DNA photolyases that catalyze light-dependent repair of cyclobutane pyrimidine dimers (CPDs) were extracted and partially purified from sorghum and cucumber. The action spectra of CPD photolyases in these plant species had a maximum at 400 nm, which differ from those in Drosophila, Escherichia coli and Anacystis.

Key words: Action spectrum — Cucumber — Cyclobutane pyrimidine dimer — DNA photolyase (EC 4.1.99.3) — Sorghum.

Ultraviolet (UV) radiation in sunlight induces DNA damage, such as the formation of cyclobutane pyrimidine dimers (CPDs). Repair of DNA damage to prevent the biological damage caused by UV light is an essential function in most organisms. Nucleotide excision repair involves endonucleolytic steps, release of damaged oligonucleotides and DNA resynthesis (Sancar 1994a). Photorepair directly reverses the dimerization of pyrimidines. The enzyme, CPD photolyase repairs CPDs by causing them to revert to normal bases using light energy (Sancar 1994b).

The genes encoding the CPD photolyases are widely distributed throughout the biological world. CPD photolyases are classified into classes I and II, based on amino acid sequence similarity. Both class I and class II CPD photolyases contain a two-electron-reduced FAD, which acts as the active-site cofactor. Class I photolyases are further subdivided into 5,10-methenyltetrahydrofolate (MTHF)-type and 8-hydroxy-5-deazaflavin (8-HDF)-type, which contain MTHF and 8-HDF as a second chromophore, respectively (Yasui et al. 1994). The photoreactivation mechanism of class I photolyases has been investigated in detail (Hearst 1995, Kim et al. 1991, 1992) and the crystal structure of MTHF-type photolyase from Escherichia coli was determined (Park et al. 1995). In contrast, investigation of the photoreactivation mechanism and cofactors of class II photolyase has been limited. The class II photolyase from Drosophila melanogaster has FAD and MTHF (Kim et al. 1996), while FAD and 8-HDF are included in the enzyme from an archaeabacterium, Methanobacterium thermoautotrophicum (Kiener et al. 1989). Yasui et al. (1994) reported that the enzyme from the eukaryote Potorous tridactylis includes FAD, although the second cofactor has not been detected in the enzyme.

DNA damage and repair in plants have been recently examined in a number of plant species, namely Arabidopsis leaves (Pang and Hays 1991), alfalfa cotyledons (Quaite et al. 1994), wheat leaves (Taylor et al. 1996) cucumber cotyledons (Takeuchi et al. 1996, 1998), rice leaves (Hidema et al. 1997), spinach leaves (Hada et al. 1998) and sorghum first internodes (Hada et al. 1999). The main pathway for the removal of CPDs in higher plants is thought to be photoreactivation. Although CPD photorepair activity has been measured in vitro in extracts of several plant systems (Langer and Wellmann 1990, Pang and Hays 1991, Buchholz et al. 1995, Hada et al. 1999), knowledge of photolyases in higher plants is limited. Recently, the photolyase gene of Arabidopsis has been reported to encode an amino acid sequence with significant homology to the class II photolyases identified in a number of prokaryotic and animal systems (Ahmad et al. 1997). However, no information is available concerning cofactors of the CPD photolyase and the photoreactivation mechanism in higher plants.

In the present study, to estimate the chromophores of CPD photolyases in higher plants, we extracted and partially purified CPD photolyases from sorghum and cucumber and determined the action spectra for photorepair of CPDs.

Seeds of Sorghum bicolor cv. Acme Broomcorn, 1991 crop from Kobe University Farm at Kasai, were soaked for 24 h in circulating tap water in a 24°C water bath, sown in vermiculite in pots, 8 x 8 x 7 (height) cm³ and grown for 3 d at 24°C. When the seedlings reached a height of ca. 7 cm, the first internodes were excised. Seeds of cu-
cucumber (Cucumis sativus L. cv. Hokushin) were germinated and seedlings were allowed to develop on wet paper towels for 5 d at 25°C in darkness. Cotyledons were excised from the seedlings.

Forty grams of the first internodes of sorghum were homogenized with 160 ml of an extraction buffer, 200 mM Tris-HCl buffer (pH 7.6) containing 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% (v/v) glycerol. The homogenate was filtrated through two layers of gauze to remove plant debris and fractionated with ammonium sulfate between 35 and 70% saturation. The ammonium sulfate precipitates were dissolved in a reaction buffer, 100 mM Tris-HCl buffer (pH 7.6) containing 100 mM NaCl, 1 mM EDTA and 1 mM DTT, desalted with a Sephadex G-25 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and further chromatographed with a linear gradient of NaCl on a blue-Sepharose column (φ1.5x8 cm, Amersham Pharmacia Biotech AB) equilibrated with the reaction buffer. In the case of cucumber photolyase, PX-buffer, 100 mM Tris-HCl buffer (pH 7.2) containing 2 mM EDTA, 20 mM 2-mercaptoethanol, 10 mM DTT and 20% (v/v) glycerol (Pang and Hays 1991) was used instead of the extraction buffer in preparation of sorghum photolyase. The ammonium sulfate precipitates were desalted with Bio-Gel P-6 DG column (φ1 x 20 cm, Bio-Rad Laboratories, CA, U.S.A.), passed through the DEAE-Sephacel column, (φ0.8 x 20 cm, Amersham Pharmacia Biotech AB) and chromatographed on a blue-Sepharose column. The active fractions from the blue-Sepharose column were combined and stored at −80°C until use and the enzyme preparations desalted with a G-25 spin column before use. To determine photolyase activity, 190 μl of the enzyme preparation was mixed with 500 ng of the substrate DNA which had been irradiated with UV light. The assay mixture was irradiated with monochromatic light at 25°C, and control samples were kept in darkness.

