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Inhibitory Effect of Natto, A Kind of Fermented Soybeans, on LDL Oxidation in Vitro

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The oxygen radical scavenging activity of natto (fermented soybeans) and its inhibitory effect on the oxidation of rat plasma low-density lipoprotein (LDL) in vitro were investigated to evaluate the usefulness of the antioxidant properties of natto, which has been shown to have antioxidant activity. Natto was separated into three water-soluble fractions: high-molecular-weight viscous substance (HMWVS; Mw > 100 000), low-molecular-weight viscous substance (LMWVS; Mw < 100 000), and soybean water extract (SWE). LMWVS had the strongest radical scavenging activity for hydroxyl and superoxide anion radicals, as assessed by electron spin resonance. The increase of conjugated dienes in LDL oxidized by copper and an azo pigment was depressed by the addition of LMWVS and SWE. These results demonstrate that natto fractions have inhibitory effects on LDL oxidation as a result of their radical scavenging activity.

KEYWORDS: Natto (fermented soybeans); antioxidant; ESR; LDL; conjugated dienes; oxygen radical scavenging activity

INTRODUCTION

Soybean has been eaten for many years in Japan. Recently, the health-promoting effects of soybean have been recognized, and their beneficial effects on a number of physiological functions, such as anti-hypertensive activity, anti-thrombotic activity, hormone-like activity, and antitumor activity, have been reported (*1*−*4*). Natto, which Japanese people have enjoyed for many years, is a kind of soybean food fermented by *Bacillus subtilis* (*natto*). It is well established that an excess of reactive oxygen and free radicals generated by various factors in the living body may act as a trigger for the lifestyle-related diseases that are becoming a major problem in present-day Japan (*5*). For example, it is known that oxidation of plasma low-density lipoprotein (LDL) plays a fundamental role in the development of arteriosclerosis (*6*). The antioxidant activity of foods and foodstuffs may help to prevent such oxidative injury, and has been the topic of recent study. It has been reported that natto, fermented soybeans, contains antioxidant components (*7*).

In this paper, the radical scavenging activities of natto water-soluble fractions (high-molecular-weight viscous substance, HMWVS; low-molecular-weight viscous substance, LMWVS; and soybean water extract, SWE), which have strong antioxidant activity by the XYZ-dish method as a new method of evaluating antioxidant activity of various foods by using chemiluminescence detection (*8*), were examined by the electron spin resonance (ESR) method. Furthermore, the inhibitory effect of these natto fractions on the oxidation of rat LDL in vitro was investigated in order to evaluate the usefulness of the antioxidant properties of natto.

MATERIALS AND METHODS

Reagents and Animals. Diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid (DTPA; Dojindo Laboratories Co., Kumamoto, Japan), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Labotec Co., Tokyo, Japan), superoxide dismutase (SOD; from bovine erythrocytes; Wako Pure Chemical Industries Ltd., Osaka, Japan), hypoxanthine (Sigma Chemical Co., St. Louis, MO), and xanthine oxidase (from buttermilk; Sigma Chemical Co., St. Louis, MO) were used for ESR. Copper(II) sulfate ($CuSO_4$), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and (−)-epigallocatechin gallate (EGCg) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) for oxidation of LDL. The highest available grade of each reagent was used. Male Wistar rats, 7 weeks old, were purchased from Clare Japan Inc. (Tokyo, Japan), and were used after acclimatization for 1 week.

Preparation of Natto Water-Soluble Fractions. The granule type of commercial natto manufactured by Taishi Foods Co., Ltd. (Towada, Japan) was used for preparation of natto water-soluble fractions at 1 day after production. Natto was suspended in 9 vol of distilled water at 4 °C for 2 h, with occasional stirring with a glass stick. After separation of the mixture into a viscous fraction and a beans fraction by filtration with gauze, the viscous fraction was separated into mycelia and viscous substance by centrifugation at 10 000 rpm for 30 min. HMWVS, having a molecular weight larger than 100 000, and LMWVS, having a molecular weight lower than 100 000, were

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separated from the viscous substance by an extra filtration at 10−20 psi in a Minitan ultrafiltration system (Millipore Co., Tokyo, Japan). The bean fraction was added to 5 vol of distilled water, and then the beans were ground in a mixer at 10 000 rpm for 1 min. After extraction at 4 °C for 14 h, the supernatant (SWE) was obtained by centrifugation at 8000 rpm for 20 min. Finally, samples were prepared by freeze-drying each fraction.

