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Inhibitory Effect of Monogalactosyldiacylglycerol, Extracted from Spinach Using Supercritical CO_2, on Mammalian DNA Polymerase Activity

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We investigated the effective extraction of monogalactosyldiacylglycerol (MGDG) from dried spinach (*Spinacia oleracea*) using supercritical fluid carbon dioxide (SC−CO_2) with a modifier/entrainer. The yield of MGDG in the SC−CO_2 extract was not influenced by increasing temperature at a constant pressure, although the total extract yield was decreased. The total extract yield and MGDG yield in the extract from commercially purchased spinach (unknown subspecies), were greatly influenced by lower pressure. In a modifier (i.e., ethanol) concentration range of 2.5−20%, both the extract and MGDG yield increased as the ethanol concentration rose. The highest total extract yield (69.5 mg/g of spinach) and a good MGDG yield (16.3 mg/g of spinach) were obtained at 80 °C, 25 MPa, and 20% ethanol. The highest MGDG concentration (76.0% in the extract) was obtained at 80 °C, 25 MPa, and 2.5% ethanol, although the total extract yield under these conditions was low (5.2 mg/g of spinach). The optimal conditions for the extraction of MGDG were 80 °C, 20 MPa, and 10% ethanol. Of the 11 subspecies of spinach tested under these conditions, "Ujyou" had the highest concentration of MGDG. The total extract yield and MGDG concentration of Ujyou were 20.4 mg of the extract/g of spinach and 70.5%, respectively. The concentration of MGDG was higher in the SC−CO_2 extract than in the extract obtained using solvents such as methanol and *n*-hexane. The extract of Ujyou, which was the optimal subspecies for the extraction of MGDG, inhibited the activity of calf DNA polymerase α with IC_{50} values of 145 μg/mL but was not effective against DNA polymerase β.

KEYWORDS: MGDG (monogalactosyldiacylglycerol); glycolipid; supercritical fluid carbon dioxide (SC−CO_2); modifier/entrainer; spinach; DNA polymerase; enzyme inhibitor

INTRODUCTION

Chloroplasts from higher plants contain thylakoid membranes, where photosynthesis takes place. The thylakoid membranes are composed of 60−65% protein and 35−45% lipid, depending upon growth conditions. The lipid composition of the thylakoids is unique among eukaryotic cellular membranes because about 77% of the lipids are neutral galactosyldiacylglycerols (*1*). Of all of the nonpigmented membrane lipids, about 51% are monogalactosyldiacylglycerol (MGDG) and about 26% are digalactosyldiacylglycerol (DGDG). MGDG and DGDG are also present in grains, roots, and fruits (*2*, *3*). These glycolipids exhibit galactosyl binding via an α-1,3 linkage to glycerol. MGDG from natural plants was reported to inhibit mammalian DNA polymerase (*4*, *5*), suppress the growth of human cancer cells (*4*), and have antitumor-promoting effects (*6*).

Extraction using supercritical fluids has received attention as an alternative to conventional means of extraction using organic solvents. A supercritical fluid possesses physical properties between those of a gas and a liquid. A liquidlike density, leading to high loadings of solutes, coupled with a pressure-dependent solvating ability, makes supercritical fluids excellent solvents for separations and reactions. A low viscosity and high molecular diffusivity, like those of a gas, in combination with a low surface tension, make supercritical fluids ideal candidates for mass-transfer solvents, allowing better penetration of the sample matrix than is achieved by liquid solvents. Carbon dioxide (CO_2) is the most often used fluid because of its nontoxicity, nonflammability, lack of chemical residue, and low critical temperature. There has been a rapid development in the application of extraction with supercritical fluid over the past 2

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Table 1. Experimental Conditions for Supercritical Fluid Extraction from Spinach[a]

experiment	temp (°C)	pressure (MPa)	EtOH flow rate (%, v/v)	total extract yield (mg)	MGDG yield (mg)
1	40	25	10	70.2 ± 4.5	26.1 ± 3.2
2	50	25	10	66.6 ± 4.6	26.9 ± 2.8
3	60	25	10	65.4 ± 2.0	25.7 ± 0.5
4	70	25	10	58.3 ± 4.0	23.7 ± 2.7
5	80	25	10	59.3 ± 2.0	25.3 ± 0.4
6	80	10	10	85.9 ± 2.2	27.9 ± 3.3
7	80	15	10	29.6 ± 2.6	11.8 ± 3.5
8	80	20	10	49.0 ± 4.5	29.5 ± 2.8
9	80	25	2.5	10.4 ± 0.6	7.9 ± 0.8
10	80	25	5	37.2 ± 3.0	20.9 ± 2.0
11	80	25	20	93.7 ± 8.9	21.9 ± 2.5

