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Inhibitory effects of glycolipids fraction from spinach on mammalian DNA polymerase activity and human cancer cell proliferation☆

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Abstract

We succeeded in purifying the fraction containing the major glycolipids in monogalactosyl diacylglycerol, digalactosyl diacylglycerol and sulfoquinovosyl diacylglycerol (SQDG) from dried vegetables. This glycolipids fraction was an inhibitor of DNA polymerase α (pol α) in vitro and also the proliferation of human cancer cells. In this study, eight common vegetables were investigated in terms of the glycolipids fraction, the amounts of major glycolipids, mammalian DNA polymerase inhibitory activity and antiproliferative activity toward human cancer cells. Green tea possessed the largest amount of glycolipids overall. Spinach contained the largest amount of SQDG, followed by parsley, green onion, chive, sweet pepper, green tea, carrot and garlic. Spinach had the strongest inhibitory effect on pol α activity and human cancer cell proliferation. A significant correlation was found between SQDG content and inhibition of DNA polymerase. Therefore, the inhibition of pol α activity by SQDG may lead to cell growth suppression. Of the six subspecies of spinach (*Spinacia oleracea*) tested, "Anna" had the largest amount of SQDG, strongest inhibitory activity toward DNA polymerase and greatest effect on human cancer cell proliferation. Based on these results, the glycolipids fraction from spinach is potentially a source of food material for a novel anticancer activity.

Keywords: Glycolipids; SQDG (sulfoquinovosyl diacylglycerol); Spinach; DNA polymerase α; Enzyme inhibitor; Anticancer agent

1. Introduction

In spite the many advances in cancer treatment, chemotherapy for solid tumors is still greatly limited by a lack of selective anticancer drugs and by the recurrence of drug-resistant tumors. Finding a source of novel chemotherapeutics continues to be a focus of effort. Diets rich in vegetables are known to reduce cancer risk, implicating edible plants as potential sources of anticancer agents.

Multiple organisms are known to contain at least 14 types of DNA polymerase [1]. DNA polymerases catalyze both DNA replication and repair [1,2]. DNA polymerase inhibitors could be employed as anticancer chemotherapy agents because they inhibit cell proliferation. Based on this idea, we have searched for and found many new DNA polymerase inhibitors over the past 9 years, for example, long-chain fatty acids and their derivatives [3–7], bile acids such as lithocholic acid [8,9], terpenoids [10–12], flavonoids [13,14], sulfate-

Abbreviations: DGDG, digalactosyl diacylglycerol; dTTP, 2′-deoxythymidine 5′-triphosphate; MGDG, monogalactosyl diacylglycerol; pol, DNA-directed DNA polymerase (EC 2.7.7.7); SQDG, sulfoquinovosyl diacylglycerol; TLC, thin-layer chromatography.

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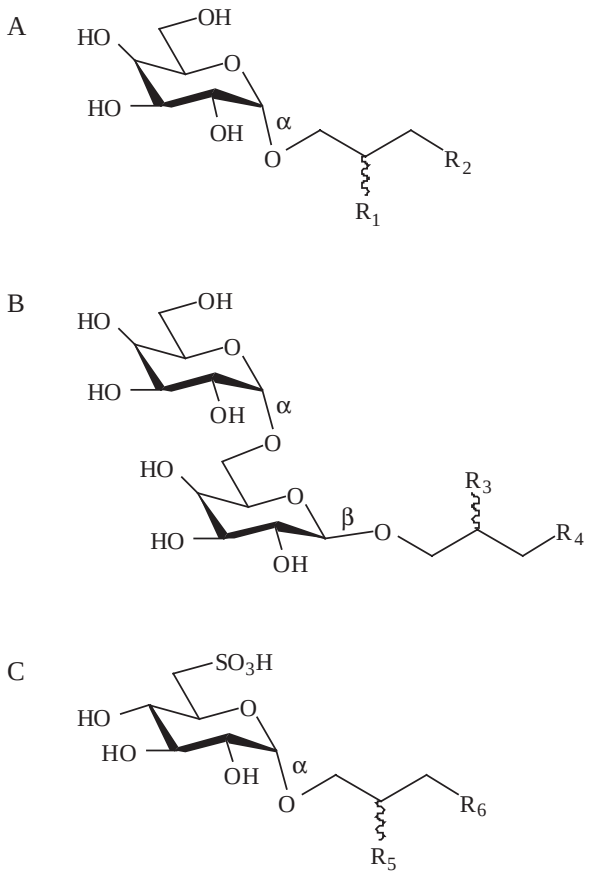

