Antioxidant Activity of Capsanthin and the Fatty Acid Esters in Paprika (*Capsicum annuum*)

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The antioxidant ability of capsanthin and the fatty acid esters was examined by measuring the free radical-oxidation of methyl linoleate. To assess radical scavenging effect, the production of methyl linoleate hydroperoxides and the decomposition of capsanthins in reaction solution were measured by HPLC. Capsanthin suppressed hydroperoxide formation as well as β-carotene, lutein, and zeaxanthin. Interestingly, capsanthin decomposed more slowly than the other carotenoids, and the radical scavenging effect of capsanthin was found to last longer. Also, the capsanthin esterified partially and/or totally with fatty acids (mono- and/or diesterified capsanthin), isolated from paprika color, suppressed oxidation of methyl linoleate in a similar manner as nonesterified capsanthin. This finding suggests that the radical scavenging ability of capsanthin was not influenced by esterification, that is, the ability would contribute to the polyene chain, especially conjugated keto group. It was first found that esterified (monoesterified and diesterified) capsanthins also were good radical scavengers.

Keywords: *Capsanthin; esterified capsanthin; esterification; antioxidant activity; radical scavenging ability*

INTRODUCTION

Ripe fruits of paprika (red pepper) are used widely as vegetables and food colorants, which are good sources of carotenoid pigments. The red carotenoids are mainly capsanthin and capsorubin (shown in Figure 1), and the capsanthin accounts for 30−60% of total carotenoids in fully ripe fruits. Also, the capsanthin is esterified partially and/or totally with fatty acids as ripening progresses (nonesterified form; 20−30%, monoesterified form; 20−30%, diesterified form; 40−50%, in fully ripe fruits) (Minguez-Mosquera and Hornero-Mendez, 1994a,b; Camara and Moneger, 1978). The fatty acids of esterified capsanthins are chiefly lauric (12:0), myristic (14:0), and palmitic (16:0) acid (Minguez-Mosquera and Hornero-Mendez, 1994b; Biacs *et al*., 1989), and major esterified capsanthins in ripe paprika exist as 3′-*O*-myristoylcapsanthin, lauroylmyristoylcapsanthin, dimyristoylcapsanthin, and myristoylpalmitoylcapsanthin (Gregory *et al*., 1987; Philip and Chen, 1988; Goda *et al*., 1995, 1996). Esterification makes the xanthophylls more liposoluble, and, therefore, it is generally believed that the esterified capsanthins accumulate more easily into the lipophilic globules of fruit chromoplastes. Although some investigations on the stabilities of esterified capsanthins toward light (Goda *et al*., 1997) and lipoxygenase from seeds (Biacs *et al*., 1989) have done, the significance of esterification and the function of esterified forms in the fruits remain obscure.

As for the function of carotenoids, it is well-known that carotenoids harvest light energy in photosynthesis and protect plants from photosensitized oxidative damage (Palozza and Krinsky, 1992). Many reports have indicated that β-carotene quenches singlet oxygen (Foote *et al*., 1970) and scavenges free radicals (Burton and Ingold, 1984). Therefore, intake of β-carotene could be expected to prevent some cancers and cardiovascular disease, and several studies have been performed to elucidate the effectiveness (Peto *et al*., 1981; Nomura *et al*., 1985) and the mechanism (Handelman *et al*., 1991; Liebler and McClure, 1996; Kennedy and Liebler, 1992). The naturally occurring xanthophylls, which show little or no pro vitamin A activity, have been also noted, and some xanthophylls have been found to be antioxidants (Terao, 1989; Miki, 1991; Hirayama *et al*., 1994). Most of these xanthophylls were in nonesterified forms; however, little is known about the antioxidant ability of esterified xanthophylls.

