Single gene retrieval from thermally degraded DNA

LIANWEN ZHANG and QINGYU WU*

Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China

**Corresponding author (Fax, 86-10-62781825; Email, qingyu@tsinghua.edu.cn)*

To simulate single gene retrieval from ancient DNA, several related factors have been investigated. By monitoring a 889 bp polymerase chain reaction (PCR) product and genomic DNA degradation, we find that heat and oxygen (especially heat) are both crucial factors influencing DNA degradation. The heat influence, mainly represented by temperature and heating time, affects the DNA degradation via DNA depurination followed by cleavage of nearby phosphodiesters. The heating time influence is temperature-dependent. By reactive oxygen species (ROS) scavenging and 1,3-diphenyl-isobenzofuran (DPBF) bleaching experiments the influence of oxygen on DNA thermal degradation was shown to occur via a singlet oxygen pathway. A comparative study of the thermal degradation of cellular DNA and isolated DNA showed that cellular lipids can aggravate DNA thermal degradation. These results confirm the possibility of gene amplification from thermally degraded DNA. They can be used to evaluate the feasibility of the retrieval of single gene from ancient remains.

[Zhang L and Wu Q 2005 Single gene retrieval from thermally degraded DNA; *J. Biosci*. **30** 599–604]

1. Introduction

The determination of nuclear DNA sequences from ancient remains would open many novel opportunities for understanding some key questions such as the resolution of phylogenies, the sexing of hominid and animal remains, and the characterization of genes involved in phenotypic traits. Fossils, as a primary form of keeping ancient remains, have been ever buried under the earth. Therefore, the influence of terrestrial heat is inevitable. To date, the best preserved ancient nucleic acids tend to stem from cold environments (Poinar *et al* 1996) though single-copy ancient nuclear DNA has been rarely recovered from warm climates (Poinar *et al* 2003). Under the earth, temperature is different at variant depth. The reported geothermal gradients throughout the upper several kilometers vary from 20°C/km to 333 ± 33°C/km (Foulger 1995; Dunlop *et al* 2000; Reiners *et al* 2000). The deeper fossils are buried, the higher temperature they would suffer.

In the past decades, heat-induced DNA degradation *in vitro* has been widely studied. Heat can induce DNA depurination, followed by cleavage of the nearby phosphodiester (Lindahl 1993). It was reported that DNA in dried seeds could not be retrieved after just one hour of heating at 150°C or above (Chalfoun and Tuross 1999).

Oxygen is another factor that influences DNA degradation. It is involved in cellular DNA degradation in dried tissues (Matsuo *et al* 1995). In artificially charred wheat seeds, DNA degradation is delayed in low-oxygen samples compared with the equivalent aerobic material (Threadgold and Brown 2003). In solution, H_2O_2, which stems from the heat-induced singlet oxygen ($^1O_2$), is involved in heat-induced DNA damage (Bruskov *et al* 2002).

Keywords. DNA thermal degradation; polymerase chain reaction (PCR) amplification; single gene retrieval; singlet oxygen.

Abbreviations used: DBPF, 1,3-diphenyl-isobenzofuran; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; ROS, reactive oxygen species.

In order to broaden our understanding of influencing factors on DNA thermal degradation in aqueous solution and the possibility to amplify a single gene from thermally degraded DNA, the damage of *Synechococus* sp. PCC 7942 genomic DNA has been investigated by using gel electrophoresis and polymerase chain reaction (PCR). We find that temperature, heating time and oxygen are all influencing factors on DNA thermal degradation, in particular the temperature. And compared with isolated DNA, cellular DNA shows more damage due to the existence of cellular lipids. It is also suggested that the oxygen influence might be via a singlet oxygen mechanism.

2. Materials and methods

2.1 *Cell culture and DNA extraction*

Cyanobacteria are the most ancient oxygenic photoautotrophic organisms dominating the microbial communities on the earth. In this work, *Synechococus* sp. PCC 7942 wild-type was grown photoautotrophically at 28°C in liquid BG11 medium (Sauer *et al* 2001). Light was provided by fluorescent lamps at a photon flux intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Cells were harvested by centrifugation when OD_{750} nm of cell suspension reached to 1·0 and were washed twice with distilled water.