Fig. 1. Chemical structures of major glycolipids in the glycolipids fraction from vegetables. (A) MGDG. (B) DGDG. (C) SQDG. R_1 to R_6 in these structures are fatty acids.

containing glycolipids [15–23], vitamin A-related compounds [24], vitamin B_6 compounds [25], vitamin D_2 and D_3 [26], and nucleotide analogs [27,28] from natural resources.

Of these, sulfoglycolipids in the class sulfoquinovosyl diacylglycerol (SQDG, Fig. 1C) from a fern [15] and an alga [16,17] were particularly potent inhibitors and we concentrated on their anticancer effects in vivo. Sulfoquinovosyl diacylglycerol was not only potent inhibitors of the DNA polymerases in vitro [15–22], but also of human lung cancer in vivo [23]. This glycolipid shows promise as an agent for cancer chemotherapy.

Sulfoquinovosyl diacylglycerol is a major glycolipid of the chloroplast membrane in plants [29]. We have widely screened for a glycolipids fraction containing SQDG from common vegetables which show such inhibitory activities. The purpose of this report is to screen for vegetables with a glycolipids fraction that can inhibit DNA polymerase activities and human cancer cell proliferation.

2. Materials and methods

2.1. Materials

Nucleotides and chemically synthesized DNA template-primers such as [^{3}H]-2′-deoxythymidine 5′-triphosphate (dTTP, 43 Ci/mmol) and poly(dA), oligo(dT)$_{12–18}$ were purchased from Amersham Biosciences (Buckinghamshire, UK). Diaion HP-20 was obtained from Mitsubishi Chemical (Tokyo, Japan). Precoated Silica-Gel 60 plates (10×20 cm, 0.25-mm layer thickness) for thin-layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Sample preparation

Fresh vegetables [carrot (*Daucus carota*), chive (*Allium tuberosum*), garlic (*Allium sativum*), green onion (*Allium fistulosum*), green tea (*Thea sinensis*), parsley (*Petroselinum crispum*), spinach (*Spinacia oleracea*) and sweet pepper (*Capsicum annuum* var. *angulosum*)] were purchased from a local supermarket (Kobe-city, Hyogo-prefecture, Japan) in March 2004. The five subspecies of spinach (*S. oleracea*) (i.e., New Anna R4, Largo, T-881, Anna and Summer keep) were grown in Kohsei-machi, Kohka-gun, Shiga-prefecture, Japan. Samples were cleaned and dried at 40°C for 48 h before extraction.

2.3. Extraction and purification of the glycolipids fraction from vegetables

The purification methods for the glycolipids fraction from vegetables are shown in Fig. 2. The water-soluble substances were extracted from dried vegetables (2 g) with 100 ml of warm water (60°C). The tissue cake was added to 100 ml of warm ethanol (60°C), and the substances containing glycolipids were extracted. The 100% ethanol extract was diluted with water to be a 70% ethanol solution. The solution was subjected to Diaion HP-20 column chromatography, a

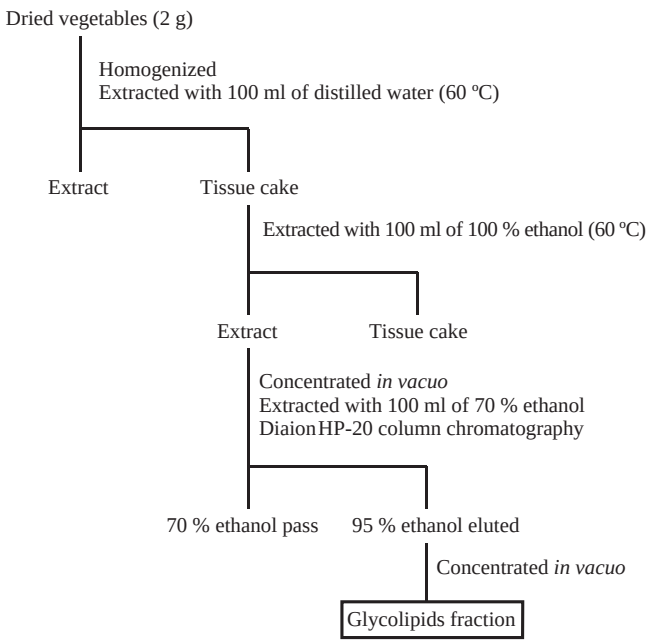

Fig. 2. The method of purifying the glycolipids fraction from dried vegetables.