In this report, we first tried to investigate the radical scavenging ability as an indicator of antioxidant effect of some esterified capsanthins. The results suggest that

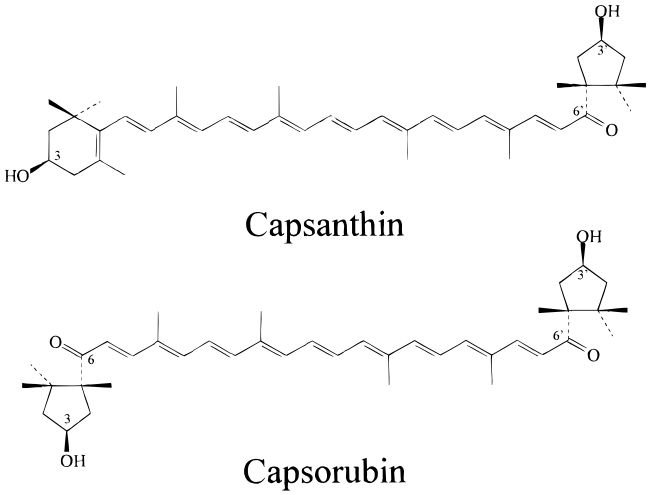

Figure 1. Structures of capsanthin and capsorubin.

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the esterified capsanthins are good radical scavengers as well as nonesterified form and the radical scavenging ability contributes to conjugated keto group and polyene chain in the molecules.

EXPERIMENTAL PROCEDURES

Materials. Capsanthin ((3*R*,3′*S*,5′*R*)-3,3′-dihydroxy-β,κ-carotene-6′-one), β-carotene (β,β-carotene), and zeaxanthin ((3*R*,3′*R*)-β,β-carotene-3,3′-diol) were purchased from Funakoshi Inc. (Tokyo, Japan) and lutein ((3*R*,3′*R*,6′*R*)-β,ϵ-carotene-3,3′-diol) was from Sigma Co. Ltd. (MO), and they were stored in the dark at −20 °C. Paprika color (paprika oleoresin DN-933) was obtained from San-Ei Gen F.F.I. Inc. (Osaka, Japan). This commercial color is a natural paprika oleoresin extract and do not contain any additives. Methyl linoleate (99%) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Other reagents and solvents were of analytical grade and used without further purification.

Reaction Conditions. An appropriate amount of carotenoids dissolved in 125 μL of chloroform was added to a solution of methyl linoleate (50 μL) and ethanol (450 μL). The solution was incubated with AMVN (3.1 mg; 12.5 μmol) in ethanol (125 μL) under the air at 37 °C in the dark. Small aliquots of the reaction solution were taken periodically for analyses of hydroperoxides from methyl linoleate by the normal-phase HPLC (Tosoh LC-8020 system, Tosoh Inc., Tokyo, Japan). For autoxidation, the reaction was carried out under above condition, except for addition of AMVN.

Assay for Antioxidant Activity. The measurement of antioxidant activity was performed by the modified method of Terao (1989, 1992), based on the monitoring of the production of four hydroperoxides from methyl linoleate. Small aliquots of the reaction solution were put directly on a normal-phase column (Shodex SIL 5B, 4.6 mm i.d. × 250 mm, Showa Inc., Tokyo, Japan) for HPLC. The column was eluted with 1.0% 2-propanol in hexane. Flow rate was maintained at 1.0 mL/min, and effluent was monitored at 235 nm. The antioxidant activity of carotenoids was estimated according to the sum of four hydroperoxides determined by the calibration curve. Also, residual carotenoids in the reaction solution were quantified by HPLC using a column of Wakosil-II $5C_{18}$-AR (4.6 mm i.d. × 250 mm, Wako Pure Chem. Co.). The column was eluted with 70% acetone for capsanthin, zeaxanthin, and lutein (90% acetone for β-carotene and esterified capsanthins). Flow rate was maintained at 0.8 mL/min, and the effluent was monitored at 460 nm.

For each estimation of antioxidant activity of carotenoids, a minimum of three replicate experiments were made.