DNA was extracted according to the routine phenol-chloroform method and dissolved in sterile deionized distilled water. OD_{260}/OD_{280} of the purified DNA was 1·84. The amount of DNA loaded on agarose gel was about 40–60 ng/well.

2.2 *Removal of lipids from alga cells*

Ten ml choloroform/methanol (2 : 1, v/v) was added to precipitated alga cells (0·6 g) (Matsuo *et al* 1995). After drastic votexing, cells were pelleted by centrifugation. Lipids extraction was repeated 3 times. Finally, the pellet was washed with 95% ethanol and dried at 37°C.

2.3 *Heat treatment process*

DNA or alga cells were bubbled with air or nitrogen gas for 10 mim in Eppendorf tubes. Then the tubes were sealed with laboratory film and heated in water bath at variant temperatures for 90 min.

2.4 *Determination of reactive oxygen species*

Reactive oxygen species (ROS) participation in heat-induced DNA degradation was determined by examining the protective effect of variant ROS scavengers such as NaN_3 and histidine (singlet oxygen quenchers), dimethyl sulfoxide (DMSO), glycerol and mannitol (hydroxyl radical scavengers) and the superoxide anion radical quencher of 2,2,4,6-tetramethyl-1-piperidinyloxy (TEMPO) (Ewing and Damasker 1995; Müller-Breitkreutz *et al* 1995; Laight *et al* 1997; Zhang *et al* 1997). By monitoring the breakage of DNA strand, it could be inferred that which ROS should be involved in the DNA degradation.

2.5 *1,3-Diphenyl-isobenzofuran bleaching experiment*

In a separate experiment, 1,3-diphenyl-isobenzofuran (DPBF) was used to detect the singlet oxygen generation in heating process. In the dark, DPBF was added into air- or nitrogen-saturated water to reach an identical final concentration (about 10^{-5} mol/l). The DPBF solutions were then incubated in water bath for 30 min at different temperature. $^1O_2$ generation was determined by measuring the DPBF amount using its maximum absorption (Young *et al* 1971; Lagorio *et al* 1989). It was repeated 3 times to obtain an average value.

2.6 *Polymerase chain reaction*

Two DNA segments were amplified using the heat-treated genomic DNA as template. The 889 bp fragment of the *arg*B gene, coding for N-acetyl glutamate kinase (Gene bank accession No. AY354518), was amplified using the primers: 5′-GGATCCGATCGCGTTCGCATTCT CAG-3′ and 5′-AAGCTTCAGGTCGACGGATCGCTC-3′. All 35 polymerase chain reaction (PCR) cycles were performed according to the following temperature regimen: 94°C for 40 s, 58°C for 30 s and 72°C for 70 s. The 128 bp segment of the *mut*S gene, coding for DNA mismatch repair protein (Gene bank accession No. AY191320·1), was amplified using the primers: 5′-CTAGTCATCGT GCGATCGAG-3′ and 5′-ACCTATTCGCTGTCGGAG TTA-3′. All 35 PCR cycles were performed according to the following temperature regimen: 94°C for 30 s, 52°C for 30 s and 72°C for 30 s.

2.7 *Determination of the DNA degradation*

Agarose gel electrophoresis was used to detect DNA degradation by observing the change of the DNA band and smear.

3. Results

3.1 *Isolated DNA degradation*

To investigate the effects of oxygen and heat on DNA

thermal degradation, heat-treated DNA was run on agarose gel. At 70°C, no evidence of DNA degradation was observed for oxygen-free sample while the air-saturated sample produced a smear. At 85°C, when air-saturated DNA was fragmented to small pieces, there were still fragments with maximum size of about 7000 bp for deoxygenated sample. But at 100°C, under both conditions, DNA was degraded to much smaller pieces to produce a similar smear (figure 1a). It is suggested that DNA is fragmented to small pieces gradually with elevated temperature and oxygen can aggravate the DNA degradation. When the temperature increased above 85°C, heat turned to be more effective in degrading DNA. At 100°C, compared with the prominent influence of heat, the oxygen effect can almost be ignored.

