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Occurrence of ELISA Inhibitors, and Inhibitor-free Determination of Cyclobutane Pyrimidine Dimers in Japanese Cypress Leaves

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DNA samples extracted from Japanese cypress leaf tissues contain isopropyl alcohol-precipitable, high molecular weight compounds, which interfere ELISA for cyclobutane pyrimidine dimers (CPD). Removal of the compounds is achieved by DEAE ion-exchange column chromatography and improves the ELISA responses of the DNA. When extracting DNA repeatedly from the same leaf tissues, the DNA samples show CPD responses which increase with the order in sequential extraction, and hence for a reliable determination of DNA lesion a thorough extraction of DNA is required. Clearing these two problems it was demonstrated that CPD level was slightly higher in the leaves of trees growing under full sunlight than in those growing under UBV-cut sunlight.

Key words: Cyclobutane pyrimidine dimer (CPD), *Chamaecyparis obtusa*, DEAE column chromatograhy, DNA lesion, ELISA inhibitors, Solar UVB

INTRODUCTION

In the current trend of the stratospheric ozone depletion, determination of ultraviolet light B (UVB)-induced DNA lesions in forest tree leaves is very important from the view points of forest production as well as global environmental protection, but only limited data is available [1]. Previously when we were determining DNA lesion in tree seedlings exposed to UVB[1] we were perplexed to find it less under highly intensified UVB than moderately intensified conditions. Another peculiar phenomenon was that when extracting DNA in sequence from the same leaf tissues, the DNA damage rate increased as it went to the later extracts. For reliable determination of DNA lesion in such tissues these problems must be settled.

As possible causes of the peculiar phenomena it is conceivable that damaged DNA may be more difficult to be extracted than less damaged or non-damaged DNA. It may also be possible that an ELISA-inhibiting substance(s) may occur and be increased by UVB, and the substance(s) is extracted earlier in sequential extraction. The aim of this paper is to report the occurrence of ELISA inhibitors, and the amount of DNA lesion determined excluding the interference of the inhibitors.

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CPD, cycobutane pyrimidine dimmer; DEAE-cellulose, diethylaminoethyl cellulose; ELISA, enzyme-linked immunosorbent assay; UVB, ultraviolet light B;

MATERIALS AND METHODS

Seedlings of Japanese cypress (*Chamaecyparis obtusa* S. et Z.) grown from seeds in the seedling cultivation field at the nursery of Faculty of Agriculture, Shinshu University, were transferred to and grown in three UVB-controlled test sections from May, 1999 to May, 2001 before harvesting leaves. Two of the three sections were covered with a sheet of cellulose acetate film (A. Warne & Co., Ltd.,) and a sheet of Lumilar film (Lumilar 100-T60, PANAC Co., Ltd.), respectively, leaving the four sides open to allow the air to pass through freely, while the rest one section remained uncovered. Harvested leaves were sent with dry ice to Kobe Women's University, Kobe and stored at -39 C until extracting DNA.

DNA was extracted according to Doyle and Doyle's CTAB method [2], except that leaves were homogenized at room temperature without heating a mortar and a pestle. Separation of ELISA inhibitors and DNA was carried out by passing through a column of DEAE cellulose. Routinely it was done with a TOYOPAK DEAE s cartridge (TOSOH Corporation, Tokyo, cat. #08591). A DNA sample dissolved in 100 ml TE buffer (10 m*M* Tris buffer, pH 7.4, and 1 m*M* EDTA 2Na salt) was loaded onto a cartridge, then eluted by 10 m*M* sodium phosphate buffer, pH 8.3. The first eluate of 800 μl contained P1, and N3 was collected by eluting with 800 μl of the buffer containing 0.5 *M* NaCl, and finally DNA was collected in the eluate of 800 μl buffer containing 1.0 *M* NaCl.

ELISA was carried out using the mouse anti-CPD monoclonal immunoglobulin, TDM-2 [3] as the primary antibody and peroxidase-conjugated rabbit anti-mouse

immunoglobulins (code No. P 0260, lot 097, DAKO, Glostrup, Denmark) as the secondary antibody . The other general procedure accorded with Mori et al. [3]. The standard DNA was salmon sperm DNA (Sigma) UVB-irradiated and supplied by Prof. E. Wellmann, Freiburg University, Freiburg, Germany.

RESULTS AND CONCLUSIONS

Figure 1 shows an elution profile from a DEAE column of a DNA sample extracted from Japanese cypress leaves. ELISA inhibitors were detected by applying eluates together with the standard damaged salmon DNA (upper curve), and DNA was located by applying eluates alone to ELISA (lower curve). Two inhibitors, P1 and N3, were separated from DNA, eluted, respectively, by plain buffer, by 0.1 *M* to 0.6 *M* NaCl, and by 0.7 *M* to 1.0 *M* NaCl. Separation between N3 and DNA was best achieved at pH 8.3 among the tested pHs 9.0, 8.3, 7.4, and 5.0 (data not shown).