Purification of Monoesterified and Diesterified Capsanthin from Paprika Color. To predict the antioxidant activity of monoesterified and diesterified capsanthin, a fractionation of paprika color by TLC was attempted. A preparative TLC was done according to the method reported by Goda *et al.* (1995). TLC plate of silica gel 60 GF_{254} (200 × 200 mm plates, thickness 0.7 mm) (Merck Co. Inc., Darmstadt, Germany) was used with 15% acetone in petroleum ether as a developing solvent. Of the obtained nine fractions, 3-*O*-monoester fraction of capsanthin (Fr.9, R_f = 0.19), 3′-*O*-monoester fraction of capsanthin (Fr. 8, R_f = 0.23), and diester fraction of capsanthin (Fr. 3, R_f = 0.50) were further subjected to a preparative HPLC to afford major esterified capsanthins. A preparative HPLC was done under the following conditions: a column of Wakosil-II $5C_{18}$-AR (20 mm i.d.× 250 mm), an elution of 87% acetone, a flow rate of 10 mL/min, and a detection of 460 nm. The eluate was rechromatographied under the same condition described above. Finally, one 3-*O*-monoesterified capsanthin from Fr. 9, two 3′-*O*-monoesteified forms from Fr. 8, and two diesterified forms from Fr. 3 were isolated.

Identification of Esterified Capsanthins. An identification of five esterified capsanthins isolated was done by mass spectrometry (MS) analysis using a JEOL JMS-SX 102A spectrometer. Each sample was dissolved in acetone and directly injected. MS analyses were done under the following conditions: positive-mode fast atom bombardment (FAB) ionization, a scan speed of 10−2000 *m*/*z* for 10 s, and an accelerator voltage of 10 kV.

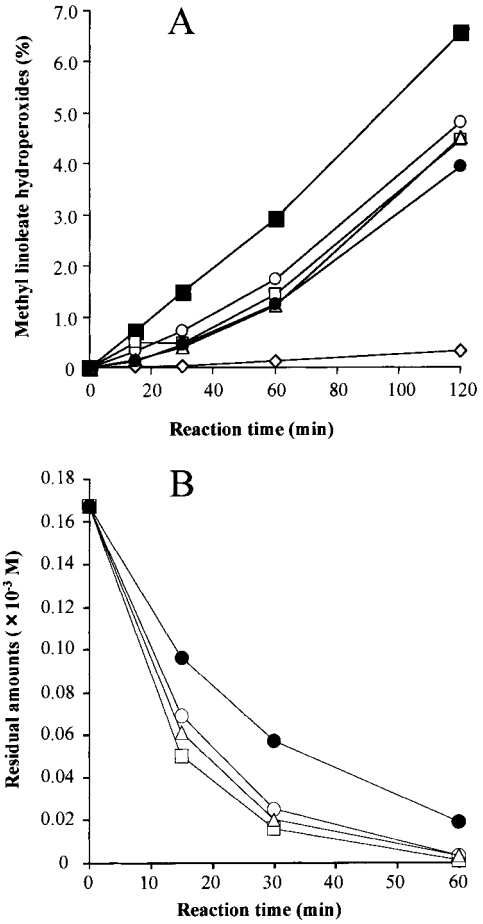

Figure 2. Formation of methyl linoleate hydroperoxides (A) and degradation of capsanthin, β-carotene, lutein, and zeaxanthin (B) in oxidation of methyl linoleate. Control (■); capsanthin (●); β-carotene (□); lutein (○); zeaxanthin (△); α-tocopherol (◇). When the carotenoids are added at 1.0×10^{-3} M, all of them suppressed the production of hydroperoxides, but no difference was observed among them. On the other hand, capsanthin was decomposed more slowly than those of the other three carotenoids significantly.