The 889 bp and 128 bp segments were amplified to clarify the feasibility of single gene retrieval from thermally degraded DNA. When genomic DNA was incubated at 85°C, the 889 bp segment could be amplified under nitrogen saturation but not under air saturation, suggesting that the aggravation of DNA thermal degradation by oxygen resulted in a PCR block. At 100°C, the 889 bp segment could not be amplified under both conditions (figure 1b). However, after incubating genomic DNA at 100°C for 3 h, the 128 bp segment could still be amplified under air saturated condition (data not shown). The result illustrates that the small DNA segment is easily amplified. Surprisingly, the 889 bp segment could not be amplified when the template DNA was heated at 85°C under air saturation though 1 kb-sized DNA fragments still existed.

An attempt was also made to evaluate the influence of heating time on DNA thermal degradation. For this purpose, isolated genomic DNA was incubated at 50°C and 70°C for different time periods. It was found that, at 50°C, no observable DNA strand breakage occurred up to 72 h. However, at 70°C, DNA damage aggravated with extended time (figure 2). This result suggests that the effect of heating time on the DNA degradation is temperature-dependent. The DNA degradation would not aggravate as time extended until it reached a certain temperature.

To investigate the influence of DNA strand length on DNA thermal degradation, the genomic DNA (about 40 kb) and the amplified 889 bp PCR product were incubated at 70°C, 85°C and 100°C for 90 min. The gel electrophoresis showed that the strand length had little effect on the DNA degradation (data not shown).

3.2 *Cellular DNA degradation*

Heat-induced cellular DNA degradation was designed to demonstrate its difference from that of isolated DNA. When samples were heated at 70°C, the DNA band could be detected for isolated DNA (figure 1a) but could not for cellular DNA. However, in lipids-removal samples, it could be detected after treatment at 85°C (figure 3a). On the other hand, the 889 bp segment could be amplified from lipids-removal samples but could not from lipid-containing samples under the same treatment (figure 3b). These results suggest that cellular DNA is more damage-

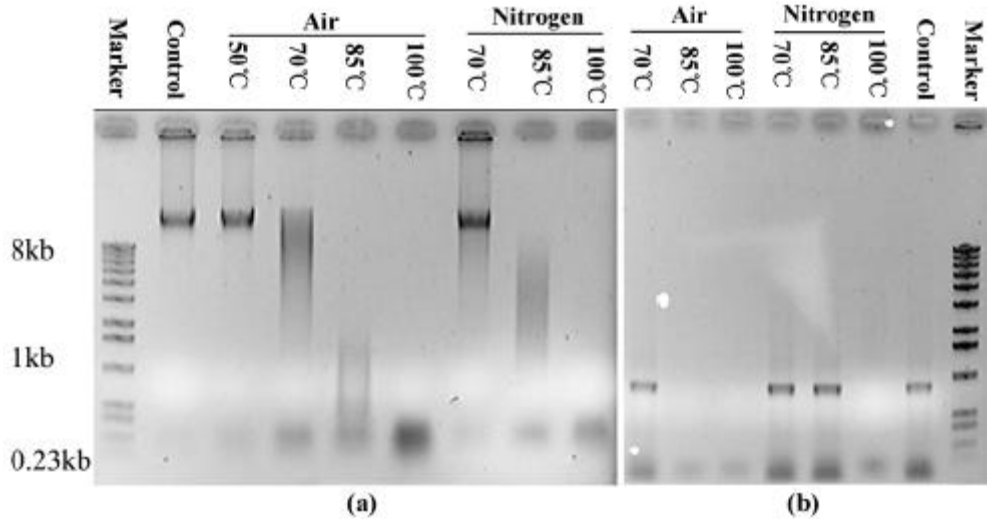

Figure 1. (**a**) Genomic DNA electrophoresis after heat treatments. (**b**) *Arg*B gene amplification from heat-treated isolated DNA. Samples were saturated with either air or nitrogen gas and incubated at variant temperature for 90 min. The genomic DNA and the 889 bp PCR product are used as control separately in (**a**) and (**b**).

able than isolated one due to the existence of cellular lipids. It is consistent with the conclusion that cellular lipids aggravate the DNA degradation in dried tissues (Matsuo *et al* 1995).

