J. Food Sci. Nutr.
Vol. 3, No. 3, p. 277~281 (1998)

Screening for Korean Vegetables with Anticarcinogenic Enzyme Inducing Activity Using Cell Culture System

Su-Mi Kim, Seung Hee Ryu, Hui-Don Choi*, Sung-Su Kim*, Jeong-Hwan Kim** and Jong-Sang Kim†

Dept. of Food Science and Nutrition, Inje University, Kimhae 621-749, Korea
**Korea Food Research Institute, Seongnam 463-420, Korea*
***Dept. of Food Technology, Kyongsang National University, Jinju 660-701, Korea*

Abstract

There is extensive evidence suggesting the protective role of fruits and vegetables against chemically induced carcinogenesis. We have tested the ability of a representative range of Korean vegetables to act as blocking agents against neoplastic initiation by determining the induction level of quinone reductase, an anticarcinogenic marker enzyme, in hepa1c1c7 cells exposed to vegetable extracts. Among thirty vegetables tested, *Arctium lappa* (Burdock), *Brassica juncea* (Mustard leaf), *Pteridium aguilinum* (Bracken) and *Chrysanthemum coronarium* (Crown daisy) caused a significant induction of quinone reductase activity with a limited increase in arylhydrocarbon hydroxylase activity. Combination of crown daisy with burdock had synergistic effect on quinone reductase induction. Quinone reductase-inducing activity was found mostly in hexane and ethylacetate fractions of MeOH extract of crown daisy while it was not detected in n-butanol and water fractions. Animal study using SD rats demonstrated that crown daisy intake induced quinone reductase activity in liver, kidney, lung, and small intestine, confirming the presence of potent QR inducer(s) in crown daisy. These data suggest that some vegetables including crown daisy induced QR merits further investigation as a potential cancer preventive agent in human.

Key words: anticarcinogenic enzyme, quinone reductase, arylhydrocarbon hydroxylase, vegetables, chemoprevention

INTRODUCTION

Cancer can be prevented by an enormous number of chemical compounds including plant food constituents (1). Screening for possible chemopreventives has been performed in vitro cell culture system, in which anticarcinogenic enzyme, NAD (P)H: (quinone-acceptor) oxidoreductase (EC1.6.99.2), from cultured cells was assayed (2,3). Quinone reductase (QR) is a widely distributed, primarily cytosolic, dicumarol-inhibitable flavoprotein that catalyzes the reduction of a wide variety of quinones and their metabolic quinoneimines. QR protects cells against the toxicity of quinones and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to hydroquinones, which are then susceptible to glucuronidation by other phase II enzyme (4). In addition, QR is induced coordinately with other phase II enzymes including glutathione *S*-transferase and UDP-glucuronosyltransferase by a variety of compounds that protect rodents from the toxic, mutagenic, and tumorigenic effects of carcinogens. There is a great deal of evidence supporting that the induction of phase II enzyme is the predominant mechanism by which many heterogeneous compounds are chemopreventive, and thus the monitoring of QR induction is a convenient method for screening anticarcinogenic activity (5-9). The hepa1c1c7 cells culture model has been successfully used in the isolation of sulforaphane from broccoli, a promising chemopreventive agent.

This study was performed to screen vegetables with the cancer protective activity by examining the ability to induce QR activity in both cell culture and *in vivo* model systems. Most of vegetables produced in Korea have never been tested for their chemopreventive potential using hepa1c1c7 cell culture system even though some were investigated for the antimutagenicity, cytotoxicity against cultured cells, and anticarcinogenicity in animal models.

Induction of arylhydrocarbon hydroxylase (AHH), which is involved in phase I metabolism of xenobiotics and bioactivation of some procarcinogens such as benzo-(a)pyrene (10), by vegetable extracts was also examined to predict the chemopreventive effect more reliably.