RESULTS

Radical Scavenging Ability of Capsanthin. Figure 2A shows the effects of the four carotenoids and α-tocopherol at 1.0×10^{-3} M (final concentrated 1.7×10^{-4} M) on AMVN-induced oxidation of methyl linoleate. In the absence of carotenoids (control), hydroperoxides from methyl linoleate increased linearly with reaction time. The additions of carotenoids and α-tocopherol suppressed the production of hydroperoxides (inhibition ratios after 120-min oxidation: capsanthin, 40 ± 2.1%; β-carotene, 36 ± 2.3%; lutein, 33% ± 2.1; zeaxanthin, 36 ± 2.8%; α-tocopherol, 92 ± 2.9%). The production behavior in the presence of capsanthin showed a similar trend to that of the other carotenoids, although the distinct induction period showed in α-tocopherol was not observed. Therefore, it was found that the capsanthin scavenged radicals as well as other carotenoids already regarded as antioxidant carotenoids. On the other hand, as for disappearance of carotenoids in the reaction solution, four carotenoids decreased with reaction time,

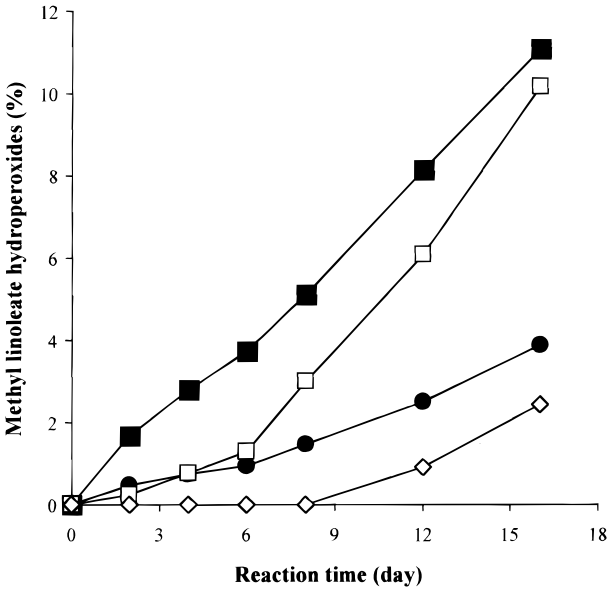

Figure 3. Effects of capsanthin, β-carotene, and α-tocopherol on autoxidation of methyl linoleate. Control (■); capsanthin (●); β-carotene (□); α-tocopherol (◇). When the capsanthin and β-carotene added at 1.0×10^{-3} M, capsanthin showed the longer antioxidant effect than β-carotene. In the reaction solution without methyl linoleate, residual capsanthin and β-carotene after 21 days were 103.7 and 92.8%, respectively.

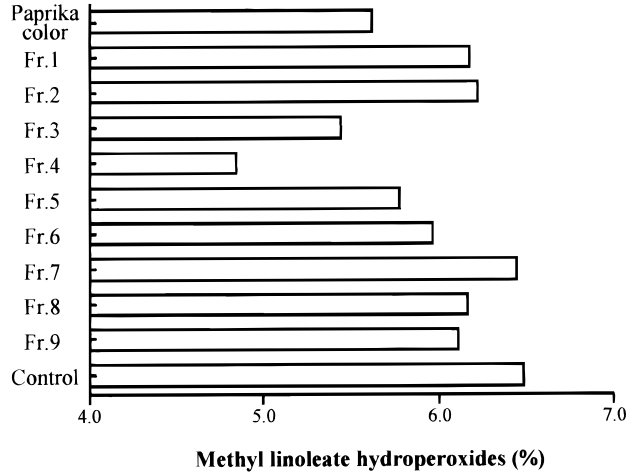

Figure 4. Antioxidant activity of TLC fraction on AMVN-oxidation of methyl linoleate. When each fraction was added at 1.0 mg mL^{-1}, their inhibition ratios after 120 min were 5.6 ± 0.2 (Fr. 1), 4.7 ± 0.4 (Fr. 2),18.7 ± 0.3 (Fr. 3), 29.3 ± 0.2 (Fr. 4), 12.7 ± 0.8 (Fr. 5), 9.4 ± 0.2 (Fr. 6), 0.8 ± 0.2 (Fr. 7), 5.9 ± 0.2 (Fr. 8), and 6.8 ± 0.2% (Fr. 9). Fr. 3 containing diesterified capsanthins mainly showed potent antioxidant activity, and Fr. 8 and 9 containing monoesterified capsanthins showed little activity. The detailed activity of monoesterified and diesterified capsanthins is shown in Figure 5.