Measurement of Hydroxyl Radical Scavenging Activity. Hydroxyl radical (•OH) scavenging activity was measured by the spin trapping technique (*9*) according to the following procedure. Into a glass test tube, 20 μL of 1/10 diluted DMPO, 37.5 μL of 40 mM iron(II) sulfate, 37.5 μL of 1 mM DTPA, 30 μL of sample solution, and 75 μL of 1 mM hydrogen peroxide were added in that order, and mixed. Deionized water was used for preparation of each reagent and sample solution. The measurement of ESR spectrum was started at 45 s after addition of hydrogen peroxide. The ESR spectra were obtained with a free radical monitor JES-FR30 (JEOL Ltd., Akishima, Japan) under the following conditions: power 4 mW, center field 335.500 mT, sweep width ±5 mT, modulation width 0.32 mT, sweep time 1 min, time constant 0.1 s, amplification 50. [I_S] was calculated from the intensity ratio of the •OH signal of the sample to the manganese (Mn) signal. [I_{DMSO}] and [I_0] were calculated from the •OH /Mn ratio of dimethyl sulfoxide (DMSO) as a standard and that of deionized water. The •OH scavenging activity of the sample was calculated as DMSO equivalent concentration from [$I_S/I_0 - 1$] using the standard curve of [$I_{DMSO}/I_0 - 1$].

Measurement of Superoxide Anion Radical Scavenging Activity. Superoxide anion radical ($O_2^{-\bullet}$) scavenging activity was measured by the following procedure, as described by Noda et al. (*10*). Into a glass test tube, 30 μL of 1/2 diluted DMPO, 50 μL of 5 mM hypoxanthine, 20 μL of 9.625 mM DTPA, 50 μL of sample solution, and 50 μL of 0.4 U/mL xanthine oxidase were added in that order, and were immediately mixed. DMPO was dissolved in deionized water, and other reagents and samples were dissolved in 100 mM phosphate buffer (pH 7.4) prepared using deionized water. The measurement of ESR spectrum was started at 45 s after addition of xanthine oxidase. The spectrum measurement conditions were: power 4 mW, center field 335.600 mT, sweep width ±5 mT, modulation width 79 μT, sweep time 1 min, time constant 0.1 s, and amplification 200. [I_S] and [I_{SOD}] were calculated from the intensity ratio of the $O_2^{-\bullet}$ signal to the Mn of sample or SOD, and [I_0] was calculated from the $O_2^{-\bullet}$/Mn ratio of phosphate buffer. The $O_2^{-\bullet}$ scavenging activity of the sample was calculated as SOD equivalent activity from [$I_S/I_0 - 1$] using the standard curve of [$I_{SOD}/I_0 - 1$].

Isolation of LDL. Blood was collected with ethylenediaminetetraacetic acid under ether anesthesia from the abdominal artery of 8-week-old male Wistar rats which had been fasted for 16 h, and plasma was obtained from the blood by centrifugation at 3000 rpm for 15 min. LDL was isolated from the plasma by the ultracentrifuge method according to Havel et al. (*11*).

Measurement of Conjugated Dienes in LDL. After dialysis of LDL against 0.15 M sodium chloride solution at 4 °C for 24 h, the change of conjugated dienes (CD) concentration in LDL oxidized by copper (Cu) or AAPH was measured according to the method of Sato et al. (*12*). The protein concentration in LDL was measured using a BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard (*13*). LDL (20 μg/mL protein), 0.15 M sodium chloride, sample solution, and 2 μM $CuSO_4$ or 0.3 mM AAPH were placed into a quartz cuvette in that order. After gentle mixing, the absorbance at 234 nm (A_{234}) of the mixture, representing CD concentration, was immediately measured with a spectrophotometer every 10 min during incubation at 37 °C for 4 h. HMWVS at 10 and 50 μg/mL and LMWVS and SWE at 50, 100, and 200 μg/mL (final concentrations) were added to the LDL. Moreover, the A_{234} of the LDL mixture with 1, 3, and 10 μM EGCg as a positive control, the mixture without sample, and the mixture without sample and radical generating agent (LDL only) were also measured. The increasing rate of CD (%) relative to the initial concentration of CD at time zero of oxidation, and the area under the curve of CD increase from 0 to 4 h (AUC_{0-4h}), were calculated.