MATERIALS AND METHODS

Preparation of vegetable extracts

Thirty kinds of vegetables used in this study were shown in Table 1. They were purchased from Karak agricultural produce market (Seoul, Korea), followed by immediate chopping and freeze-drying to minimize the possible loss of active component (s). Freeze-dried vegetables were powdered using cyclotec mill (Tecator, Sweden). Portions (100g) of these powders were extracted with 2 liters of 80% methanol for 12 hr at room temperature, and filtered through Whatman No. 1 paper. The filtrates were evaporated to less than 50 ml in a rotary evaporator (<40°C), and freeze-dried to give

†Corresponding author

Table 1. Vegetables used in the study

Root vegetables	Leafy vegetables
Burdock, *Arctium lappa*	Braken, *Pteridium aguilinum var. japonicum*
Carrot, *Daucus carota var.sativa*	Cabbage, *Brassica oleracea var. capitata*
Do-dok, *Codonopsis lanceolata*	Celery, *Apium graviolens*
Doraji, *Platycodon grandiflorum*	Chinese cabbage, *Brassica pekinensis gp.*
Garlic, *Allium sativum*	Chwi, *Pueraria thunbergiana*
Ginger root, *Zingiber officinale*	Crown daisy, *Chrysanthemum coronarium*
Korean Radish, *Raphanus sativus*	Green onion, *Allium fistulosum*
Lotus root, *Nelumbo nucifera*	Korean lettuce, *Lactuca sativa*
Mungbean sprout, *Pisum aureus*	Leek, *Allium tuberosum*
Onion, *Allium cepa*	Lettuce, *Lactuca sativa var. capitata*
	Mugwort, *Artemisia spp.*
	Mustard leaf, *Brassica juncea*
	Perilla leaf, *Sesamum indicum*
	Pumpkin young leaf, *Curcurbita moschata*
	Red cabbage, *Brassica oleracea var. capitata*
	Royal fern, *Osmunda japonica*
	Shepherd's purse, *Capsella bursapast*
	Spinach, *Spinacia oleracea*
	Water dropwort, *Nasturtium officinale*
	Wild garlic, *Allium monanthum*

powders. The powders were redissolved in 80% MeOH for cytotoxicity and enzyme assays.

Cell culture

Hepa1c1c7 cells were plated at density of 5×10^5 cells/plate in 10 ml of α-MEM supplemented with 10% charcoal-treated fetal bovine serum (α-MEM/FBS). The plates were grown for 3~4 days in humidified incubator in 5% CO_2 at 37°C. Hepa1c1c7 cells were cultured in the absence and presence of 80% methanol extract of freeze-dried vegetables, and then detoxifying enzyme activities were measured.

Cytotoxicity assay

5×10^3 cells/well were plated in wells of a 96-well plate with 200 ul α-MEM/FBS medium, and allowed to attach to the plate for 4 hr. Various levels of MeOH extract were added to duplicate wells. After 72 hr, the plates were gently shaken free of media and submerged in a vat containing 0.2% crystal violet and 2% ethanol for 10 min. The plates were then washed by soaking into water 3 to 5 times, and the retained dye was solubilized by warming the plates at 37°C incubator for 120 min with 200 ul/well of 0.5% SDS and 50% ethanol, measuring absorbance at 610 nm with a multiwell plate reader (UV max, MD, USA) (3).

Enzyme assays

QR activity was measured by the method described by Benson et al. (11) with a slight modification (8). Briefly, hepa1c1c7 cells were plated, grown, and exposed to MeOH extract (0.5~5.0 mg/ml) for 24 hr before cell harvest. The cells were washed with ice-cold 0.15 M KCl-10 mM potassium phosphate (pH 7.4), removed from the plates by scraping with rubber policeman, and disrupted for 5 sec using ultrasonic cell disrupter (50W, Kontes, Vineland, NJ, USA). Two 200 ul aliquots of the resulting 1.0 to 1.5 ml suspension were assayed for AHH as described previously (12). The remaining samples were centrifuged at 9,000×g for 20 min and assayed for QR activity by measuring the rate of oxidation of 2,6- dichlorophenolindophenol at 600 nm in the assay system containing 25 mM Tris-HCl (pH 7.4), 0.7 mg crystalline bovine serum albumin at pH 7.4, 0.01% Tween 20, 5 uM FAD, 0.2 mM NADH, 0 or 10 uM dicoumarol, and 200 ul cell extract in a final volume of 3.0 ml. Protein concentrations were determined by the method of Lowry (13).

Animal experiment

SD rats were obtained from Dae-Han Laboratory Animal Reserarch Center (Seoul, Korea) at 7 weeks of age. Each animal was housed in a stainless cage in a room at a temperature of 24±2°C and 55% humidity with a 12 hr light-dark cycle. They were maintained on Chow (ICN, Costa Mesa, CA, USA) and tap water *ad libitum* for 2 weeks before the start of the experiment. Starting at the age of 9 weeks, two groups of 10 rats each received AIN-76 powdered basal diet either alone or containing 10% crown daisy powder for 7 days. Animals were weighed and food intake was measured everyday. At the end of experimental period, animals were sacrificed by drawing blood from heart after being anesthetized. Liver, lung, kidney, and small intestine were removed, frozen in liquid nitrogen and stored at -70°C until the enzyme assays. QR and AHH activities of rat organs were measured by the same method described for cells.