[a] Data are expressed as the mean ± SD; $n = 3$.

decades, for example, decaffeination of green coffee with supercritical fluid CO_2 ($SC-CO_2$) (*7*), and the extraction of hops (*8*), spices (*9*), fruit aromas (*10*), cholesterol from edible animals fats (*11*), perfumes and flavors from natural products (*12*), and unsaturated fatty acids from fish oil (*13*).

We previously reported that the amount of glycolipids such as MGDG and DGDG in spinach was larger than in other vegetables tested (*4*). In this paper, we investigate the influences of extracting pressure, temperature, and modifier concentration on the MGDG yield extracted from spinach using $SC-CO_2$ and the inhibitory effect of extracts from spinach on mammalian DNA polymerase activity.

MATERIALS AND METHODS

Materials. Spinach (*Spinacia oleracea*) was purchased in May, 2005, from a supermarket (Cooperative Union Co., Kobe, Hyogo prefecture, Japan). The 11 subspecies of spinach (*S. oleracea*) (i.e., Allright, Anna, Esper, Houyou, Largo, Minstarland, Nobel, Nippon, Try, Ujyou, and Venture R5) were grown in Kohsei-machi, Kohka-gun, Shiga prefecture, Japan. The HPLC-grade solvents were all purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Pure carbon dioxide was purchased from Kobe-Sanso Co. (Hyogo, Japan). MGDG as a standard was purified from spinach using HPLC.

Supercritical Fluid Extraction: Apparatus and Operating Procedures. All experiments on the extraction with $SC-CO_2$ were performed with a SCF-Get $SC-CO_2$ extraction system (JAS.CO, Tokyo, Japan) in a laboratory, using a 10 mL extraction vessel. The freeze-dried spinach was ground in a food mill for efficient extraction, and 2.0 g of the ground spinach powder was charged into an extraction vessel. Extractions were performed using different pressures, temperatures, and modifier [ethanol (EtOH)] volumes for 1 h. The total flow rate was 5.0 mL/min. Experimental conditions for the extraction from spinach using $SC-CO_2$ are shown in **Table 1**. First, to remove oils with a lower polarity than glycolipids, extraction with $SC-CO_2$ was carried out at experimental temperatures and pressures for 1 h. These extracts did not contain glycolipids (data not shown). Second, to extract the glycolipids, the procedure was conducted for 1 h with $SC-CO_2$ and a modifier at various experimental temperatures, pressures, and modifier volumes. The extracts were obtained by removing the solvent with a rotator evaporator. The extracts (2 mg) were dissolved in a mixture of 2 mL of chloroform and 2 mL of methanol for HPLC analyses.

HPLC Analyses. MGDG was quantified using a high-performance liquid chromatography (HPLC) system that consisted of two pumps (Shimadzu LC-10AD and LC-10AT, Shimadzu Co. Ltd., Kyoto, Japan) and an evaporative light-scattering detector (ELSD) (ELSD model 200, Softa Co., Tokyo, Japan). The silica column was composed of LiChrospher Si 60 (5 μm, 125 × 4 mm i.d., Merck KGaA, Darmstadt, Germany). The mobile phase was made up of chloroform (solvent A) and 95:5 methanol/water (solvent B). The following binary gradient profile was employed: 99:1 (A/B, v/v) at 0 min, 75:25 (A/B, v/v) at 15 min, 10:90 (A/B, v/v) at 20 min, and maintained until 25 min. The flow rate was 1.0 mL/min.

Organic Solvent Extraction. The components of the $SC-CO_2$ extract were compared with those of the solvent extract. The freeze-dried spinach was ground in a food mill for efficient extraction. A total of 2 g of the ground spinach powder was extracted using *n*-hexane or methanol (300 mL) in an ultrasonic bath. After 1 h, each extract was decanted and filtered. The extracts were obtained by removing the solvent with a rotator evaporator. Next, 2 mg of extract was dissolved in a mixture of 1 mL of chloroform and 1 mL of methanol for HPLC.