Statistical Analysis. Data were obtained as the mean ± standard deviation (SD), and were analyzed by the Scheffe test after a one-way analysis of variance (ANOVA) using the Stat View System (SAS Institute Inc., Cary, NC). A significant difference in the mean values was assumed at $P < 0.05$.

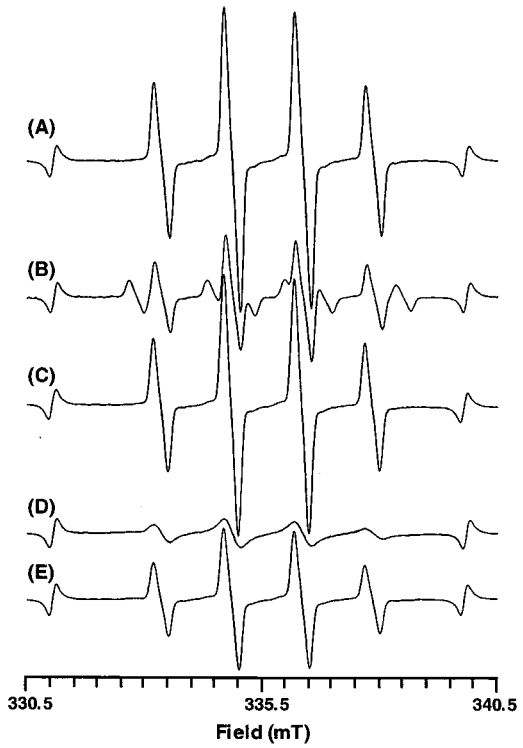

Figure 1. ESR spectra of spin adducts of •OH observed using DMPO as a spin trapping reagent in the Fenton reaction with deionized water (A), 0.3 mM DMSO (B), 10 mg/mL HMWVS (C), 10 mg/mL LMWVS (D), and 10 mg/mL SWE (E). Measurement conditions are described in the text.

RESULTS AND DISCUSSION

The ESR spectra of •OH generated by the Fenton reaction in the presence of deionized water, 0.3 mM DMSO, or 10 mg/mL HMWVS, LMWVS, and SWE are shown in **Figure 1**. As expected, the spectrum with deionized water showed a strong •OH signal (**Figure 1A**), and decrease and splitting of the •OH signal were found with addition of DMSO (**Figure 1B**). In the spectra with the natto fractions, a methyl radical adduct was not found, and •OH signal intensities were reduced in the order HMWVS (strongest signal) > SWE > LMWVS (**Figure 1C**, **D**, **E**).

Figure 2 shows the ESR spectrum of $O_2^{-\bullet}$ generated by the hypoxanthine−xanthine oxidase reaction in the presence of phosphate buffer, 10 U/mL SOD, or 10 mg/mL HMWVS, LMWVS, and SWE. Generation of $O_2^{-\bullet}$ was confirmed in the spectrum with phosphate buffer, because 12 split signals, which were assigned to the DMPO−O_2H adduct, were found in the ESR spectrum (**Figure 2A**). Addition of SOD decreased the $O_2^{-\bullet}$ signal intensity (**Figure 2B**). Addition of natto fractions caused weakening of the intensity of the $O_2^{-\bullet}$ signal, in the order SWE (strongest signal) > HMWVS > LMWVS (**Figure 2C**, **D**, **E**).

Table 1 shows the •OH and $O_2^{-\bullet}$ scavenging activities of natto fractions calculated from the ratio of radical signal to Mn signal in the ESR spectra after standardization against DMSO and SOD. The •OH scavenging activity (mM DMSO equivalent) increased in the following order: HMWVS < SWE < LMWVS. The $O_2^{-\bullet}$ scavenging activities (U SOD equivalent) of LMWVS and HMWVS were similar, and were stronger than that of SWE. These results suggest that LMWVS has the strongest radical

