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We have shown that a chlorophyll sample from *Chlorella* inhibits direct-acting mutagenicity of *N*-hydroxyl Trp-P-2 in the Salmonella assay and that it suppresses the wing spot formation in *Drosophila* caused by Trp-P-2 (Negishi et al., 1989). In this experiment, the chlorophyll sample was given to *Drosophila* orally, together with the mutagen; it is a model closer to the real setting for humans than the bacterial assay system. Therefore, we decided to extend the study on chlorophylls using the *Drosophila* assay, and now report here the results of exploring the effect of several chlorophyll preparations on the genotoxic action of 4-nitroquinoline 1-oxide (4NQO).

2. Materials and methods

2.1. General

4NQO was purchased from Wako Chemicals (Osaka, Japan). Copper chlorophyllin Na₃ was a product of Nacalai Tesque (Kyoto, Japan).

2.2. Chlorophyll samples

Finely cut spinach (1 kg) was extracted with acetone-H₂O (8:2, 5.5 liters). The acetone solution was subjected to liquid-liquid extraction with hexane (1 liter). The hexane phase, into which chlorophylls were transferred from the acetone, was washed with acetone-H₂O (1:1, 1 liter × 2) and was evaporated to dryness to give an oily 'crude chlorophylls'. This sample (4.5 g) contained 12.7% chlorophyll *a* and 3.8% chlorophyll *b*, as determined spectroscopically; the remaining mass consisted of carotenoids and degradation products from chlorophylls, such as pheophytin. This spectroscopic analysis was carried out according to the method described in the Official Methods of Analysis (AOAC, 1990).

This crude sample was subjected to a series of resin open-column fractionations: (1) Passage through an ion-exchanging Dia-ion WK-11 column to remove polar substances, and (2) absorption of chlorophylls to a Duolite S-761 column, which was then eluted successively with toluene (to elute pheophytin) and acetone to obtain chlorophylls. The purified sample thus prepared contained 63.4% chloro-

phyll *a* and 22.8% chlorophyll *b*. The yield of the 'purified chlorophylls' was 0.35 g from 2 g 'crude chlorophylls'.

Crude and purified samples of chlorophylls from *Chlorella vulgaris* were prepared similarly. The chlorophyll contents of *Chlorella* 'crude chlorophylls' were chlorophyll *a* 18.4% and *b* 1.2%, and those of 'purified chlorophylls' were chlorophyll *a* 60.7% and *b* 20.0%.

2.3. *Drosophila* wing spot test

The assay was done with *Drosophila melanogaster* in which wing-hair mutants resulting from somatic chromosomal recombinations and gene mutations are detected (Graf et al., 1983; Graf et al., 1984; Würgler and Vogel, 1986). Larvae were obtained by mating virgin females (*mwh* *ju*; *spa*^{mut}) and males (*flr*³/*TM3, Ser*) as described previously (Negishi et al., 1991). These lines of flies were generous gifts from Dr. K. Fujikawa of Kinki University. 150-200 larvae (84 ± 12 h old; the 3rd instar) were placed on the feeding medium in a culture bottle (Ø 25 mm × 105 mm). The bottle was allowed to stand at 25°C in the dark, until adult flies emerged. During this period, the larvae were fed the medium for 1 or 2 days before pupation. The chlorophyll-containing feeding medium was prepared as follows: to 1.5 g Formula 4-24 *Drosophila* medium of Carolina Biological Supply Co. (Burlington, NC, USA) was added 200 mg chlorophyll dissolved in a small amount of ethanol, and the mixture was homogenized by being ground in an agate mortar with a pestle. This food was then added to a 5-ml aqueous solution containing 3.8 mg 4NQO (dispersed into water from a stock ethanol/Tween 80 (2/1)-solution) that had been placed in the culture bottle, and the resulting slurry was thoroughly mixed. Other feeding media including the one with added 200 mg copper chlorophyllin (an aqueous solution of copper chlorophyllin was used) were prepared similarly.

Among the hatched flies, those with normal shape-wings were harvested. The wings were collected and mounted with Faure's solution (gum arabic 30 g, glycerol 20 ml and chloral hydrate 50 g in 285 ml water) on glass slides. Mutant cell clones on the wings were scored under a microscope. The spots

were classified into three groups, i.e., small singles, large singles, and twins, according to Graf et al. (1984). The results were interpreted by using the statistical analysis described by Frei and Würgler (1988).

3. Results and discussion

All the chlorophyll preparations showed inhibitory actions toward the spot formation by 4NQO with statistical significance ($P < 0.01$) (Table 1). There was no significant difference in the inhibitory activity among 'crude' and 'purified' samples of chlorophylls, and both spinach and *Chlorella* were effective to similar extents. Chlorophyllin showed inhibition at an extent similar to that of chlorophylls.