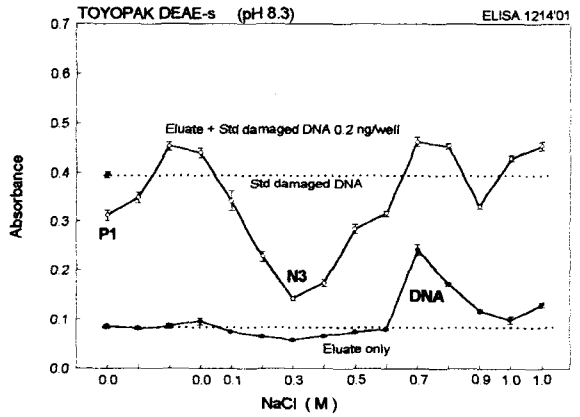

Figure 1. Elution profile of DEAE anion exchange column chromatography with a DNA sample extracted from Japanese cypress leaves. Eluted by 10 m*M* sodium phosphate buffer (pH 8.3) containing NaCl at the concentrations indicated on the abscissa. Column, TOYOPAK DEAE s (TOSOH Corporation, Tokyo, Japan); Eluate of 160 µl each was collected. An aliquot of each eluate, 164 ng DNAeq/well, was applied alone (lower curve), or with 0.2 ng/well of damaged std. DNA (upper curve). DNAeq. implies the relative amount of an inhibitor indicated by the amount of DNA which coexisted before separation. Short bars at each data point, S.E. from values of 4 wells.

The above-stated findings allowed us to establish the routine separation method as described in Materials and Methods. The efficiency of the method was confirmed by re-chromatographing each of the eluates by 0.5 *M* and 1.0 *M* NaCl, followed by ELISA in the same manner as above (data not shown), and the absence of significant cross-contamination between N3 and DNA was confirmed.

P1 and N3 in eluates from a DEAE column could be desalted and concentrated with a Centricon-10 (MW 10,000-cut-off, AMICON, Inc., Beverly, MA, USA) and be precipitated by addition of an equal volume of isopropyl alcohol. Recovery of both inhibitors were almost 100% in both methods.

Next, effects of P1 and N3 on the CPD response of cypress DNA as well as of the standard DNA were tested (Fig. 2). Comparison of the shapes of the curves suggests that the modes of inhibition are different between cypress DNA and std. DNA as well as between P1 and N3. Both inhibitors are more effective against std. DNA than cypress DNA. On std. DNA the effect of N3 is thoroughgoing, whereas that of P1 is only partial.

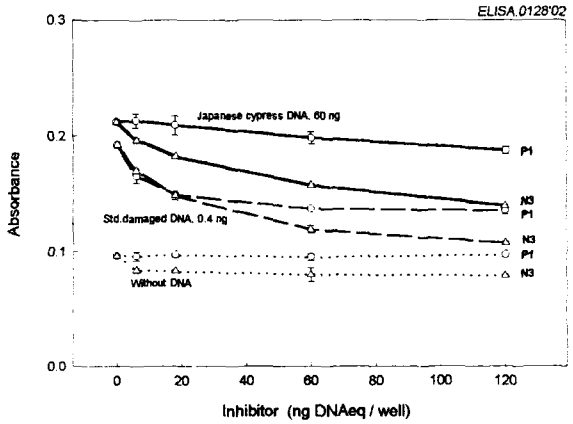

Figure 2. Effects of inhibitors P1 (○) and N3 (△) on CPD responses of 60 ng/well of purified cypress DNA (solid line) and 0.4 ng/well of std. DNA (broken line), and in the absence of DNA (dotted line). Abscissa, doses of P1 and N3 in DNAeq/well.

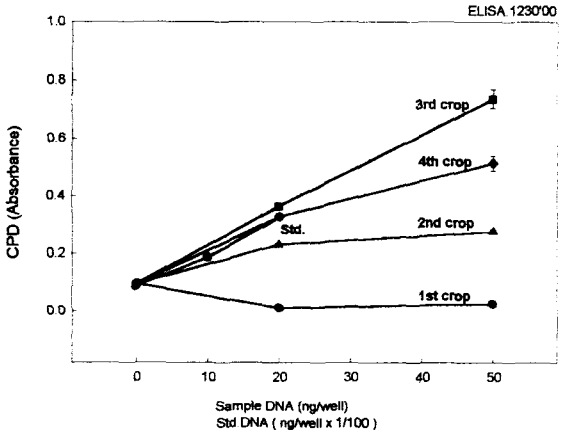

Figure 3. CPD responses of crude DNA samples extracted sequentially from the same cypress leaf tissues. The curve std. represents CPD response of std. DNA. Abscissa, real amount of DNA/well.