3.3 *Oxygen-mediated DNA thermal degradation*

In an attempt to characterize the oxygen influence on DNA thermal degradation, the participation of ROS in this process was investigated by examining the protective effect of variant ROS scavengers. Sodium azide and histidine could completely protect DNA from thermal degradation, but no detectable protective effect was found in the presence of glycerol, DMSO, mannitol and TEMPO (figure 4), suggesting that oxygen takes part in DNA thermal degradation via singlet oxygen but not other ROS such as hydroxyl radical and superoxide anion radical.

Possible formation of singlet oxygen in heating process was further investigated by DPBF bleaching experiment. DPBF, as a high efficient trapping agent of singlet oxygen,

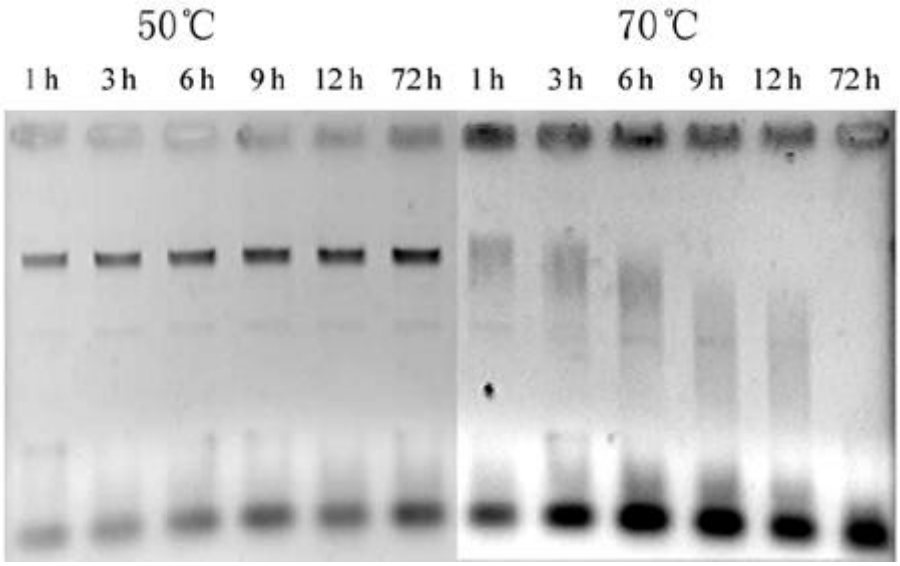

Figure 2. Time effect upon DNA thermal degradation. Isolated genomic DNA was incubated at 50°C and 70°C for variant time periods: 1 h, 3 h, 6 h, 9 h, 12 h and 72 h.