DNA Polymerase Assays. DNA polymerase α was purified from calf thymus by immunoaffinity column chromatography as described previously (*14*). DNA polymerase β was purified from a recombinant plasmid expressing rat DNA polymerase β (*15*). The activities of the DNA polymerases were measured by the methods described previously (*16*, *17*). For the DNA polymerases, poly(dA)/oligo$(dT)_{12-18}$ (A/T = 2:1) and 2′-deoxythymidine 5′-triphosphate (dTTP) were used as the DNA template−primer and nucleotide substrate, respectively. These compounds were dissolved in dimethyl sulfoxide at various concentrations and sonicated for 30 s. A total of 4 μL of each sonicated sample was mixed with 16 μL of each enzyme (final 0.05 units) 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. A total of 1 unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleotide triphosphates into synthetic DNA template−primers at 37 °C for 60 min.

RESULTS AND DISCUSSION

Supercritical Fluid Extraction. Pressure and temperature are important physical factors when extracting compounds using supercritical fluids because they define the density of the $SC-CO_2$. A lower temperature at constant pressure results in an increase in the density and solvent power of $SC-CO_2$.

The total extract yield from spinach purchased in May, 2005 (unknown subspecies), under various temperatures at 25 MPa is shown in **Figure 1A**. The total extract yield decreased slightly with increasing temperature, reflecting the reduced density of the solvent; however, the MGDG yield was constant under various temperatures. The concentration of MGDG in the extracts was observed to increase slightly with increasing temperature (**Figure 1B**).

The total extract yield from spinach obtained with $SC-CO_2$ and 10% EtOH under various pressures at 80 °C is shown in **Figure 2A**. A higher pressure at constant temperature resulted in an increase in the density and solvent power of $SC-CO_2$. At a pressure of 15−25 MPa, the total extract yield increased with increasing pressure; however, it peaked at 10 MPa (43.0 mg/g of dried spinach). At 15−25 MPa, as the solvent power of EtOH decreased, the solvent power of $SC-CO_2$ increased. Although the MGDG yield at 10, 20, and 25 MPa showed the same tendency as the total extract yield, the MGDG concentration was maximum at 20 MPa (60.2% of extract) and minimum at 10 MPa (32.4% of extract) (**Figure 2B**). At 10 MPa, other compounds with relatively strong polarity were extracted because of their preferential dissolution in EtOH. At 25 MPa, compounds with weak polarity were extracted by the increased solvent power of $SC-CO_2$.

The effect of the concentration (2.5−20%) of EtOH on the extract from spinach (unknown subspecies) was examined at