although their rates were quite different (Figure 2B). The capsanthin was decomposed more slowly than those of the other carotenoids significantly ($p < 0.05$), and the initial rate of disappearance was 4.7×10^{-6} M·min^{-1} (β-carotene, 7.9×10^{-6}; lutein, 6.5×10^{-6}; zeaxanthin, 7.1×10^{-6} M·min^{-1}). Thus, these findings imply that the radical scavenging ability per one molecule of capsanthin might be more potent than that of the other carotenoids, that is, the antioxidant effect of capsanthin might last longer than that of the others.

Then, to confirm the prolonged effect of the capsanthin, the effect of capsanthin on autoxidation of methyl linoleate was investigated. Figure 3 shows the effects of capsanthin and β-carotene at 1.0×10^{-3} M on the autoxidation. During 6-day oxidation, the production curve of hydroperoxides from methyl linoleate in the presence of capsanthin was similar to that of β-carotene. However, thereafter the capsanthin suppressed the oxidation of methyl linoleate more potently than β-carotene (inhibition ratios after 16-day oxidation: capsanthin, 65 ± 5.5%; β-carotene, 8 ± 1.6%). As for disappearance of carotenoids in the reaction solution after 6-day oxidation, capsanthin was only decomposed 75%, although more than 90% of β-carotene was done (data not shown). Thus, these results suggest that the capsanthin would be a more effective radical scavenger than β-carotene.

Radical Scavenging Ability of Paprika Color. To survey antioxidant ability of esterified capsanthins, the effect of paprika color at 1.0 mg mL^{-1} (final concentrated 0.17 mg mL^{-1}) containing esterified capsanthins mainly on AMVN-induced oxidation of methyl linoleate was investigated. The paprika color suppressed formation of hydroperoxide on 120-min oxidation (inhibition ratio: 15.4 ± 4.3%). When the paprika color at higher concentrations (final concentrated 0.85 and 1.7 mg mL^{-1}) was added to the reaction solution, the inhibitory effect markedly increased with increasing concentration (inhibition ratios: 24.8 ± 7.1 and 43.8 ± 4.7%, respectively). Thus, it was assumed that esterified capsanthins also would have radical scavenging ability. Then, to confirm the effect of esterified capsanthins, the paprika color was fractionated by a preparative TLC. It has already been reported that the fractionation by TLC under the foregoing condition made it possible to obtain separately the monoester and diester fractions (Goda *et al.*, 1995). Of the obtained nine fractions, the fraction 1 (Fr. 1, $R_f = 0.75$) contains β-carotene mainly, the Fr. 3 ($R_f = 0.50$) is the diesterified capsanthins mainly, the Fr. 4 ($R_f = 0.42$) is the diesterified capsorubins mainly, the Fr. 8 ($R_f = 0.23$) is the 3′-*O*-monoesterified capsanthins mainly, and the Fr. 9 ($R_f = 0.19$) is 3-*O*-monoesterified capsanthins mainly. Figure 4 shows the effect of the each fraction at 1.0 mg mL^{-1} (final concentrated 0.17 mg mL^{-1}) on 120-min oxidation of methyl linoleate. In the Fr. 3 and Fr. 4, the potent antioxidant effects were observed. On the other hand, the Fr. 8 and 9 showed little antioxidant activity. Generally, a paprika extract is known to include a very high amount of triglycerides containing unsaturated fatty acids (Philip *et al.*, 1971). Thus, to evaluate the ability of esterified capsanthins in more detail, the further purification of these fractions (Fr. 3, 8, and 9) by HPLC was done.