Monochromatic light was provided by the large spectrograph at the National Institute for Basic Biology, Okazaki, Japan (Watanabe et al. 1982). The enzyme sample was poured in a glass vial (φ1.4 x 1 cm) and each vial was irradiated with vertical incident light in the threshold sample box (Watanabe et al. 1982). Photon fluence rates were determined with a photon density meter (PFDM-200LX, Rayon Industrial Co., LTD, Tokyo, Japan). After irradiation, the reaction was stopped by adding 180 μl of the assay mixture to 570 μl of DNA extraction buffer [100 mM Tris-HCl, pH 8.0, 2% (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA and 0.2% 2-mercaptoethanol], which was then heated for 30 min at 60°C. An equal volume of chloroform-isoamylalcohol (24:1, v/v) was added to the reaction mixture, and the samples were mixed and centrifuged at 10,000 x g for 5 min. An aliquot (600 μl) of the upper phase was taken and mixed with an equal volume of isopropanol to precipitate DNA. After centrifugation, the pellet was washed with 70% ethanol, dried, and re-suspended in 1 ml of 10 mM phosphate-buffered saline, pH 7.4. The remaining amounts of CPDs in the substrate DNA were measured by enzyme-linked immunosorbent assay according to Mori et al. (1991) with a monoclonal antibody specific to CPDs, TDM-2.

For substrates, calf thymus DNA (Sigma Chemical Co., MO, U.S.A.) irradiated with a germicidal lamp (GL15, Toshiba, Tokyo, Japan; 50 J m−2) was used in the cucumber experiments. Salmon sperm DNA (1 mg ml−1, Sigma Chemical Co.) that had been irradiated with a UV-B lamp (TL40W/12, Philips Electronics, Amsterdam, Netherlands) through a WG295 cutoff filter (Schott Glass, Mainz, Germany) at a distance of 10 cm for 24 h was used in the sorghum experiments.

Ahmad et al. (1997) showed that CPD photolyase in Arabidopsis thaliana has a sequence similarity to class II photolyase in animal cells. Partial sequences of CPD photolyase genes from several higher and lower plants, including sorghum, had high homology with class II type photolyase (personal communication from G. Buchholz and E. Wellmann). From these findings, it has been widely assumed that plants have a class II CPD photolyase. However, there have been no reports on the action spectrum of CPD photolyases and their cofactors in higher
Amounts of CPDs repaired with sorghum photolyase were proportional to fluences at 400 nm at various fluence rates (18.5, 13.2 and 6.01 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and durations (20-120 min, Fig. 1), indicating that action spectra produced under these conditions obey the reciprocity law. Similar results were obtained with cucumber photolyase (data not shown). Dark repair was not observed in this experimental condition. From these results, the duration of irradiation producing fluence-response curves at each wavelength between 300 and 500 nm was set at 60 min.

Figure 2 shows typical data for fluence-response curves for CPD photorepair with sorghum photolyase at several wavelengths. No activity was observed at 300 nm and 500 nm. Similar results were obtained with cucumber photolyase. From these data, action spectra of these two photolyases were constructed and shown to be similar, having peaks at 400 nm (Fig. 3). We previously reported that the response spectrum for CPD photorepair in vivo in cucumber cotyledons had a broad peak from 375 to 425 nm (Takeuchi et al. 1998). This in vivo response spectrum was not exactly consistent with the action spectrum of cucumber photolyase shown in Fig. 3. The difference is probably due to scattering and absorption of irradiation light by cellular components other than DNA in the in vivo experiments.

Action spectra of CPD photolyase of the MTHF type in class I have been reported in \textit{E.coli} (Sancar et al. 1987), \textit{Saccharomyces cerevisiae} (Madden and Werbin 1974), \textit{Bacillus firmus} (Malhotra et al. 1994) and \textit{Neurospora}.
Arabidopsis CPD photolyase overexpressed in E. coli

The photorepair of damage to DNA have been reported for *M. thermoautotrophicum* that in class II from these findings, action spectra of the 8-HDF type in class I Arabidopsis blue light receptor CRY1 (Malhotra et al. 1999). In conclusion, we presumed that CPD photolyases in higher plants may have a second chromophore other than 8-HDF or MTHF, which remains to be identified.

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**References**


DNA photolyase from Arabidopsis thaliana contains FAD as a cofactor.


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