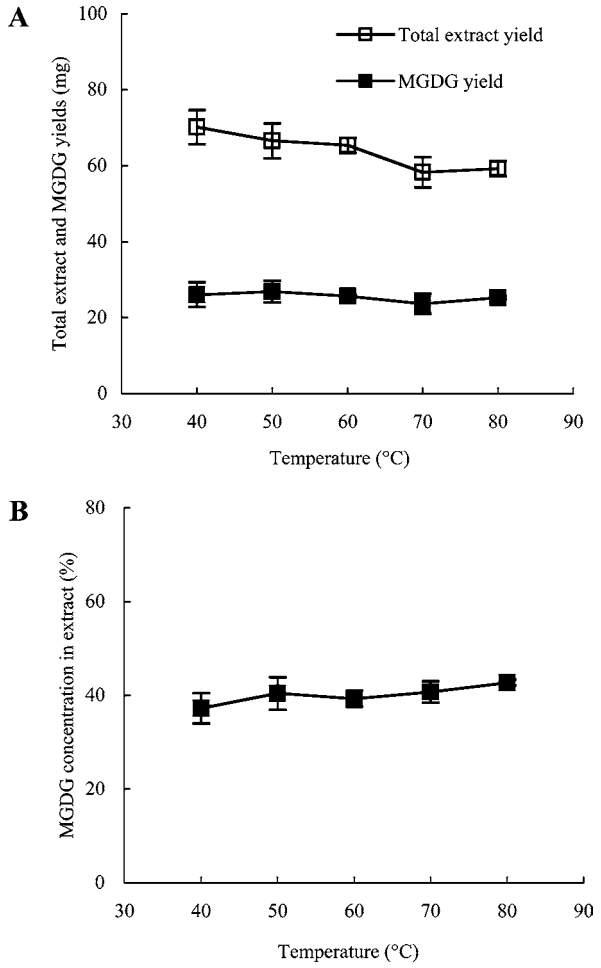

Figure 1. Effect of temperature on the (A) total extract and MGDG yield and (B) MGDG concentration with supercritical fluid CO_2 extraction at a constant pressure of 25 MPa. Data are shown as the means ± standard error of the mean (SEM) for three independent experiments.

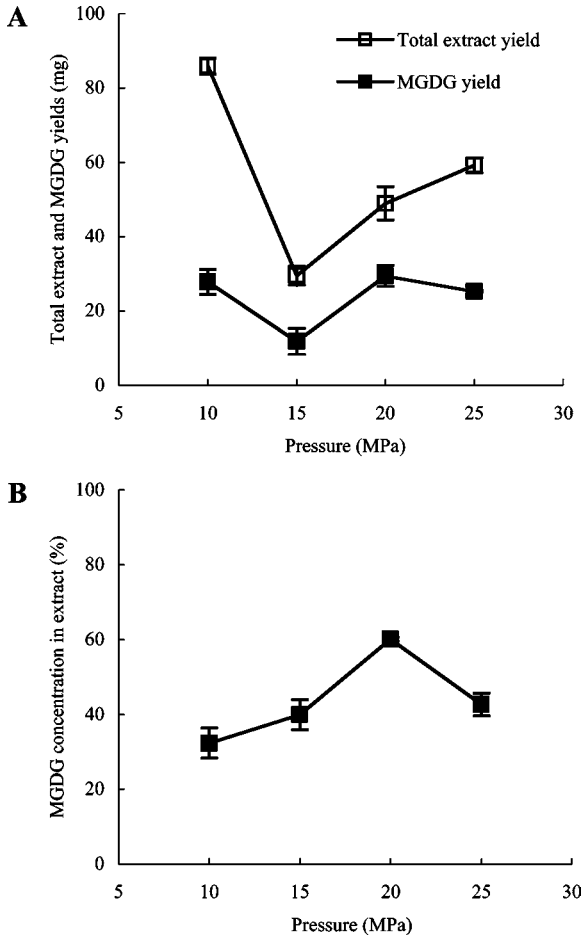

Figure 2. Effect of pressure on the (A) total extract and MGDG yield and (B) MGDG concentration with supercritical fluid CO_2 extraction at a constant pressure of 80 °C. Data are shown as the means ± SEM for three independent experiments.

80 °C and 25 MPa for 1 h (**Figure 3A**). The total extract yield increased with increasing EtOH concentration and peaked at 20% EtOH (69.5 mg/g of dried spinach). As a result, the MGDG concentration decreased as the EtOH concentration rose (**Figure 3B**), because the increase in the concentration and solvent power of EtOH resulted in an increase in other compounds with greater polarity than MGDG. The concentration of MGDG in the extract at 2.5% EtOH was 76.0% of the highest concentration under the conditions tested, although the total extract yield was 10.4 mg, the lowest value. The data of **Figure 3A** indicated that amounts of MGDG from the 2.0 g of dried spinach using SC−CO_2 extraction containing 2.5, 5, 10, and 20% ethanol were 7.9, 20.9, 25.3, and 21.9 mg, respectively. Therefore, we considered that the optimal conditions for the extraction of MGDG using SC−CO_2 were 80 °C, 20 MPa, and 10% ethanol.

HPLC Analyses of the SC−CO_2 and Organic Solvent Extracts. The effect of SC−CO_2 was compared with extraction using an organic solvent (**Table 2**). The methanol extract (351.7 mg/g of dried spinach) was large but contained little MGDG (2.7% of the extract). Although the SC−CO_2 extract (24.5 mg/g of dried spinach) was smaller, it had the highest concentration of MGDG (14.8 mg/g of dried spinach, 60.2% of the extract) of all of the extracts tested. A chart of the data from the HPLC analysis of the SC−CO_2 extract is shown in **Figure 4**. The peaks of MGDG were smaller on extraction with *n*-hexane and methanol than with SC−CO_2, and many other peaks were detected. Therefore, the extraction of MGDG using SC−CO_2 with an entrainer is more effective than other methods.