Table 1
Glycolipid composition of vegetables (μg/1 g of dried vegetable)

Vegetable	MGDG	DGDG	SQDG	Total
Green tea	941 (0.305)[a]	2063 (0.668)[b]	84 (0.027)[c]	3088
Parsley	933 (0.326)	1482 (0.517)	450 (0.157)	2865
Spinach	469 (0.204)	1010 (0.438)	824 (0.358)	2303
Green onion	338 (0.257)	616 (0.469)	359 (0.274)	1313
Chive	207 (0.309)	315 (0.471)	147 (0.220)	669
Sweet pepper	126 (0.231)	285 (0.522)	135 (0.247)	546
Garlic	78 (0.266)	169 (0.577)	46 (0.157)	293
Carrot	47 (0.187)	151 (0.599)	54 (0.214)	252

Vegetables: (1) green tea (*T. sinensis* L.), (2) parsley (*P. crispum* L.), (3) spinach (*S. oleracea* L.), (4) green onion (*A. fistulosum* L.), (5) chive (*A. tuberosum* L.), (6) sweet pepper (*C. annuum* var. *angulosum*), (7) garlic (*A. sativum* L.), (8) carrot (*D. carota* L.).

[a] Ratio of MGDG in total glycolipids fraction.

[b] Ratio of DGDG in total glycolipids fraction.

[c] Ratio of SQDG in total glycolipids fraction.

hydrophobic type of chromatography, and washed with 100 ml of 70% ethanol and then eluted using 95% ethanol. The 95% ethanol solution was the glycolipids fraction.

2.4. Measurement of contents and amounts of glycolipids

The major glycolipids such as monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and SQDG (Fig. 1) in the glycolipids fraction were separated by TLC [chloroform/methanol (3:1, v/v)] and then detected by 50% sulfuric acid spray. In the TLC, the rate of flow of MGDG, DGDG and SQDG were 0.62, 0.52 and 0.21, respectively [see Fig. 3 of Ref. [30]]. Zero-D scan (version 1.0, M & S Instruments Trading) was used for densitometric quantitation of each spot of glycolipid in TLC. Completely purified MGDG, DGDG and SQDG were used as the calibration curves.

2.5. Enzymes

DNA polymerase α (pol α) was purified from calf thymus by immunoaffinity column chromatography as described previously [31]. Pol β was purified from a recombinant plasmid expressing rat pol β [32]. Pol I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi et al. [33]. The Klenow fragment of pol I and human immunodeficiency virus type-1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical (Freehold, NJ, USA). Calf thymus terminal deoxynucleotidyl transferase, T7 RNA polymerase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA, USA). Taq DNA polymerase, T4 DNA polymerase and T4 polynucleotide kinase were purchased from Takara (Tokyo, Japan).