Solvent fractionation of QR inducers from crown daisy

MeOH extract of crown daisy was fractionated with hexane, ethylacetate, n-butanol, and water sequentially. Each fraction was rotary-evaporated to dryness and redissolved in a small amount of 80% MeOH. Hepa1c1c7 cells were exposed for 24 hr to the each fraction at the concentration of 0.25~5.0 mg/ml medium and cytosolic QR activities assayed as described above.

RESULTS AND DISCUSSION

Cytotoxicity of vegetable extracts

The cytotoxicity of thirty kinds of vegetable extracts against hepa1c1c7 cells is shown in Table 2. The cells exposed continously to different concentrations of vegetable extracts showed a wide variation of sensitivity depending on vegetables, with highest sensitivity for green onion, perilla leaf, and wild garlic. However, most of MeOH extracts of vegetables showed a relatively low cytotoxicity. ED_{50} values,

Table 2. Cytotoxicity of vegetable extracts against Hepa1c1c7 cells

Root vegetables	ED_{50} (mg/ml)	Leafy vegetables	ED_{50} (mg/ml)
Burdock	>5.0	Bracken	1.25~2.5
Carrot	>5.0	Cabbage	2.5~5.0
Do dok	2.5~5.0	Celery	>5.0
Doraji	0.63~1.25	Chinese cabbage	>5.0
Garlic	2.5~5.0	Chwi	1.25~2.5
Ginger root	0.31~0.63	Crown daisy	>5.0
Lotus root	>5.0	Green onion	0.08~0.16
Mungbean sprout	>5.0	Korean lettuce	>5.0
		Leek	2.5~5.0
Onion	2.5~5.0	Lettuce	>5.0
Turnip	2.5~5.0	Mugwort	2.5~5.0
		Mustard leaf	>5.0
		Perilla leaf	0.08~0.16
		Pumpkin young leaf	>5.0
		Red cabbage	>5.0
		Royal fern	1.25~2.5
		Shepherd's purse	0.31~0.63
		Spinach	1.25~2.5
		Water dropwort	2.5~5.0
		Wild garlic	0.16~0.31

the concentration of the extracts required to inhibit cell growth to 50% of the untreated control, were used as an index for determining concentrations of the extracts to be added into cell culture for QR induction study.

Anticarcinogenic enzyme induction

Thirty kinds of vegetables were evaluated for chemopreventive potentials by determining quinone reductase induction, which is one of the cancer preventive markers, in hepa1c1c7 cells. As shown in Table 3, turnip, burdock, braken, cabbage, crown daisy, mustard leaf, red cabbage, and royal fern induced QR activity over 140% when cells were exposed to their 80% methanol extracts at 2.5 or 5.0 mg/ml for 24 hrs.

Since CYP 1A enzyme induction is possibly undesirable due to its association with the activation of many carcinogens to ultimate electrophiles, and hence counteract chemoprotection, AHH induction was examined for nine vegetable extracts induced QR significantly. Turnip, royal fern, cabbage and red cabbage led to over 4-fold induction in AHH activities (Fig. 1). Four vegetables including burdock, braken, crown daisy, and mustard leaf induced QR significantly and AHH in a relatively minor degree, were further investigated for dose-response relationship in hepa1c1c7 cells (Fig. 2). Among four kinds of vegetables examined, crown daisy showed the highest QR induction, with 1.6-and 1.7-fold increase at 2.5 and 5.0 mg/ml, respectively. Combination of crown daisy extract with burdock extract showed a synergistic effect on QR induction while the mixtures of mustard leaf and crown daisy extracts, burdock and mustard leaf extracts were not significantly effective (Table 4).

QR induction in rat fed crown daisy

Fig. 3 shows the induction pattern of QR and AHH at different organs of rats fed crown daisy powder at 10% level.