MGDG Analysis of SC−CO_2 Extract of the Subspecies of Spinach. We cultivated and prepared 11 subspecies (i.e., Allright, Anna, Esper, Houyou, Largo, Minstarland, Nobel, Nippon, Try, Ujyou, and Venture R5) of spinach (*S. oleracea*) in the ground. The extraction conditions using SC−CO_2 were 80 °C, 20 MPa, and 10% ethanol. Each total extract yield and MGDG concentration using SC−CO_2 is shown in **Table 3**. Largo had the largest total extract yield (28.5 mg/g of dried spinach) of all subspecies. Ujyou had the highest concentration of MGDG (70.5% of the extract) of all subspecies tested; therefore, this was the best subspecies for the isolation of MGDG using SC−CO_2. It was interesting that the total extract yield and MGDG concentration were different among subspecies.

Effect of the SC−CO_2 Extract of "Ujyou" Spinach on the Activities of Mammalian DNA Polymerases. Figure 5 shows the dose−response curves of the inhibition of the SC−CO_2 extract of Ujyou, which was the optimal subspecies for the extraction of MGDG (70.5% MGDG in the extract), against mammalian DNA polymerases α and β. The SC−CO_2 extract could clearly inhibit the activity of calf DNA polymerase α, which is a replicative polymerase, in a dose-dependent manner, and IC_{50} values were 145 μg/mL; however, the extract could not influence rat DNA polymerase β activity.

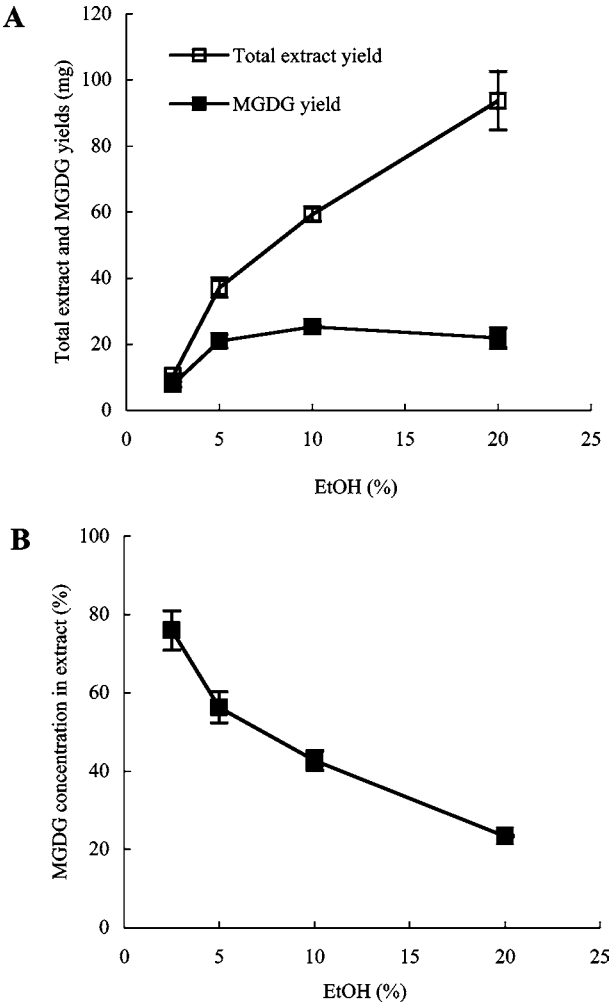

Figure 3. Effect of the modifier EtOH on the (A) total extract and MGDG yield and (B) MGDG concentration with supercritical fluid CO_2 extraction at 25 MPa and 80 °C. Data are shown as the means ± SEM for three independent experiments.

