solution between glass plates. This causes a photo-induced chemical damage of DNA molecules. It was reported that YOYO causes double-strand breaks of DNA through the accumulation of single-strand breaks [11]. The mechanism of photocleavage in single-strand breakage or nicking was found to depend on the binding ratio; at higher YOYO binding, the reaction is mediated by singlet oxygen [11]. It was suggested that at low binding radical species were involved in the breakage reaction. The mechanism of the accumulation of single-strand breaks before double-strand breakage has been supported by Lyon et al. [16] based on a study on T4 DNA using an intercalating dye, POPO.

Thus, it is expected that, during light irradiation, single-strand breakage will be induced in a stochastic manner along long double-strand DNA. Even when breakage on either strand of DNA is generated, the DNA molecule still maintains its full length if the complementary strand is safe in the neighborhood of the breakage on the damaged strand. The double-stranded chain is cut into fragments only when both sides of the DNA backbone are broken. If either strand of long DNA is broken through the effect of photo-irradiation, a local deformation or stress at the nick will be caused. This implies that reactive oxygen or some other chemical species will attack such a local defect on double-stranded DNA and then break the surviving strand. Thus, it is plausible that the breakage reaction in long DNA is accomplished by a chemical attack at a nick in the double-stranded structure, which is caused by breakage of the other strand by photo-illumination (see Scheme 1).

This scheme shows a plausible two-step mechanism for double-strand breakage, based on the above discussion. In the presence of YOYO, single-strand breakage accumulates along the long DNA molecule, and double-strand breakage is then caused by the attack of a reactive species such as reactive oxygen. With this mechanism in mind, we will consider a kinetic equation to interpret the time-dependence of the breakage reaction.



Scheme 1.

By denoting the number of nicks per single DNA molecule as n, the rate of the increase in nicks (step I of Scheme 1) can be written as,

$$dn/dt = \alpha I \quad (1)$$

where I is the intensity of illuminated light, or the strength of photon flux, and α is a constant. For simplicity, we assume that variation in the base composition along the DNA chain has a negligible effect on the possibility of breakage. As the initial condition, we take $n = 0$ when $t = 0$. Thus, Eq. (1) is integrated as,

$$n = \alpha I t \quad (2)$$

The reaction kinetics of step II in Scheme 1, i.e., the probability of chemical attack at the nick, is represented as dx/dt, where x is the number of DNA molecules surviving from fragmentation (see, e.g., Fig. 4). By introducing a rate constant k, dx/dt is given as,

$$dx/dt = -knx = -k\alpha I t x \quad (3)$$

After integration of Eq. (3),

$$\ln x = -(1/2)k\alpha I t^2 + \text{Const.} \quad (4)$$

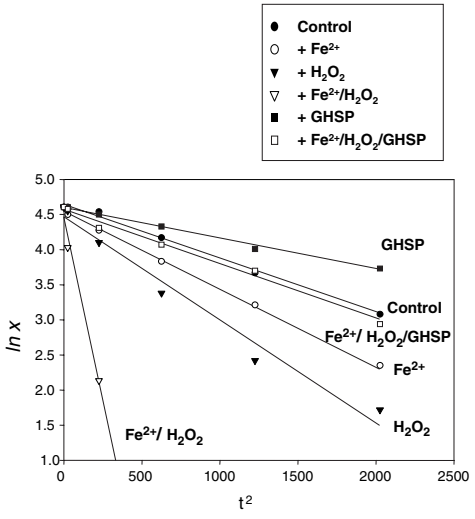


Fig. 5. Linear relationships between t^2 and $\ln x$, where x is the percentage of surviving DNA molecules and is calculated as 100% − (percentage of damaged DNA). The experiments were the same as in Figs. 3 and 4.

By introducing an effective kinetic constant, $A = (1/2)k\alpha I$, Eq. (4) becomes

$$\ln x = -At^2 + \text{Const.} \quad (5)$$

Thus, when double-strand breakage is induced in a two-step reaction with stochastic single-strand damage and subsequent attack by reactive oxygen, a plot of $\ln x$ vs. t^2 should provide a straight line, where the slope of the linear relationship is proportional to the rate constant k of the chemical breakage reaction on long DNA molecules, if the reaction rate α of stochastic photo-breakage remains almost constant.

Fig. 5 shows linear relationships between t^2 and $\ln x$, suggesting that the above two-step mechanism of double-strand breakage explains the experimental trend in a satisfactory manner. In addition, Fig. 5 shows that the effective kinetic constant A changes significantly depending on the presence or absence of GHSP. The kinetic constant A in the experiment with reactive oxygen is 12 times greater than that in the control experiment. In the presence of reactive oxygen with hydrogen peroxide and Fe^{2+}, the kinetic constant A with GHSP is $12.2/0.9 \cong 13$ times smaller than that without GHSP (see Table 1).

4. Future development

Much of the recent information concerning reactive oxygen-induced double-strand breaks comes from studies at the oligomer level. On the other hand, genomic DNA molecules are generally very large, which suggests that a single event of double-strand breakage along a long DNA can have a fatal effect. The present methodology may provide useful insights into the mechanism and kinetics of DNA double-strand breaks.

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