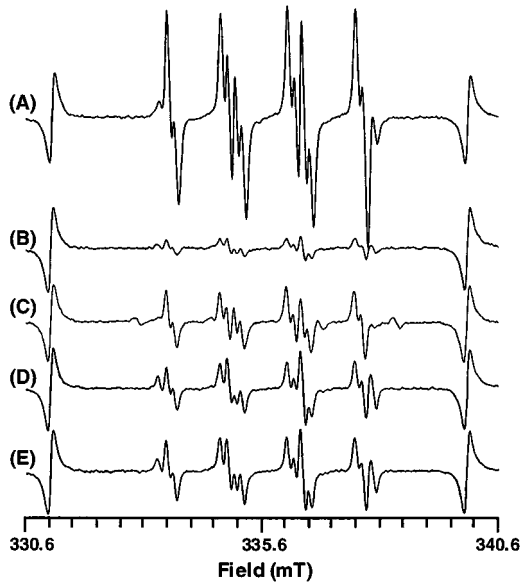

Figure 2. ESR spectra of spin adducts of $O_2^{-\bullet}$ observed using DMPO as a spin trapping reagent in the hypoxanthine–xanthine oxidase reaction with phosphate buffer (A), 10 U/mL SOD (B), 10 mg/mL HMWVS (C), 10 mg/mL LMWVS (D) and 10 mg/mL SWE (E). Measurement conditions are described in the text.

Table 1. •OH and $O_2^{-\bullet}$ Scavenging Activities[a] of Natto Fractions by ESR

	•OH (mmol DMSO eq/mg)	$O_2^{-\bullet}$ (U SOD eq/mg)
HMWVS	0.058 ± 0.004	5.995 ± 0.063
LMWVS	0.708 ± 0.492	6.301 ± 1.145
SWE	0.142 ± 0.032	2.508 ± 0.401

[a] Data represent the mean ± SD of three measurements.

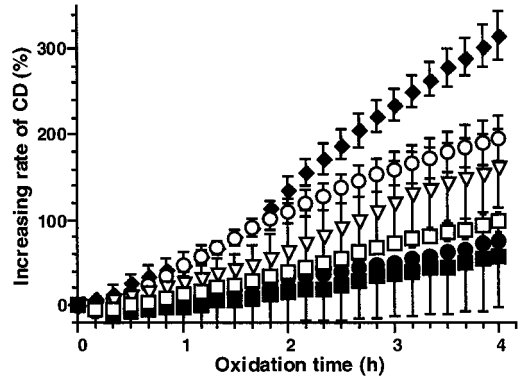

Figure 3. Change of increasing rate of CD in Cu-oxidized LDL with natto fractions. LDL (■) was oxidized by addition of 2 μM $CuSO_4$ (◆) with 3 μM EGCg (●), 50 μg/mL HMWVS (○), 50 μg/mL LMWVS (□), and 50 μg/mL SWE (▽) in a final volume of 2 mL at 37 °C. Each point represents the mean ± SD of three measurements.

scavenging activities in vitro among the natto fractions. The reason why the order of radical scavenging activities differed by radical species is uncertain.

Figure 3 shows the increasing rate of CD concentration, which represents the oxidation of LDL, in Cu-oxidized LDL in the presence of 3 μM EGCg or 50 μg/mL HMWVS, LMWVS, or SWE in vitro. In this study, the increasing rate of CD concentration was assumed to represent the time course of LDL oxidation. The increase of CD concentration in LDL only (20 μg/mL protein) was gradual, with a 50% increase in the initial concentration after 4 h. However, LDL oxidation was accelerated by addition of 2 μM $CuSO_4$, with 3× the initial concentration reached after 4 h. Addition of 3 μM EGCg to LDL + Cu reduced the rate to almost the same level as that of LDL only. Although HMWVS depressed the increase of CD in LDL + Cu after 2 h of oxidation, there was a higher increase than that with LDL only. On the other hand, LMWVS and SWE suppressed the increase of CD in LDL + Cu. Furthermore, the effects of LMWVS and SWE on the increase of CD were dose-dependent, and the addition of 200 μg/mL LMWVS and SWE depressed the increasing rate of CD concentration to less than that with LDL only (data not shown).

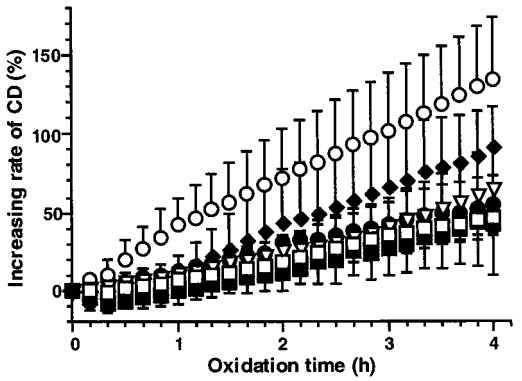

Figure 4. Change of increasing rate of CD in AAPH-oxidized LDL with natto fractions. LDL (■) was oxidized by addition of 0.3 mM AAPH (◆) with 3 μM EGCg (●), 50 μg/mL HMWVS (○), 50 μg/mL LMWVS (□), and 50 μg/mL SWE (▽) in a final volume of 2 mL at 37 °C. Each point represents the mean ± SD of three measurements.