With respect to the mechanisms of the inhibition by chlorophylls and chlorophyllin, there seem to be several possibilities: (1) trapping of 4NQO by complex formation resulting in an accelerated passage of the mutagen through the digestive tract, and (2) disturbance of metabolic activation of 4NQO inside the body, either through enzyme inhibition or through degradation of activated species of the mutagen. In the case of inhibition against Trp-P-2, we presented spectroscopic data showing molecular interactions between chlorophyll and the heterocyclic amine (Negishi et al., 1989) and suggested that the trapping mechanism would be the one operating in this inhibition. Our recent experiments using an insoluble chlorophyllin-chitosan complex (Arimoto et al., 1993; and unpublished work) have shown that

chlorophyllin can adsorb 4NQO, although not as efficiently as for Trp-P-2.

Chlorophyllin is an inhibitor against *P*-450 enzymes in general (Yun et al., 1995). For metabolic activation of 4NQO, enzymes other than *P*-450s are involved (Bailleul et al., 1989). Whether chlorophylls and chlorophyllin can interfere with this enzymatic activity is subject to future studies. Furthermore, it is also to be studied whether chlorophyll or portions of its molecule are absorbed in the body at all.

As we have shown in this work, preparing quantities of crude chlorophylls is possible, although somewhat laborious. It would be important to study further the effects of chlorophylls towards mutagens and carcinogens.

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Table 1
Inhibition of *Drosophila* wing spots induced by 4-nitroquinoline 1-oxide (4NQO) by chlorophylls

Treatment ^a	No. of wings examined	No. per wing (% inhibition)			
		Small single spot	Large single spot	Twin spot	Total spot
4NQO only	173	1.89 (0)	2.25 (0)	1.16 (0)	5.31 (0)
4NQO + spinach 'crude chlorophylls'	139	1.37 (35)	0.72 (69)	0.24 (81)	2.32 (62)
4NQO + spinach 'purified chlorophylls'	212	1.38 (34)	1.25 (45)	0.64 (46)	3.27 (42)
4NQO + <i>Chlorella</i> 'crude chlorophylls'	186	1.21 (46)	1.06 (54)	0.39 (68)	2.66 (55)
4NQO + <i>Chlorella</i> 'purified chlorophylls'	182	1.25 (43)	1.19 (48)	0.42 (65)	2.86 (51)
Chlorophylls only ^b	100	0.33	0.01	0.02	0.36
4NQO + Cu-chlorophyllin	202	1.17 (48)	0.64 (73)	0.33 (73)	2.15 (65)
No addition	168	0.40	0.04	0.02	0.47

^a The doses of reagents were, 3.8 mg 4NQO (20 μmol), and 200 mg green pigments per bottle.

^b Purified chlorophylls from *Chlorella vulgaris* was used.



Antigenotoxic activity of natural chlorophylls

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Abstract

Chlorophyllin, a man-made water-soluble form of chlorophyll, is a focus of intensive studies from many laboratories for its antimutagenic and anticarcinogenic properties. Natural chlorophylls, in contrast, have been little studied in this regard. Since yellow-green vegetables are implicated to be protective against human cancers by epidemiological studies, it is important to explore the antigenotoxic properties of natural chlorophylls. Previously, we reported that a chlorophyll sample prepared from *Chlorella vulgaris* inhibited the mutagenicity of 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3-*b*]indole, a direct-acting mutagen, in *Salmonella*, and that the chlorophyll also showed inhibition of wing spot formation in *Drosophila* induced by 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2). We have now prepared several samples of chlorophyll from spinach and chlorella, and studied their effect on the genotoxicity of 4-nitroquinoline 1-oxide (4NQO) in *Drosophila*. The results showed that the genotoxicity of orally given 4NQO was suppressed by simultaneous administration of the chlorophylls. The mechanisms of this inhibition are discussed.

Keywords: Chlorophyll; 4-nitroquinoline 1-oxide; *Drosophila* wing spots; Antigenotoxicity

1. Introduction

Epidemiological studies have shown that ingestion of vegetables is protective against cancer in humans (Phillips, 1975). Carotenoids, vitamin E and fibers in plants have been implicated as anticarcinogenic agents (Committee on Diet, 1982; Hayatsu et al., 1988). Plant cells contain green and yellow pigments, the former being chlorophylls and the latter being carotenoids and xanthophylls. The ratio of the total green to total yellow pigments is approximately 3:1 (Kephart, 1955). The contents of chloro-

phylls in green vegetables are high: for example, spinach contains as much as 1% chlorophylls on a dry-weight basis (Chipchase, 1961). Early work by Lai et al. (1980) and Terwel and van der Hoeven (1985) has shown that vegetable extracts containing chlorophylls are antimutagenic in bacterial assay systems. Chlorophyllin, a stable, soluble derivative of chlorophyll, has then been intensively studied as an inhibitor to various mutagens (see Hayatsu et al., 1988 and Sarkar et al., 1994, for review). Recent work from several laboratories has demonstrated that chlorophyllin protects animals from reagent-induced tumorigenesis (Breinhold et al., 1995; Guo et al., 1995; Hasegawa et al., 1995; Park and Surh, 1996). In contrast to these extensive studies on chlorophyllin, little is known about chlorophylls regarding their actions as antimutagens and anticarcinogens.

Abbreviations: 4NQO, 4-nitroquinoline 1-oxide; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole

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