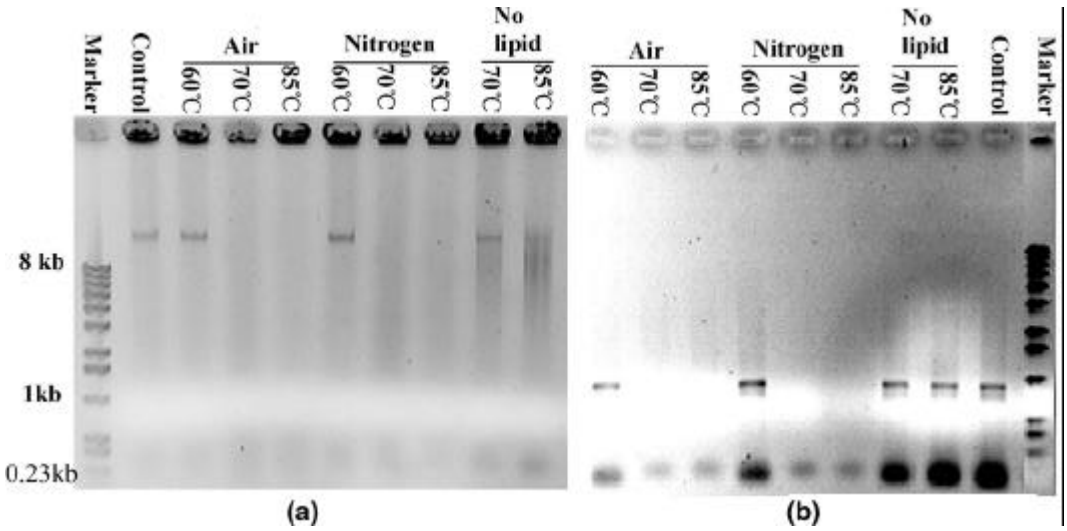

Figure 3. (**a**) Electrophoresis of heat-treated cellular DNA. (**b**) *Arg*B gene segment amplification from heat-treated cellular DNA. Air or nitrogen gas saturated samples were incubated at variant temperature for 90 min. The genomic DNA and the 889 bp PCR product are used as control separately in (**a**) and (**b**).

has a maximum absorption at 419 nm in water (data not shown). As temperature increases, the absorption decreased quickly under air saturation compared with that under nitrogen saturation (figure 5). It is suggested that at low temperature, the quantum yield of singlet oxygen is low, and the yield increases concomitantly with elevated temperature. The decline of the absorption under nitrogen saturation probably results from the existence of residual oxygen.

4. Discussion

In this work, we have investigated several related factors influencing DNA degradation in aqueous solution. Compared with other influencing factors on DNA thermal degradation, the influence of temperature appears to be the central one (figure 1–3).

Bases release or bonds breakage in DNA strands need definite level of energy (e.g. depurination active energy, *Ea* = 127 kJ/mol) (Lindal and Nyberg 1972) above which the electron vibration can overcome the nuclear bondage and result in bond breakage. And below that level, no bond breakage would happen even that DNA is heated for a long time. This can explain the result that DNA degradation aggravates with extended heating time at 70°C but not at 50°C (figure 2).

In our experiment, the strand length does not show any influence on DNA thermal degradation (data not shown). It is probably due to heat-induced random strand breakage and random distribution of the frangible bases.

ROS-mediated lipid peroxidation is often the late stage and has been considered to be closely related to DNA degradation (Zastawny *et al* 1995). Lipid peroxides and its further products can induce nucleic DNA damage. Furthermore, the lipid peroxidation is particularly more damaging than ROS because the products of the lipid peroxidation lead to a facile propagation of free radical reactions (Yang *et al* 2003). This can account for the enhancement of the DNA thermal degradation by cellular lipids (figure 3).

Since other ROS quenchers cannot protect DNA from thermal degradation except the well known singlet oxygen quenchers (figure 4). Singlet oxygen is affirmatively involved in heat-induced DNA degradation though ROS other than singlet oxygen cannot be ruled out.

Singlet oxygen has an excitation energy of 0·98 eV (Chan *et al* 2003), so its generation must be temperature-dependent in heating process. At low temperature, little singlet oxygen can be generated as suggested in our experiment (figure 5).

Using PCR, the 889 bp segment could be retrieved from genomic DNA incubated at 70°C but could not from that incubated at 85°C (figure 1). It might be explained by the severe DNA damage at high temperature which results in the scarcity of the required template. We failed to amplify the 889 bp segment in the presence of the 1 kb-sized template, probably due to the bases damage or