In the oxidation of LDL, water-soluble azo pigments such as AAPH are also used as radical generating agents (*12*). In this study, oxidation of LDL by AAPH was used to investigate whether the antioxidant activity of natto fractions was based on radical scavenging activity. The changes in increasing rate of CD concentration in AAPH-oxidized LDL with EGCg, HMWVS, LMWVS, and SWE are shown in **Figure 4**. As before, LDL only (20 μg/mL protein) was oxidized gradually, with a 40% increase of initial CD concentration after 4 h. Addition of 0.3 mM AAPH caused acceleration of the increase of CD concentration, with a 90% increase of the initial concentration after 4 h. Addition of EGCg to LDL + AAPH decreased LDL oxidation to a rate similar to that of LDL only. HMWVS increased the CD to higher than that of LDL + AAPH, reaching 130% of initial concentration after 4 h. On the other hand, addition of LMWVS and SWE to LDL + AAPH decreased the increasing rate of CD. The decrease was found to be dependent on the concentration of LMWVS and SWE (data not shown). The order of the inhibitory effect of natto fractions on LDL oxidation was parallel with the order of •OH scavenging activity. These results suggest that LMWVS, with molecular weight less than 100 000, and SWE have strong inhibitory effects on oxidation of LDL by Cu and AAPH, and HMWVS has a pro-oxidant action.

Figure 5 shows the AUC_{0-4h} of the increasing rate of CD in LDL oxidized by Cu and AAPH for 4 h. In this study, the AUC_{0-4h} of the increasing rate of CD was calculated to compare the inhibitory effects of the natto water-soluble fractions on LDL oxidation, allowing for variations in the lag time for inhibition of LDL oxidation. In the Cu-oxidized LDL (**Figure 5A**), the AUC_{0-4h} of LDL + Cu increased significantly to 8× that of LDL only. Addition of EGCg (3 and 10 μM), LMWVS (50, 100, and 200 μg/mL), and SWE (100 and 200 μg/mL) significantly decreased the AUC_{0-4h} compared with that of LDL

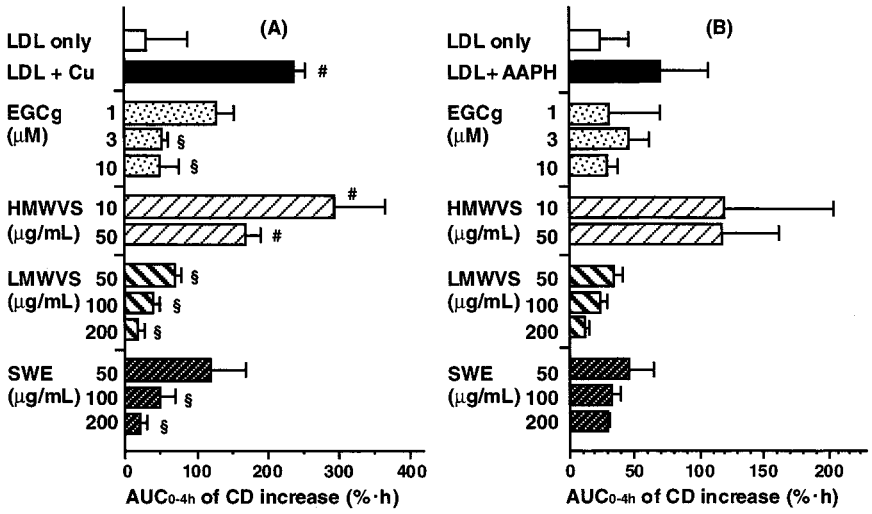

Figure 5. AUC_{0-4h} of increasing rate of CD in Cu-oxidized (A) and AAPH-oxidized (B) LDL with natto fractions. LDL (20 μg/mL protein) was oxidized by addition of 2 μM $CuSO_4$ or 0.3 mM AAPH with EGCg, HMWVS, LMWVS, or SWE in a final volume of 2 mL at 37 °C. Data represent the mean ± SD of three measurements. Significant differences from LDL only (#) and LDL + Cu (§) were assumed at $P < 0.05$.