Table 3. Quinone reductase induction in Hepa1c1c7 cells by vegetable extracts

Root vegetables	Conc. (mg/ml)	Relative QR activity (%)
Burdock	5.0	145
Carrot	5.0	137
Do dok	2.5	121
Doraji	0.63	74
Garlic	2.5	90
Ginger root	0.31	102
Lotus root	5.0	78
Mungbean sprout	5.0	121
Onion	2.5	105
Turnip	2.5	161
Leafy vegetables	**Conc. (mg/ml)**	**Relative QR activity (%)**
Bracken	2.5	182
Cabbage	2.5	153
Celery	5.0	120
Chinese cabbage	5.0	147
Chwi	1.25	109
Crown daisy	5.0	160
Green onion	0.05	101
Korean lettuce	5.0	123
Leek	2.5	102
Lettuce	5.0	90
Mugwort	2.5	120
Mustard leaf	5.0 (2.5)	143 (185)
Perilla leaf	0.16	111
Pumpkin young leaf	5.0	113
Red cabbage	5.0	160
Royal fern	2.5	142
Shepherd's purse	0.31	105
Spinach	1.25	113
Water dropwort	5.0 (2.5)	183 (133)
Wild garlic	0.16	118

Table 4. Combination effects of vegetable extracts on cellular quinone reductase induction

Sample	Conc. (mg/ml)	QR induction (%)
Control	-	100
Mustard leaf / Crown daisy	5.0 / 0.0	153
	2.5 / 2.5	136
	3.3 / 1.7	133
	1.7 / 3.3	155
	0.0 / 5.0	168
Mustard leaf / Burdock	5.0 / 0.0	153
	2.5 / 2.5	163
	3.3 / 1.7	130
	1.7 / 3.3	174
	0.0 / 5.0	165
Bracken / Burdock	5.0 / 0.0	106
	2.5 / 2.5	138
	3.3 / 1.7	139
	1.7 / 3.3	170
	0.0 / 5.0	165
Bracken / Crown daisy	5.0 / 0.0	106
	2.5 / 2.5	133
	3.3 / 1.7	114
	1.7 / 3.3	127
	0.0 / 5.0	168
Crown daisy / Burdock	5.0 / 0.0	168
	2.5 / 2.5	166
	3.3 / 1.7	179
	1.7 / 3.3	203
	0.0 / 5.0	165

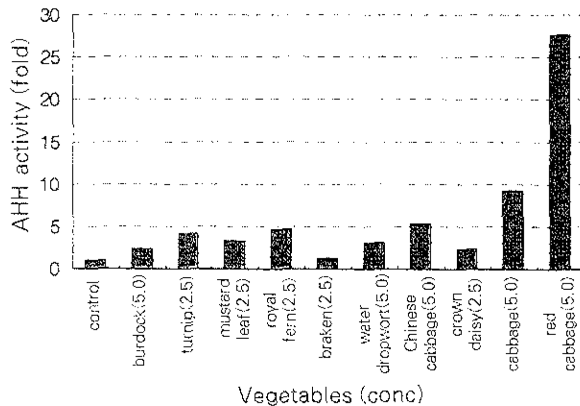

Fig. 1. Arylhydrocarbon hydroxylase induction by vegetable extracts. Hepa1c1c7 cells precultured for 72 hr were exposed to the extract at the concentration of 2.5 or 5.0 mg/ml for 24 hr, followed by the determination of AHH activity. Values in parentheses represent the concentrations of the extracts as expressed in mg/ml.

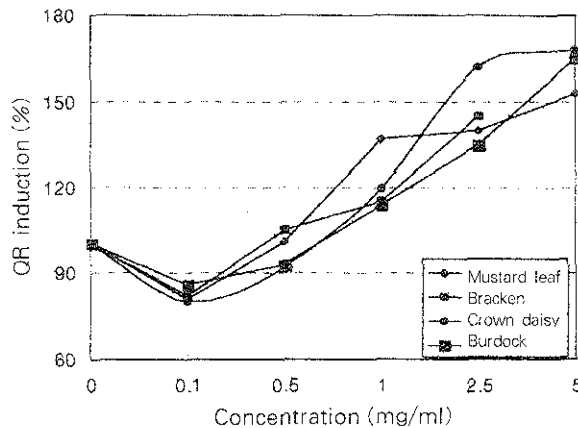

Fig. 2. Quinone reductase induction at different concentrations of vegetable extracts. Hepa1c1c7 cells precultured for 72 hr were exposed to the extract at the concentration of 0 to 5.0 mg/ml for 24 hr, followed by the determination of QR activity.