In repeated extraction of the same sample of cypress leaves, the sequential extracts had CPD response which increased from the first to third extract, followed by a decrease in the fourth extract (Fig. 3), although the yields of DNA was the highest in the first extraction, and decreased as it came to the later extraction. In view that the low responsiveness of earlier extracts in a sequential extraction may be ascribable to the presence of ELISA inhibitors, the first and second extracts were, respectively, deprived of the inhibitors to test for CPD response (Fig. 4). As a result, the CPD responses of both DNA samples were raised, but the level of the first extract could not reach that of the second extract and hence, only the occurrence of inhibitors is not the cause of the low CPD response of the first extract, but besides, there exists the difference in CPD response among the DNA samples repeatedly extracted from the same tissues.

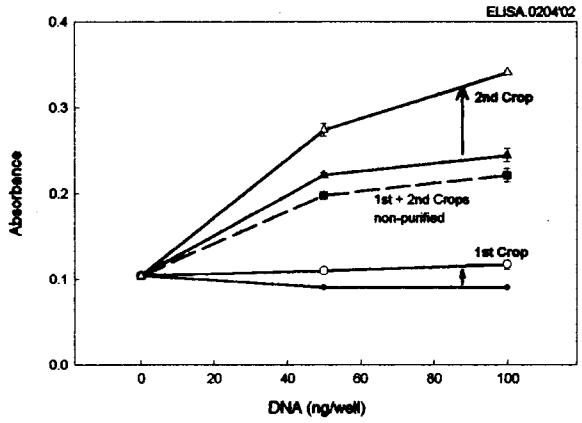

Figure 4. (1) Improvement by removal of the ELISA inhibitors of CPD responses of the first and second crops of DNA samples extracted from cypress leaf tissues. Indicated by arrows. (2) Lowering of CPD response of the non-purified second crop by addition of the non-purified first crop (broken line).

The heterogeneity of DNA is an interesting, but perplexing findings. As a possible cause of it, damaged DNA may be localized in cells which are more lignified by UVB action [4] and more difficult to crush. In order to determine CPD content representing a tissue, all the DNA of the tissue must be extracted.

Taking account of these findings we attempted to estimate the CPD in cypress leaves due to the solar UVB. A thorough extraction of DNA was made by repeating extraction four times, the last extraction giving less than 3% of the total amount of DNA. The four extracts were combined and deprived of the ELISA inhibitors before being applied to ELISA. The results were (Fig. 5) that the CPD response was slightly higher in the full sunlight section than in the sections covered with cellulose acetate film or Lumilar film. Between the latter two sections no difference was found. Whether the determined CPD responses are truly CPD needs to be confirmed by, for example, examining the susceptibility to CPD photolyase, but the observed different CPD responses due to the different UVB treatments may indicate that the CPD responses represent in some way the effects of prolonged solar UVB exposure on DNA in cypress leaves.

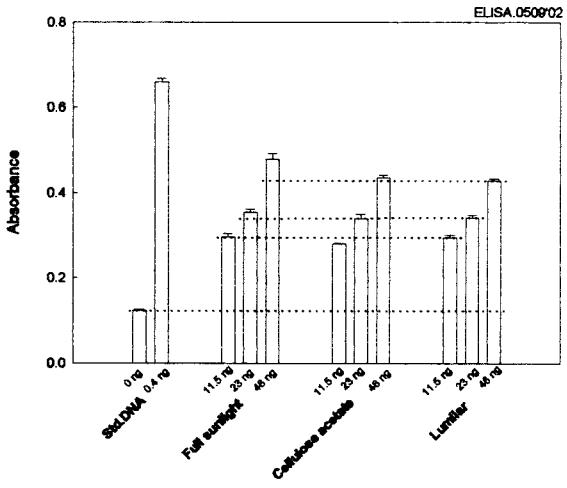

Figure 5. Effect of the solar UVB on the CPD responses of DNAs extracted from the leaves of cypress trees grown under full sunlight, covered with cellulose acetate film, and covered with Lumilar film. Left-most bars, background and std. DNA. Numerals under each bar, the amount of DNA/well.

In conclusion, the leaves of Japanese cypress trees growing under sunlight contain high molecular weight ELISA inhibitors, which suppress CPD responses on ELISA. The inhibitors can be separated from DNA with a DEAE ion-exchange column. For reliable CPD determination by ELISA, a thorough extraction of DNA and removal of the inhibitors are required. In this way, the CPD response of DNA was shown to be slightly, but definitely higher in cypress trees grown under the full sunlight than those under UVB-cut sunlight.

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