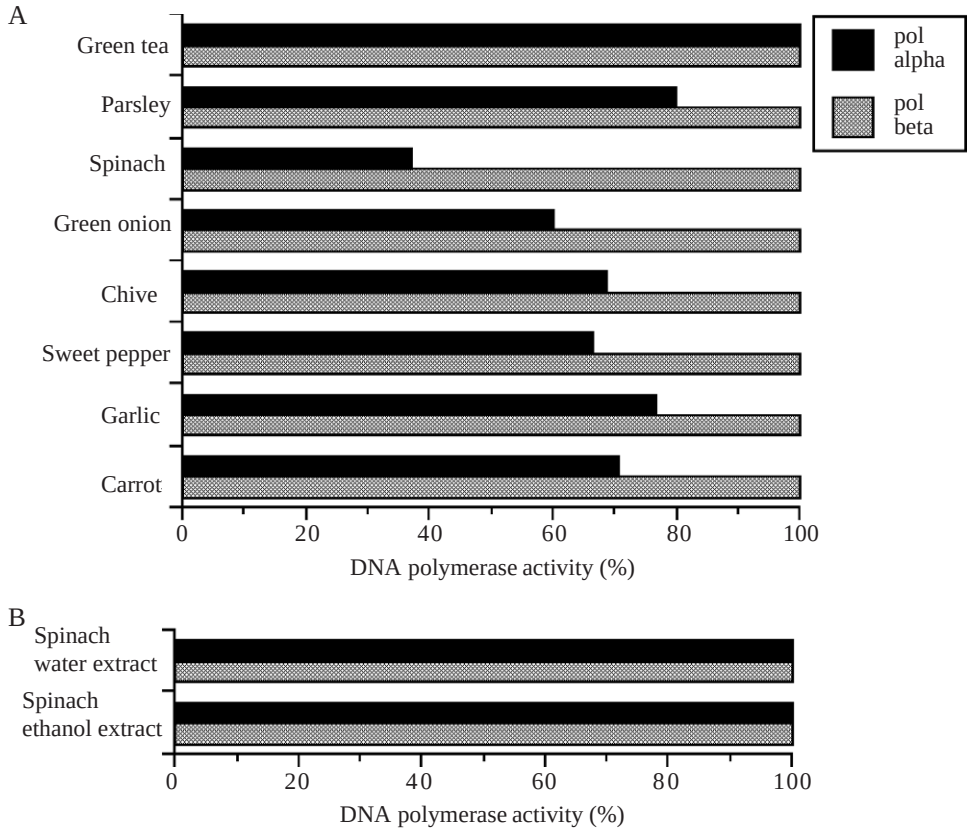

Fig. 3. Effects of the glycolipids fraction from vegetables on the activities of pol α and β. (A) The purified glycolipids fraction from vegetables. (B) The 60°C water and 60°C ethanol extract from spinach. The compounds (50 μg/ml each) were incubated with each enzyme (0.05 U). The enzymatic activity was measured as described in Materials and methods. Enzyme activity in the absence of compounds was taken as 100%.

2.6. DNA polymerase and other DNA metabolic enzyme assays

The activities of the DNA polymerases were measured by methods described previously [3,4]. For the DNA polymerases, poly(dA)/oligo(dT)$_{12-18}$ (A/T=2/1) and dTTP were used as the DNA template-primer and nucleotide substrate, respectively. The glycolipids fractions were dissolved in dimethyl sulfoxide at various concentrations and sonicated for 30 s. Four microliters of each sonicated sample was mixed with 16 μl of each enzyme (final 0.05 U) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0°C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures, and incubation was carried out at 37°C for 60 min. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleoside triphosphates into synthetic DNA template-primers at 37°C for 60 min [3,4].

2.7. Other enzyme assays

Activities of calf DNA primase of pol α, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in each of the standard assays according to the manufacturer's specifications as described by Koizumi et al. [34], Nakayama and Saneyoshi [35], Soltis and Uhlenbeck [36], and Lu and Sakaguchi [37], respectively.

2.8. Cell culture and measurement of cell proliferation

A human gastric cancer cell line, NUGC-3, and human promyelocytic leukemia cell line, HL-60, were obtained from the Health Science Research Bank (Osaka, Japan). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humid atmosphere of 5% CO_2/95% air. For the cell proliferation assay, NUGC-3 and HL-60 cells were plated at 3×10^5 cells into each well of 96-well microplates with various concentrations of the glycolipids fractions. These compounds were dissolved in phosphate-buffered saline at a concentration of 10 mM as a stock solution. The stock solutions were diluted to the appropriate final concentrations with growth medium just before use. The cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [38].

3. Results and discussion

3.1. Glycolipid contents of vegetables

As briefly described in the Introduction, we screened for and found many DNA polymerase inhibitors from natural resources [3–17,22–28]. Some of the natural glycolipids such as SQDG (Fig. 1C) were found to strongly inhibit the activities of DNA polymerases. This is the reason we investigated the effects of the glycolipids fraction from common vegetables on DNA polymerase activity and human cancer cell proliferation.