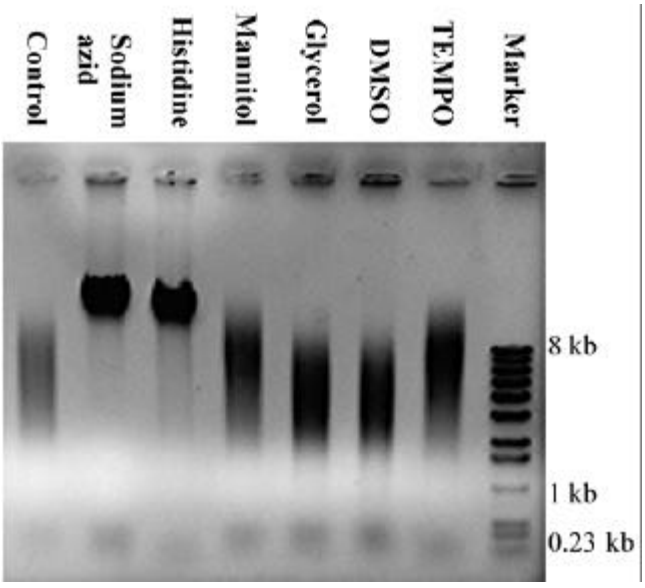

Figure 4. ROS determination in heat-induced DNA degradation. Samples mixed with the following substances were all incubated at 75°C for 90 min: control, sodium azide (20 mmol/l), histidine (20 mmol/l), mannitol (50 mmol/l), glycerol (80% v/v), DMSO (80% v/v) and TEMPO (0·5 mmol/l).

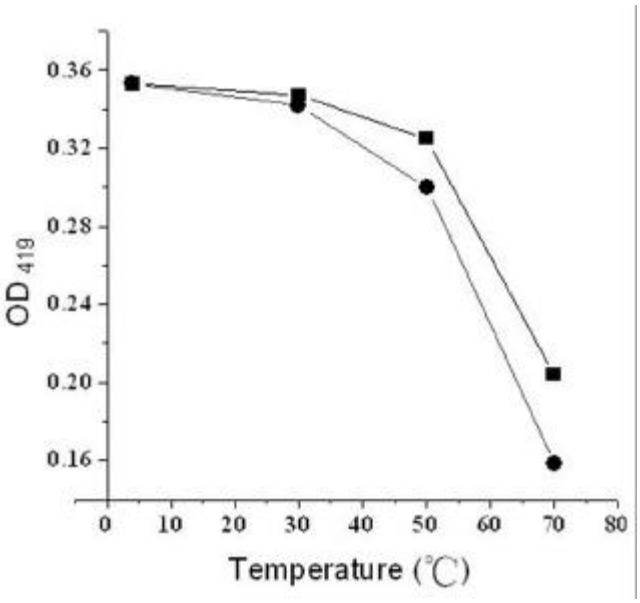

Figure 5. DPBF bleaching experiment. DPBF solution saturated with nitrogen gas or air were incubated for 30 min at variant temperature. (■) Under nitrogen saturation; (●) under air saturation.

bases loss that further resulted in PCR blockage. In contrast to the failure to amplify the 889 bp DNA segment, amplification of the 128 bp segment from genomic DNA after incubation at 100°C for 3 h indicated that a small DNA segment can still be amplified (data not shown). However, to retrieve too small a segment is of little significance since a vast amount of sequence information is needed to resolve key questions such as those of phylogenies and characterization of genes. The evidence to amplify the 889 bp segment from thermally degraded DNA can shed some light on single gene retrieval from ancient remains.

PCR is a powerful method to amplify a DNA segment, but it would be very difficult to retrieve a DNA segment from ancient remains especially those deeply buried. Heat can trigger DNA base release (Lindahl 1993) or oxidative damage to bases (Bruskov *et al* 2002) that can result in a blockage or a false result in DNA amplification. The fidelity with which thermally damaged DNA is amplified should be further investigated even though ancient DNA could be extracted under good conditions, especially at high temperature.

To sum up, temperature has a significant effect on the recovery of ancient DNA, though other factors also affect DNA degradation. Under the earth, temperature is closely related with depth. So, based on known geothermal gradients and the depth of ancient remains, one can try to estimate the chances of retrieval of single genes.

Acknowledgements

We thank Prof. Gerard Peter Latawiec (University of Reading, UK) for his critical reading of the manuscript. This work was supported by the NSFC key project 40332022.