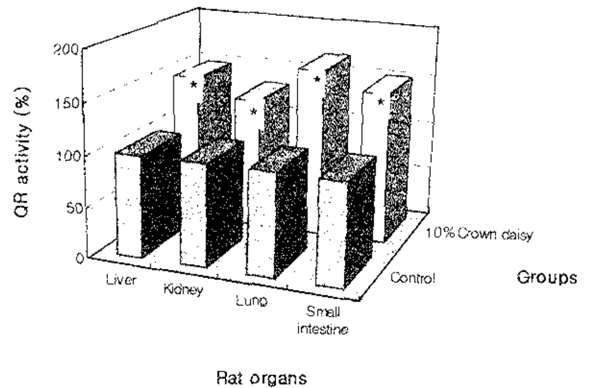

Fig. 3. Quinone reductase activities in liver, kidney, lung, and small intestine from rats fed crown daisy-containing diet. SD rats at 9 weeks of age were fed AIN-76 diet either containing or not containing 10% crown daisy powder for 7 days, followed by sacrifice and the assays of quinone reductase activities in organs. Crown daisy-containing diet was adjusted to have the same composition as the control.

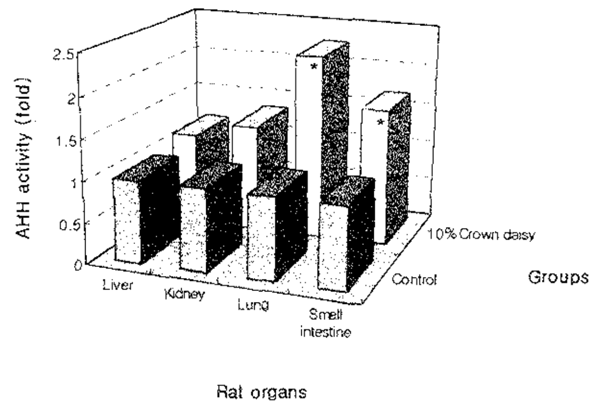

Fig. 4. Arylhydrocarbon hydroxylase activities in liver, kidney, lung, and small intestine from rats fed crown daisy-containing diet. See the note under Fig. 3 for experimental protocol.

Cytosolic QR activity in all organs examined was elevated by 30 to 67 percent over those of the control group while AHH activity was significantly increased in lung and small intestine alone (Fig. 4). These findings are consistent with the observation made in cell culture system.

The protective action of some vegetables against chemically induced carcinogenesis in rodents is well established (14). For instance, cruciferrous vegetables such as broccoli, cauliflower, brussel sprouts, and cabbage, are unique in their high content of dithiolthiones and isothiocyanates which have been shown to increase the activity of enzymes involved in the detoxification of carcinogens and other xenobiotics. Sulphoraphane, initially isolated from broccoli, appears to be one of the most potent inducers of phase 2 enzymes without affecting phase 1 enzymes (1). Resveratrol, a natural product derived from grape, was postulated to exert cancer-chemopreventive activity via induction of phase 2 enzyme as well as inhibiting cyclooxygenase and hydroperoxidase functions (15). Induction of phase 1 enzyme has been a conflicting theme because its advantageous and disadvantageous effects were reported. For instance, CYP1A1 is involved in not only bioactivation of procarcinogens to ultimate carcinogens but detoxification of lipophilic substrates that might otherwise accumulate to toxic concentrations within the cell. Considering the possible adverse effect of phase 1 enzyme induction, the vegetables excessively induced AHH were excluded from further study. Dose-response experiment demonstrated that crown daisy were the most active in QR induction with a limited induction of AHH in cell culture system. Animal experiment confirmed that crown daisy had a strong potential to induce QR in rats and thus might be a good candidate for cancer prevention. Even though rats in treatment group were fed relatively high dose (10% of diet) of crown daisy powder, rats did not show any apparent toxic symptoms in terms of food intake and weight

Table 5. Solvent fractionation of quinone reductase inducer(s) from crown daisy extract[1)]

Solvent fraction	Relative content(%)	Concentration (mg/ml)	Relative QR activity(%)
Control	-	-	100
Hexane	1.4	0.25[2)]	224
Ethylacetate	2.6	1	331
n-Butanol	25.6	5	108
Water	70.4	5	103

[1)]Hepa1c1c7 cells pre-cultured for 72 hr were exposed to each fraction for 24 hr, followed by QR assay.
[2)]The concentration above 0.25 mg/ml was too cytotoxic to perform enzyme assay.