Two grams of dried vegetables was homogenized with warm ethanol (60°C) using a Waring blender for 5 min and fat-soluble compounds were extracted. The glycolipids fraction was purified using hydrophobic column chroma-

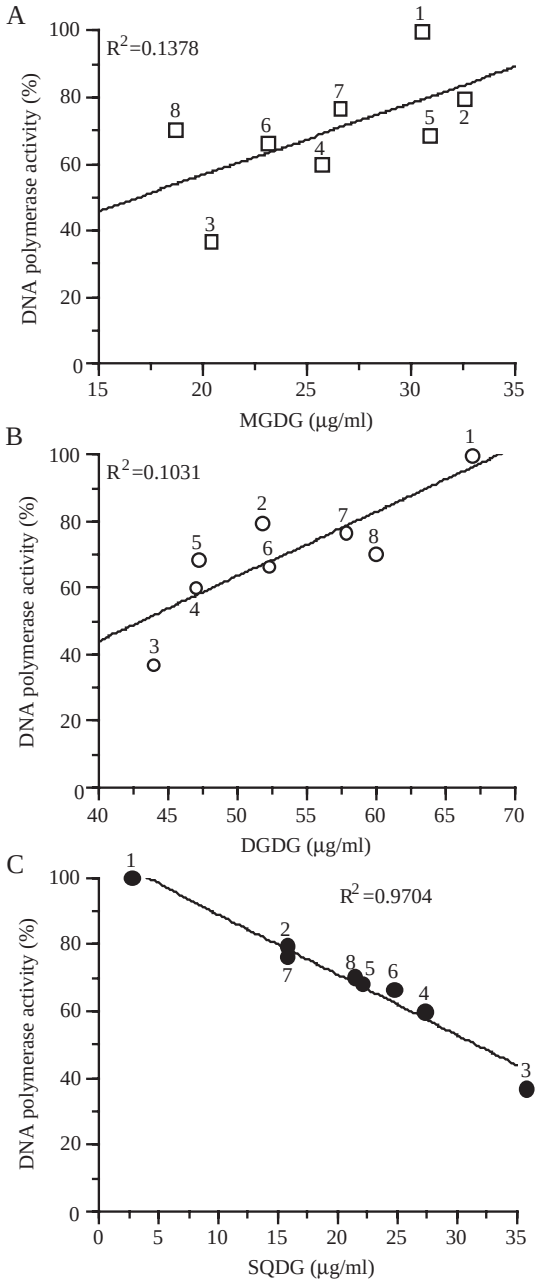

Fig. 4. The relationship between glycolipid amounts and the inhibitory activity toward pol α by the glycolipids fraction from vegetables. (A) MGDG contents in the glycolipids fraction vs. relative activity of pol α. (B) DGDG contents in the glycolipids fraction vs. relative activity of pol α. (C) SQDG contents in the glycolipids fraction vs. relative activity of pol α. The numbers (1–8) in the figures were green tea, parsley, spinach, green onion, chive, sweet pepper, garlic and carrot, respectively. The values of correlation coefficients are shown in each figure.