Radical Scavenging Ability of Esterified Capsanthin. The radical scavenging effects of major esterified capsanthins in Fr. 3, 8, and 9, which were isolated by HPLC, were investigated. The esterified capsanthins obtained from three fractions were confirmed by FAB-MS, and they were found to be 3-*O*-lauroylcapsanthin (*m/z* 767 $[M + H]^+$, *m/z* 566 $[M - CH_3(CH_2)_{10}COO]^+$), 3′-*O*-lauroylcapsanthin (*m/z* 767 $[M + H]^+$, *m/z* 567 $[M - CH_3(CH_2)_{10}COO + H]^+$), 3′-*O*-myristoylcapsanthin (*m/z* 795 $[M + H]^+$, *m/z* 567 $[M - CH_3(CH_2)_{12}COO + H]^+$), lauroylmyristoylcapsanthin (*m/z* 993 $[M + H]^+$, *m/z* 794 $[M - CH_3(CH_2)_{10}COO]^+$, *m/z* 765 $[M - CH_3(CH_2)_{12}COO + H]^+$), and dimyristoylcapsanthin (*m/z* 1021 $[M + H]^+$, *m/z* 794 $[M - CH_3(CH_2)_{12}COO]^+$). Figure 5A shows the radical scavenging effects of these esterified capsanthins on AMVN-

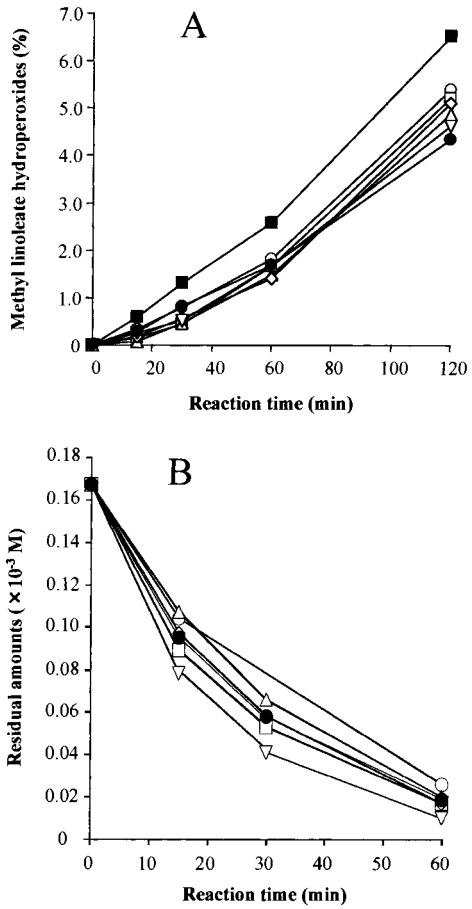

Figure 5. Formation of methyl linoleate hydroperoxides (A) and degradation of capsanthin and the fatty acid esters (B) in oxidation of methyl linoleate. Control (■); capsanthin (●); 3-*O*-lauroylcapsanthin (▽); 3′-*O*-lauroylcapsanthin (△); 3′-*O*-myristoylcapsanthin (◊); lauroylmyristoylcapsanthin (□); dimyristoylcapsanthin (○). All esterified capsanthins used in this experiment suppressed the production of hydroperoxides, but no difference was observed among them.

induced oxidation of methyl linoleate. The production curve of hydroperoxides in the presence of these esterified capsanthins showed a nearly same trend to that of nonesterified form. Considering that monoesterified capsanthins suppressed the oxidation of methyl linoleate, little activity of TLC fractions (Fr. 8 and 9) would be caused by contaminants with triglycerides. Therefore, it was first found that monoesterified and diesterified capsanthins also had the same radical scavenging ability as nonesterified form. Also, no significant difference between disappearances of these esterified capsanthins and that of nonesterified form was observed (Figure 5B). Thus, these findings suggest that the radical scavenging ability of capsanthin was not influenced by esterification.