Table 2. Total Extract, MGDG Yield, and MGDG Concentration Using Supercritical CO_2 and the Organic Solvents *n*-Hexane and Methanol[a]

extraction	total extract yield (mg of extracts/ g of dried spinach)	MGDG yield in extracts (mg of MGDG/ g of dried spinach)	MGDG concentration (%)
n-hexane	28.8 ± 1.8	5.6 ± 0.7	19.3 ± 1.2
methanol	703.3 ± 12.6	18.7 ± 0.9	2.7 ± 0.4
supercritical CO_2[b]	49.0 ± 4.5	29.5 ± 2.8	60.2 ± 0.5

[a] Data are expressed as the mean ± SD; $n = 3$. [b] Supercritical CO_2 was applied at experimental temperatures and pressures for 1 h, and 10% EtOH was added at 20 MPa and 80 °C.

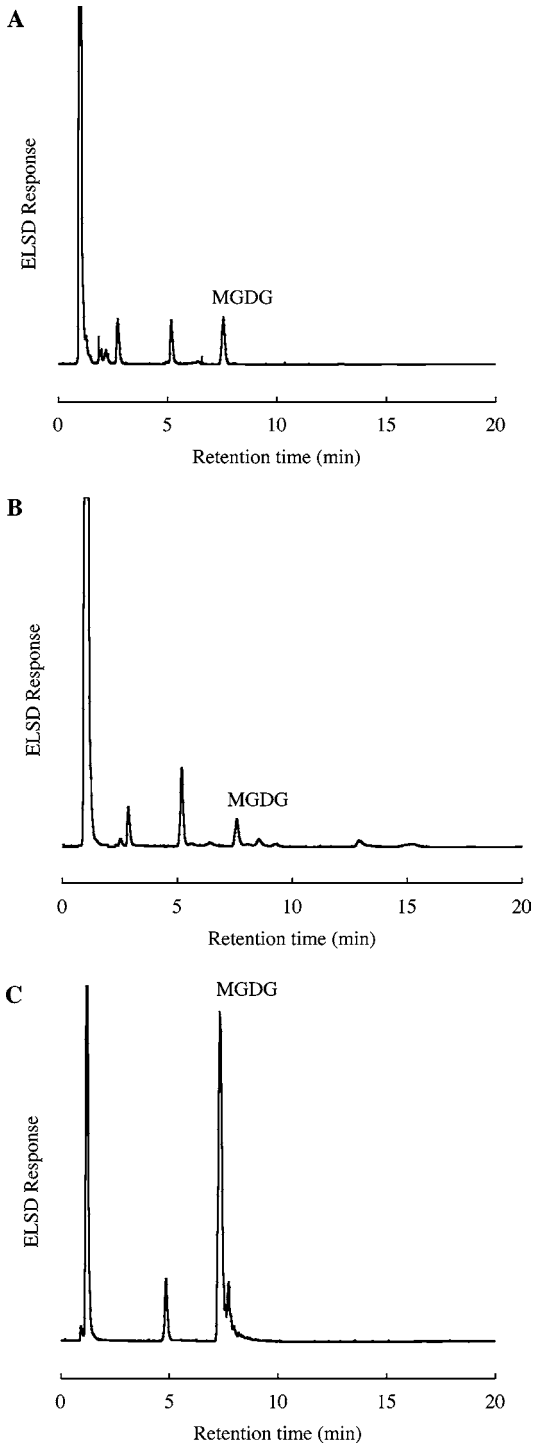

Figure 4. Chart of the HPLC analysis of the solvent and supercritical fluid CO_2 extracts: (A) *n*-hexane, (B) methanol, and (C) supercritical fluid CO_2.

We reported previously that MGDG was isolated from a sea alga, *Petalonia bingbamiae* (*5*), and vegetables (*4*, *18*) as a selective inhibitor of mammalian DNA polymerase. Eukaryotic cells reportedly contain three replicative DNA polymerases (α, δ, and ϵ), a mitochondrial DNA polymerase such as γ, and at least 13 repair-type DNA polymerases such as β, δ, ϵ, ζ, η, θ, κ, λ, μ, σ, ϕ, pol1-like I, and pol1-like II (*19*, *20*). Replicative DNA polymerases are regarded as a target of some anticancer drugs because they play central roles in DNA replication, which is indispensable for the proliferation of cancer cells. It is well-known that DNA polymerase α is overexpressed in rapidly propagating cancer cells. In a previous paper, we described that purified MGDG from spinach inhibited the activities of DNA polymerase α, δ, and ϵ with IC_{50} values of 24, 24 and 21 μg/mL, respectively, but was not effective against DNA polymerase β (*4*). We screened for a glycolipid fraction containing MGDG extracted from dried vegetables and found that spinach (*S. oleracea*) was the strongest inhibitor of DNA polymerase α and cancer cell proliferation (*18*).