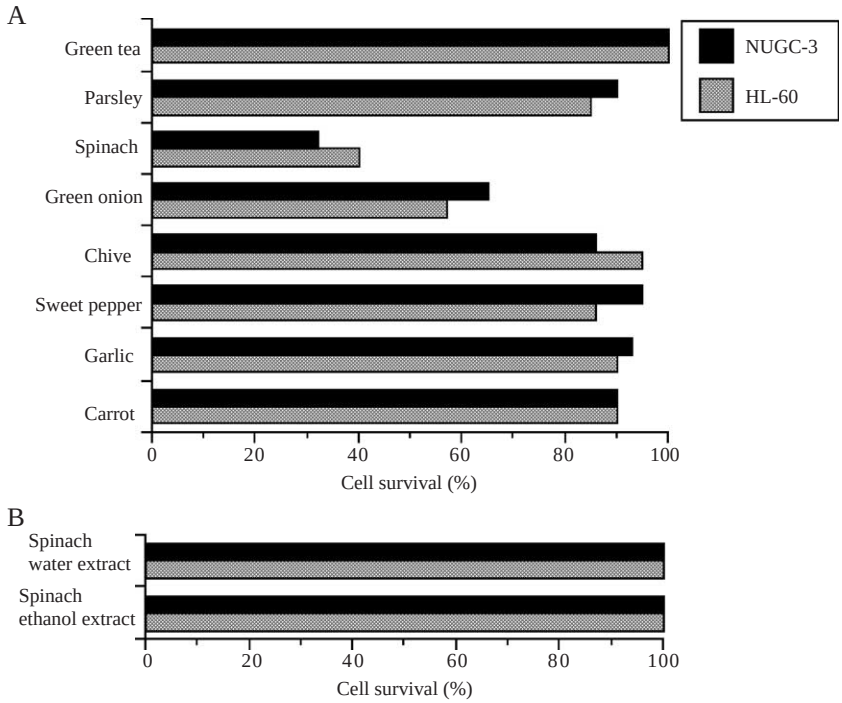

Fig. 5. Effect of the glycolipids fraction from vegetables on the proliferation of human cancer cells. (A) The purified glycolipids fraction from vegetables. (B) The 60°C water and 60°C ethanol extract from spinach. NUGC-3 and HL-60 cells were treated with 100 μg/ml of the compounds for 48 h. Cell proliferation was determined by MTT assay [38].

tography from the extraction material as in Fig. 2. In the glycolipids fraction, three major compounds were analyzed by TLC, and no other compounds were detected. Each of these compounds was completely purified by silica gel column chromatography, and their chemical structures were determined by ^{1}H-, ^{13}C- and distortionless enhancement by polarization transfer NMR spectroscopic analyses. These compounds were glycolipids such as MGDG (Fig. 1A), DGDG (Fig. 1B) and SQDG (Fig. 1C). The compounds could theoretically have stereoisomers of two configurations, α or β type, between the sugar and the glyceride. Natural MGDG and SQDG had the α type and DGDG had α and β types (Fig. 1).

The amount of each glycolipid content in the glycolipids fraction of eight vegetables is shown in Table 1. Values are expressed as micrograms per gram of dried weight of the edible part of vegetables. Green tea had the largest amount of total glycolipids (3088 μg/1 g of sample), whereas carrot had the smallest (252 μg/1 g of sample). As for MGDG and DGDG, green tea had the largest amount (941 and 2063 μg/1 g of sample, respectively). On the other hand, spinach had the most SQDG (824 μg/1 g of sample) among the vegetables tested. Spinach also contained the highest percentage of SQDG (35.8%), followed by green onion, sweet pepper, chive, carrot, parsley, garlic, and green tea.

3.2. Effects of the glycolipids fraction of vegetables on the activities of mammalian DNA polymerases

As briefly described in the Introduction, some of the glycolipids, which strongly inhibit DNA polymerase activities, might be suitable anticancer agents. This is the reason we screened for the glycolipids fraction from common vegetables. In this section, therefore, we investigated the effects of the fraction containing major natural glycolipids such as MGDG, DGDG and SQDG on calf pol α and rat DNA polymerase β (pol β). Pol α and β are representative replicative and repair-related DNA polymerases, respectively. The effects of 50 μg/ml of the glycolipids fraction on mammalian pol α and β are depicted in Fig. 3A. Spinach had the strongest inhibitory activity toward pol α, showing significant inhibition compared with other vegetables. The glycolipids fraction from spinach dose-dependently inhibited pol α activity, and the IC_{50} value was 41.0 μg/ml. Green tea did not inhibit pol α

Table 2
Glycolipid composition of spinach subspecies (μg/1 g of dried spinach)

Glycolipid	Spinach subspecies					
	S-1	S-2	S-3	S-4	S-5	S-6
MGDG	395 (0.131)[a]	174 (0.062)	81 (0.030)	469 (0.204)	389 (0.178)	140 (0.075)
DGDG	1842 (0.611)[b]	2038 (0.711)	1990 (0.734)	1010 (0.438)	880 (0.402)	1110 (0.598)
SQDG	778 (0.258)[c]	649 (0.227)	638 (0.236)	824 (0.358)	918 (0.420)	607 (0.327)
Total	3015	2861	2709	2303	2187	1857

Species of spinach (*S. oleracea*): S-1, New Anna R4; S-2, Largo; S-3, T-881; S-4, unknown (purchased at March, 2004, in Kobe-city, Hyogo-prefecture, Japan); S-5, Anna; S-6, Summer keep.

[a] Ratio of MGDG in total glycolipids fraction.

[b] Ratio of DGDG in total glycolipids fraction.

[c] Ratio of SQDG in total glycolipids fraction.