gain (data not shown). Many substances present in vegetables and fruits are reported to help protect against cancer; these include dithiolthiones, isothiocyanates, allium compounds, isoflavones, protease inhibitors, saponins, folic acids, D-limonene, beta-carotene, polyphenols, lycopene, selenium, vitamin E, flavonoids, and dietary fiber (14). The majority of compounds including sulforaphane, oltipraz and ellagic acid exert chemopreventive effect by inducing phase 2 enzyme (1,16,17). Crown daisy is unique in terms that it belongs to *chrysanthemum* family, while most vegetables reported to have chemopreventive effect fell into *cruciferrous* and *allium* families. Although the component(s) responsible for QR induction in crown daisy remains to be identified, it must be highly hydrophobic as most QR-inducing activity was recovered in hexane and ethylacetate fractions (Table 5). Further fractionations using column chromatography and semi-preparative high performance liquid chromatography are in progress, and the identification of active component (s) responsible for QR induction will be performed.

ACKNOWLEDGEMENTS

This research was supported by SGRP/PTDP Fund (1994 ~1996) of Korean Ministry of Agriculture and Forestry.

REFERENCES

1. Zhang, Y. S., Talalay, P., Cho, C. G. and Posner, G. H. : A major inducer of anticarcinogenic protective enzymes from broccoli-Isolation and elucidation of structure. *Proc. Natl. Acad. Sci. USA*, **89**, 2399 (1992)
2. Prochaska, H. J. and Santamaria, A. B. : Direct measurement of NAD(P)H: Quinone reductase from cells cultured in microtiter wells: A screening assay for anticarcinogenic enzyme inducers. *Anal. Biochem.*, **169**, 328 (1988)
3. Prochaska, H. J. : Screening strategies for the detection of anticarcinogenic enzyme inducers. *J. Nutr. Biochem.*, **5**, 360 (1994)
4. De Long, M. J., Prochaska, H. J. and Talalay, P. : Induction of NAD(P)H:quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes, and other chemoprotectors: A model system for the study of anticarcinogens. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 787 (1986)
5. Prestera, T., Holtzclaw, W. D., Zhang, Y. S. and Talalay, P. : Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. USA*, **90**, 2965 (1993)
6. De Long, M. J., Prochaska, H. J. and Talalay, P. : Tissue-specific induction patterns of cancer-protective enzymes in mice by tert-butyl-4-hydroxyanisole and related substituted phenols. *Cancer Res.*, **45**, 546 (1985)
7. Talalay, P., De Long, M. J. and Prochaska, H. J. : Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **85**, 8261 (1988)
8. Prochaska, H. J. and Talalay, P. : Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res.*, **48**, 4776 (1988)
9. Prochaska, H. J. and Talalay, P. : Purification and characterization of two isofunctional forms of NAD(P)H: quinone reductase from mouse liver. *J. Biol. Chem.*, **261**, 1372 (1986)
10. Bjeldanes, L. F., Kim, J.-Y., Grose, K. R. and Bartholomew, J. C. : Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*-Comparison with 2,3,7,8-tetrachlorodibenzo-paradioxin. *Proc. Natl. Acad. Sci. USA*, **88**, 9543 (1991)
11. Benson, A. M., Hunkeler, M. J. and Talalay, P. : Increase of NAD(P)H: Quinone reductase by dietary antioxdants; Possible role in protection against carcinogenesis and toxicity. *Proc. Natl. Acad. Sci. USA*, **77**, 5216 (1980)
12. Nebert, D. W. : Genetic differences in microsomal electron transport: the *Ah* locus. *Methods Enzymol.*, **52**, 226 (1978)
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. : Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265 (1951)
14. Steinmetz, K. A. and Potter, J. D. : Vegetables, fruits, and cancer prevention: review. *J. Am. Diet. Assoc.*, **96**, 1027 (1996)
15. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Metha, R. G., Moon, R. C. and Pezzuto, J. M. : Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, **275**, 218 (1997)
16. De Long, M. J., Dolan, P., Santamaria, A. B. and Bueding, E. : 1,2-Dithiol-3-thione analogs: Effects on NAD(P)H quinone reductase and glutathione levels in murine hepatoma cells. *Carcinogenesis*, **7**, 977 (1986)
17. Barch, D. and Rundhausen, L. M. : Ellagic acid induces NAD(P)H:quinone reductase through activation of the antioxidant responsive element of the rat NAD(P)H: quinone reductase gene. *Carcinogenesis*, **15**, 2065 (1994)

(Received June 4, 1998)