References

Bruskov V I, Malakhova L V, Masalimov Z K and Chernikov A V 2002 Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA; *Nucleic Acids Res*. **30** 1354–1363

Chalfoun D J and Tuross N 1999 Botanical Remains: Utility in Protein and DNA Research; *Ancient Biomolecules* **3** 67–79

Chan S P, Chen G, Gong X G and Liu Z F 2003 Oxidation of carbon nanotubes by singlet O_2; *Phys. Rev. Lett*. **90** 086403

Dunlop D J, Özdemir Ö, Clark D A and Schmidt P W 2000 Time–temperature relations for the remagnetization of pyrrhotite (Fe_7S_8) and their use in estimating paleotemperature; *EPSL* **176** 107–116

Ewing D and Damasker K E 1995 Hydroxyl radicals and DNA double-strand breaks in X-irradiated *Escherichia coli*.; *Biochem. Biophys. Res. Commun*. **207** 957–962

Foulger G R 1995 The hengill geothermal area, iceland: variation of temperature gradients deduced from the maximum depth of seimogenesis; *J. Volc. Geotherm. Res*. **65** 119–133

Lagorio M G, Dicelio L E and San Román E A 1989 Quantum yield of singlet molecular oxygen sensitization by copper(II) tetracarboxyphthalocyanine; *J. Photochem. Photobiol. B* **3** 615–624

Laight D W, Andrews T J, Haj-Yehia A I, Carrier M J and Änggård E E 1997 Microassy of superoxide anion scavenging activity in vitro; *Environ. Toxicol. Pharmacol*. **3** 65–68

Lindahl T 1993 Instability and decay in the primary structure of DNA; *Nature* (*London*) **362** 709–715

Lindal T and Nyberg B 1972 Rate of depurination of native deoxyribonucleic acid; *Biochemistry* **11** 3610–3618

Matsuo S, Toyokuni S, Osaka M, Hamazaki S and Sugiyama T 1995 Degradation of DNA in dried tissues by atmospheric oxygen; *Biochem. Biophys. Res. Commun*. **208** 1021–1027

Müller-Breitkreutz K, Mohr H, Briviba K and Sies H 1995 Inactivation of viruses by chemically and photochemically generated singlet molecular oxygen; *J. Photochem. Photobiol. B* **30** 63–70

Poinar H, Kuch M, McDonald G, Martin P and Pääbo S 2003 Nuclear gene sequences from a late pleistoncene sloth coprolite; *Curr. Biol*. **13** 1150–1152

Poinar H N, HÖss M, Bada J L and Pääbo S 1996 Amino acid racemization and the preservation of ancient DNA; *Science* **272** 864–867

Reiners P W, Brady R, Farley K A, Fryxell J E, Wernicke B and Lux D 2000 Helium and argon thermochronometry of the Gold Butte block, south Virgin mountains, Nevada; *EPSL* **178** 315–326

Sauer J, Schreiber U, Schmid R, Völker U and Forchhammer K 2001 Nitrogen starvation induced chlorosis in *synechococcus* PCC 7942, low-level photosynthesis as a mechanism of long-term survival; *Plant Physiol*. **126** 233–243

Threadgold J and Brown T A 2003 Degradation of DNA in artificially charred wheat seeds; *J. Archaeol. Sci*. **30** 1067–1076

Yang Y S, Sharma R, Sharma A, Awasthi S and Awasthi Y C 2003 Lipid peroxidation and cell cycle signaling: 4-hydroxynonenal, a key molecule in stress mediated signaling; *Acta Biochim. Pol*. **50** 319–336

Young R H, Wehrly K and Martin R L 1971 Solvent effects in dye-sensitized photooxidation reactions; *J. Am. Chem. Soc*. **93** 5774–5779

Zastawny T H, Altman S A, Randers-Eichhorn L, Madurawe R, Lumpkin J A, Dizdaroglu M and Rao G 1995 DNA-base modifications and membrane damage in cultured-mammalian-cells treated with iron ions; *Free Radical Biol. Med*. **18** 1013–1022

Zhang X S, Rosenstein B S, Wang Y, Lebwohl M and Wei H C 1997 Identification of possible reactive oxygen species involved in ultraviolet radiation induced oxidative DNA damage; *Free Radical Biol. Med*. **23** 980–985

MS received 9 December 2004; accepted 3 September 2005

*e*Publication: 11 October 2005

Corresponding editor: VIDYANAND NANJUNDIAH