Table 3
IC_{50} values of glycolipids fraction of spinach subspecies on the calf pol α activity

Spinach subspecies	S-1	S-2	S-3	S-4	S-5	S-6
IC_{50} value (μg/ml)	64.1	89.1	79.6	41.0	29.2	43.8

Subspecies of spinach (*S. oleracea*): S-1, New Anna R4; S-2, Largo; S-3, T-881; S-4, unknown (purchased at March, 2004, in Kobe-city, Hyogo-prefecture, Japan); S-5, Anna; S-6, Summer keep.
Glycolipids fraction was incubated with 0.05 U of pol α. The activity of pol α was measured as described in Materials and methods. Enzyme activity in the absence of the glycolipids fraction was taken as 100%.

activity. On the other hand, the glycolipids fraction of all vegetables had no effect on pol β (Fig. 3A). We reported previously that purified SQDG from plants inhibited the activities of pol α and β [15,16]. The glycolipids fraction from plant might be containing not only SQDG but also other compounds which interfered with the pol β inhibitory activity by SQDG. The 60°C water extract containing water-soluble fraction and the 60°C ethanol extract containing fat-soluble fraction from spinach did not inhibit the activities of pol α and β (Fig. 3B). Therefore, the purification of the glycolipids fraction using HP-20 column chromatography was important for DNA polymerase inhibition.

Next, the inhibition of pol α by the vegetable glycolipids fraction was compared with the amounts of each glycolipid such as MGDG, DGDG and SQDG. Fig. 4 indicates the relationship between the pol α inhibition and glycolipid content in the fraction from vegetables. The amount of SQDG had the highest correlation (correlation coefficient=0.9704) with the inhibitory activity for pol α (Fig. 4C), and the amounts of MGDG and DGDG were not

Table 4
IC_{50} values of glycolipids fraction from S-5 (Anna) subspecies of spinach for the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} value of glycolipids fraction (μg/ml)
Mammalian DNA polymerases	
Calf pol α	29.2
Rat DNA polymerase β	>200
Plant DNA polymerases	
Cauliflower DNA polymerase I (α-like)	>200
Cauliflower DNA polymerase II (β-like)	>200
Prokaryotic DNA polymerases	
E. coli DNA polymerase I (Klenow fragment)	>200
Taq DNA polymerase	>200
T4 DNA polymerase	>200
Other DNA metabolic enzymes	
Calf DNA primase of pol α	>200
Calf terminal deoxynucleotidyl transferase	>200
HIV-1 reverse transcriptase	>200
T7 RNA polymerase	>200
T4 polynucleotide kinase	>200
Bovine deoxyribonuclease I	>200

Glycolipids fraction was incubated with each enzyme (0.05 U). The enzymatic activity was measured as described in Materials and methods. Enzyme activity in the absence of the compound was taken as 100%.

Table 5
LD_{50} values of glycolipids fraction of spinach subspecies for the proliferation of human cancer cells

Spinach subspecies	S-1	S-2	S-3	S-4	S-5	S-6
LD_{50} value (μg/ml)						
NUGC-3 cells	105	192	116	66.8	58.0	76.7
HL-60 cells	100	156	125	70.1	63.2	87.1

Subspecies of spinach (*S. oleracea*): S-1, New Anna R4; S-2, Largo; S-3, T-881; S-4, unknown (purchased at April, 2003, in Kobe, Japan); S-5, Anna; S-6, Summer keep.
The survival rate was determined by MTT assay.

related to the pol α inhibition at all (Fig. 4A and B). These results suggested that SQDG could be a pol α inhibitor.

3.3. Effect of the glycolipids fraction on human cancer cell proliferation

Next, we tested their influence on human stomach cancer cells (NUGC-3) and promyelocytic leukemia (HL-60). The cells were incubated with 100 μg/ml of the glycolipids fractions for 48 h. As shown in Fig. 5A, more spinach efficiently inhibited the cancer cell proliferation than other vegetables. The inhibitory effect of NUGC-3 was almost the same as that of HL-60. The inhibition by the spinach glycolipids fraction was dependent on dose, and the LD_{50} value was 66.8–70.1 μg/ml. Green onion had the second strongest effect, but the other vegetables hardly inhibited the cell growth at all. The water extract containing water-soluble fraction and the ethanol extract containing fat-soluble fraction from spinach did not influence the cell proliferation of human cancer cells (Fig. 5B).