Table 3. Total Extract, MGDG Yield, and MGDG Concentration Using Supercritical CO_2[a] from Spinach Subspecies[b]

spinach subspecies	total extract yield (mg of extracts/ g of dried spinach)	MGDG yield in extracts (mg of MGDG/ g of dried spinach)	MGDG concentration (%)
Esper	51.3 ± 3.4	12.9 ± 1.2	25.2 ± 1.3
Nobel	43.8 ± 1.8	11.4 ± 0.9	25.9 ± 1.2
Anna	42.3 ± 2.4	13.0 ± 1.0	30.6 ± 1.8
Nippon	51.7 ± 2.8	20.4 ± 1.5	39.4 ± 1.4
Minstarland	41.3 ± 1.8	21.6 ± 1.7	52.2 ± 1.8
Venture R5	34.1 ± 1.9	19.1 ± 1.0	56.2 ± 2.4
Houyou	41.9 ± 1.5	23.9 ± 1.3	57.0 ± 2.9
Largo	56.9 ± 3.8	32.8 ± 2.1	57.6 ± 1.8
unknown[c]	49.0 ± 2.6	29.5 ± 1.8	60.2 ± 3.0
Allright	51.7 ± 3.2	32.9 ± 2.0	63.6 ± 2.4
Try	44.2 ± 2.6	28.3 ± 1.9	64.1 ± 3.5
Ujyou	40.7 ± 1.5	28.7 ± 1.6	70.5 ± 2.8

[a] Supercritical CO_2 with 10% EtOH added at 20 MPa and 80 °C. [b] Data are expressed as the mean ± SD; $n = 3$. [c] The unknown subspecies was purchased in May, 2005, in Kobe, Hyogo prefecture, Japan.

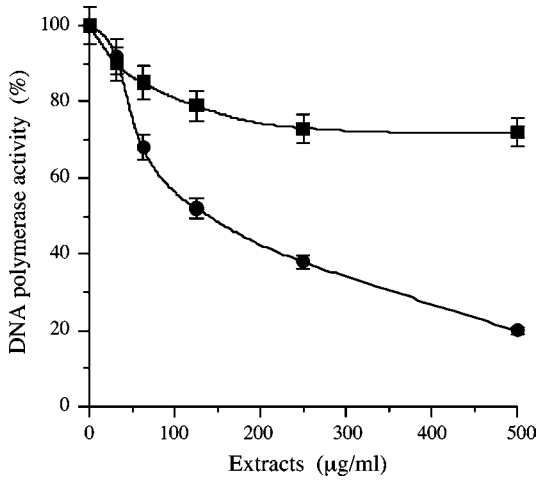

Figure 5. Inhibition of mammalian DNA polymerase activities by supercritical fluid CO_2 extract. The extract of spinach (Ujyou) containing 70.5% MGDG was obtained at 80 °C, 20 MPa, and 10% ethanol. The mixture contained 0.05 unit of enzyme. DNA polymerase activity in the absence of the compounds was taken as 100%. The mammalian DNA polymerase was tested, and the symbols used are as follows: calf DNA polymerase α (●) and rat DNA polymerase β (■). Data are shown as the means ± SEM for three independent experiments.

In this paper, it was found that the SC−CO_2 extract of the "Ujyou" subspecies had the highest concentration of MGDG. A vegetable with a large amount of glycolipid fraction containing MGDG could be an anticancer functional food; however, the water and ethanol extracts from spinach (i.e., water-soluble and fat-soluble fractions, respectively) did not inhibit the activities of replicative DNA polymerases or cancer cell growth, although the ethanol extract contained MGDG (*21*). Therefore, it was suggested that there were some compounds that could avoid the bioactivities of MGDG. It is important to effectively extract the MGDG fraction, particularly extracted from the "Ujyou" spinach subspecies using SC−CO_2, because it could help prevent cancer and be a functional food with anticancer activity.

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