DISCUSSION

A capsanthin is one of the major carotenoids of red paprika fruits and occurs esterified with fatty acids in ripe fruits. The esterified capsanthins increase as the fruit is ripened, and they account for 70−80% of total capsanthins (Minguez-Mosquera and Hornero-Mendez, 1994b). Biacs *et al.* (1989) have reported that esterified capsanthins were more stable toward lipoxygenase from seeds than nonesterified capsanthin, and the greater stability was found in the diesterified capsanthins. On the other hand, Goda *et al.* (1997) have reported that esterified and nonesterified capsanthin did not differ in photostability; therefore, it is thought that esterified capsanthins would be the same as nonesterified capsanthin or have any more function.

Contrary to studies of antioxidant ability on the β-carotene and nonesterified xanthophylls, the ability of esterified xanthophylls has been scarcely investigated in detail. Miki (1991) has reported that diesterified astaxanthin, which was a major ovarian carotenoid, had no radical scavenging ability, despite the potent ability of nonesterified form. In our data, the esterified capsanthins showed the same radical scavenging effect as that of nonesterified form (Figure 5), although the chemicals and the assay method used in our study were different from those of his report. However, judging from the reaction solution (50% aqueous ethanol) used in his study, no activity of diesterified astaxanthin was observed as a result of the insolubility in the solution. Our data suggests that radical scavenging ability of capsanthin would be not influenced by hydroxyl groups at the 3- and 3′-positions in β-ionone and cyclopentane rings, respectively. Namely, this finding led to the suggestion that the ability would contribute to the polyene chain only.

Several investigators (Di Mascio *et al.*, 1989) have reported that the antioxidant effects of carotenoids depended on the number of conjugated double bonds, the chain structure, and functional groups. β-Carotene seems to scavenge a radical by a mechanism in which the conjugated polyene chain trapped the radical, followed by resonance-stabilized carbon-center radical (Burton and Ingold, 1984). Miki (1991) and Terao (1989) have reported that canthaxanthin and astaxanthin with oxo groups at 4- and 4′-positions in the β-ionone of β-carotene and zeaxanthin, respectively, were more potent antioxidants than β-carotene and zeaxanthin and concluded that the oxo group would enhance the stability of the trapped radical. Also, Hirayama *et al.* (1994) have investigated the singlet oxygen quenching abilities of 18 carotenoids and reported that the conjugated keto group enhanced quenching, while hydroxy, epoxy, and methoxy groups showed lesser effects. Thus, the result that the radical scavenging effect of capsanthin lasted longer than β-carotene (Figure 2) indicates that the conjugated keto group (the carbonyl at 6′-position in the polyene chain) of capsanthin would enhance the effect, in addition to radical scavenging ability of conjugated polyene chain. Therefore, the result that the TLC fraction of diesterified capsorubin, which possesses two carbonyl groups at 6- and 6′-positions, showed more potent activity than that of esterified capsanthins (Figure 4) supports this speculation. At present, we are attempting to confirm the antioxidant activity of capsorubin.

In conclusion, it was found that capsanthin was the more effective antioxidant than β-carotene, and the esters had the same radical scavenging ability. In paprika fruits, as ripening progresses, capsanthin is progressively esterified with fatty acids and more easily incorporated into the structure of membranes. Thus, esterified capsanthins may serve as the antioxidant in hydrophobic regions such as the lipophilic globules of chromoplates, although further studies are needed to clarify the function. Also, the fact that esterified capsanthins (paprika color) had antioxidant ability should be discussed as a possible food additives.

ABBREVIATIONS USED

AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC, high performance liquid chromatography.

Registry No. Supplied by the Author: Capsanthin ((3*R*,3′*S*,5′*R*)-3,3′-dihydroxy-β,κ-carotene-6′-one), 465-42-9; β-carotene (β,β-carotene), 7235-40-7; lutein ((3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol), 127-40-2; zeaxanthin ((3*R*,3′*R*)-β,β-carotene-3,3′-diol), 144-68-3; α-tocopherol, 59-02-9.

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