It is of interest that the glycolipid compound from a healthy green vegetable was most active against the cancer cells. The IC_{50} value for pol α inhibitory activity of the glycolipids fraction from spinach was approximately twofold stronger than the LD_{50} value for cancer cell growth, and SQDG might cause to inhibit the activity of pol α. These results suggested that SQDG was able to penetrate the cancer cells and reached the nucleus inhibiting pol α activity.

3.4. Glycolipid composition of spinach subspecies

As described above, the spinach glycolipids fraction was the strongest inhibitor of pol α activity, and then, human cancer cell proliferation of the vegetables was tested (Figs. 3 and 5). We cultivated and prepared five subspecies (i.e., S-1, New Anna R4; S-2, Largo; S-3, T-881; S-5, Anna; S-6, Summer keep) of spinach (*S. oleracea*) in the ground (Kohsei-cho, Kohka-gun, Shiga-prefecture, Japan). The amount of each glycolipid in the glycolipids fraction of six subspecies is shown in Table 2. Values are expressed as micrograms per gram of dried weight of the edible part of spinach. We named the six subspecies S-1 to S-6 according to the total amount of glycolipids. “New Anna R4” (S-1) and “Summer keep” (S-6) had the largest and smallest amount of total glycolipids with 3015 and 1857 μg/L g of sample, respectively. S-1 had 1.62-fold more than S-6. Of the six subspecies of spinach, S-4, S-2 and S-5 had the largest

amount of MGDG, DGDG and SQDG, respectively, and S-4, S-3 and S-5 had the highest percentage of MGDG, DGDG and SQDG in the glycolipids fraction, respectively. It is of interest that the subspecies with the largest amount of total glycolipids was different from that with the largest amount of each glycolipid.

3.5. Effects of the glycolipids fraction of spinach subspecies on the activities of mammalian DNA polymerases and human cancer cell proliferation

We screened for the glycolipids fraction from subspecies of spinach to develop anticancer functional foods. IC_{50} values of the fraction for the effect on mammalian pol α and β are shown in Table 3. "Anna" (S-5) was the strongest inhibitor of pol α, followed by S-4, S-6, S-1, S-3 and S-2. No glycolipids fraction of the subspecies of spinach had an effect on pol β (Table 3).

The effects of the glycolipids fraction from the subspecies "Anna" on various DNA polymerases and other DNA metabolic enzymes are depicted in Table 4. This fraction inhibited the activities of mammalian pol α, but had no inhibitory effect on plant pol α and β, prokaryotic DNA polymerases and other DNA-metabolic enzymes such as DNA primase of pol α, calf terminal deoxynucleotidyl transferase, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase and calf deoxyribonuclease I. The glycolipids fraction was a selective mammalian pol α inhibitor.

LD_{50} values of the glycolipids fraction on human cancer cell proliferation (NUGC-3 and HL-60 cells) are shown in Table 5. "Anna" (S-5) exhibited the strongest suppression of NUGC-3 and LH-60, followed by S-4, S-6, S-1, S-3 and S-2. On the other hand, S-5 had the highest percentage of SQDG in the glycolipids fraction, followed by S-4, S-6, S-1, S-3 and S-2. These results suggested that SQDG, which could inhibit pol α activity, might suppress human cancer cell proliferation.

A vegetable containing a large amount of SQDG (i.e., a high percentage of SQDG in the glycolipids fraction) could be a material for an anticancer functional food. However, the water extracts at 60°C and ethanol extracts at 60°C from spinach (i.e., water-and fat-soluble fractions, respectively) did not inhibit the activities of pol α or cancer cell growth, although the 60°C ethanol extract contained SQDG [30]. Therefore, it was suggested that there were some compounds which could avoid the bioactivities of SQDG. It must be important to purify the glycolipids fraction containing SQDG. We screened for the glycolipids fraction extracted from dried vegetables and found that spinach was the strongest inhibitor of pol α and cancer cell proliferation. In the spinach (*S. oleracea*), the subspecies "Anna" had the highest percentage of SQDG, greatest inhibiting effect on pol α and strongest anticancer activity. The glycolipids fraction from the "Anna" subspecies of spinach could help to prevent cancer disease and be a functional food with anticancer activity.

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