Table 2. Inhibitory Effect of Natto Fractions on Increase of Conjugated Dienes Concentration in Oxidized LDL (IC_{50})

fraction		Cu oxidation	AAPH oxidation
EGCg	(μM)	1.70	6.86
	(μg/mL)	0.80	3.14
HMWVS	(μg/mL)	86.45	1720.3
LMWVS	(μg/mL)	58.36	65.93
SWE	(μg/mL)	76.14	71.16

+ Cu. The AUC_{0-4h} values of 200 μg/mL LMWVS and SWE were similar to that of LDL only. On the other hand, the AUC_{0-4h} of HMWVS showed a tendency to be lower than that of LDL + Cu, but was significantly larger than that of LDL only. This result showed that LMWVS and SWE had dose-dependent inhibitory effects on oxidation of LDL by Cu. In the oxidation of LDL by AAPH (**Figure 5B**), the AUC_{0-4h} of LDL + AAPH increased to 3× that of LDL only, similar to the oxidation by Cu. The AUC_{0-4h} of LDL + AAPH with EGCg, LMWVS, or SWE showed a tendency to be lower than that of LDL + AAPH, with dose-dependent decreases of AUC_{0-4h} by LMWVS and SWE. On the other hand, the AUC_{0-4h} of LDL + AAPH with HMWVS was larger than that of LDL only, and HMWVS had no effect on the oxidation of LDL by AAPH.

Table 2 shows the median inhibitory concentration (IC_{50}) of natto water-soluble fractions for the oxidation of LDL, as calculated from the AUC_{0-4h}. In both types of oxidation of LDL, the inhibitory effect on the oxidation was in the order LMWVS > SWE > HMWVS. The IC_{50} values of the natto fractions for the oxidation of LDL by Cu were lower than those for the oxidation of LDL by AAPH, suggesting that the strong inhibitory effects of natto fractions on oxidation by Cu could be caused by the dual actions of radical scavenging and metal chelating.

It has been reported that the viscous substance of natto contains crude proteins as well as γ-polyglutamic acid, and that the protein content increases with fermentation time (*14*). It was found that most of the crude proteins existed as peptide polymers having molecular weights larger than 60 000. LMWVS, which showed the strongest inhibitory effect on LDL oxidation in our study, is a crude fraction having a molecular weight less than 100 000, which was prepared by an extra filtration. Moreover, it has also been shown that the antioxidant activity of LMWVS, assessed by the XYZ-dish method, increases with the fermentation time of natto (*8*). Therefore, it is expected that highly polymerized peptides, with molecular weight less than 100 000, and amino-carbonyl reactive substances contribute to the inhibitory effect of LMWVS on LDL oxidation.

It is known that tocopherols and isoflavones participate in the antioxidant activity of soybean. Esaki et al. (*7*, *15*) reported that the antioxidant activity of natto was dependent on the increase of water-soluble fractions of natto with fermentation time, rather than on the increase of isoflavone aglycons, such as daizein and genistein. Tocopherols and alcohol-soluble isoflavones would probably not be present in the SWE, which was found to inhibit LDL oxidation, because it is a water-soluble extract of a soybean fraction excluding the viscous substances. Although proteins, peptides, and water-soluble flavonoids are expected to be active ingredients of our fractions, isolation and identification of the antioxidative substances are now in progress.

We investigated the inhibitory effect of natto water-soluble fractions on LDL oxidation, a process related to arteriosclerosis, to evaluate the antioxidant properties of natto. EGCg, which was reported to depress LDL oxidation strongly (*16*), was used as a positive control and showed marked inhibition of LDL oxidation induced by Cu and AAPH, in agreement with previous reports. Although the activity of natto fractions, as measured by IC_{50}, is weaker than that of EGCg, the antioxidant activities of natto fractions may be useful to inhibit LDL oxidation, because it is expected that the natto fractions are mixtures of crude water-soluble proteins and peptide polymers, as mentioned above. Furthermore, the chain-breaking and radical scavenging effects demonstrated in the studies of AAPH-oxidized LDL, and in the ESR studies, appear to be responsible for the inhibitory effect of natto fractions on LDL oxidation.

ABBREVIATIONS USED

HMWVS, high-molecular-weight viscous substance; LMWVS, low-molecular-weight viscous substance; SWE, soybean water extract; ESR, electron spin resonance; LDL, low-density lipoprotein; CD, conjugated dienes; EGCg, (−)-epigallocatechin gallate; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; •OH,

hydroxyl radical; $O_2^{-\bullet}$, superoxide anion radical; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid; AUC, area under the curve.

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