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Antioxidative Effect of Crude Anthocyanins in Water-in-Oil Microemulsion System

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Abstract Antioxidative ability of anthocyanins in water-in-oil microemulsion was examined. Microemulsion was prepared by solubilizing crude anthocyanins extracted from grape skin (*Cambell early*) in organic solvent (hexane) containing anionic surfactant [bis (2-ethylhexyl) sodium sulfosuccinate, AOT] and linolenic acid (10%, w/v). Lipid oxidation significantly decreased with increasing concentration of anthocyanins (5-20 μM) at micellar phase, and increasing micelle size (Wo=5-20 μM). At given micelle size (Wo=10), lipid oxidation decreased as number of micelles decreased. These results indicate antioxidative ability of anthocyanins is critically affected by water core and micelle structure formed by surfactant. Interactions between AOT and anthocyanins decreased antioxidative ability of anthocyanins. Antioxidative ability of anthocyanins significantly increased when α-tocopherol was added into organic phase. This indicates of synergism between the two antioxidants.

Keywords: anthocyanins, antioxidative effect, anionic surfactant, water-in-oil microemulsion

Introduction

Lipid oxidation is one of the main causes of quality deterioration in many foods, because organoleptical and nutritional qualities of foods can be significantly altered by lipid oxidation products (1). To prevent or delay undesirable lipid oxidation, extensive studies have been performed on the mechanism of lipid oxidation and efficacy of various antioxidants (2, 3). Recently, interest in lipid oxidation of multiphase system has greatly increased, because a large number of the actual food products are in the emulsified forms or contain multicomponent matrices (4). In partitioned medium such as an emulsion system, the rate of lipid oxidation is affected by the location of antioxidants and properties of lipid interface (5). Silvestre *et al.* (6) also reported that the characteristics of surfactant polar head groups were critically influenced the oxidative stability of oil-in-water emulsion and thickness of the interfacial membrane played an important role in determining the ability of lipid peroxides to oxidize fatty acids. However, the understanding of physicochemical interactions between antioxidants and surfactants in emulsion system, especially for water-in-oil emulsion, is fairly limited.

Reverse micelle, a water-in-oil microemulsion, is an interesting system to examine the interaction between surfactant and water-soluble solutes, because solutes can be incorporated into the polar core of micelles dispersed in the organic solvent without any phase separation. The aggregate of surfactant molecules are spontaneously generated in the organic solvent as a result of molecular self-assembly, and these aggregates accommodate water-soluble solutes in their polar cores (7). In addition, structural dimensions such as size and number of micelles can be easily controlled by molar ratios of water to surfactant (Wo) (8). Of surfactants, anionic bis (2-ethylhexyl) sodium sulfosuccinate (AOT) has been widely used for the formation of reverse micelles due to its stability and the capacity to solubilize large amount of water (100 mol water / mol AOT) (9).

Anthocyanins are water-soluble natural pigments widely used in food industry. In addition to their attractive color quality, anthocyanins are becoming important as bioactive components. The potential health benefits of anthocyanins were reviewed by Wrolstad (10). The biological activities of anthocyanins have been correlated to the free radical-scavenging ability and the inhibition of lipid peroxidation (11, 12). Previously, we reported the color expression of anthocyanins in an apolar medium. The color intensity of anthocyanins in reverse micelles was primarily affected by the interaction between sulfonate head of AOT and flavilium cation of anthocyanins (13). This finding suggests antioxidative activity of anthocyanins could be changed by various microenvironments including the size and number of micelles.

The objective of this study was to examine the effect of anthocyanins on lipid oxidation in a model water-in-oil microemulsion. The antioxidative activity changes in various microenvironments and synergistic effects between anthocyanins and α-tocopherol were also determined.

Materials and Methods

Chemicals Sodium bis (2-ethylhexyl) sulfosuccinate (AOT) and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Linolenic acid was supplied by ICN Biomedicals, Inc. (Tokyo, Japan). All other chemicals were from Fischer Scientific (Springfield, NJ, USA.) and were HPLC or analytical grade unless otherwise stated.

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Extraction of crude anthocyanins Fresh grape skin (*Cambell early*) was obtained from local supermarket (Seoul, Korea), and grape skins were collected and frozen at -50°C until use. Anthocyanins were extracted with methanol solution containing 3% formic acids (methanol: formic acid:water=70:3:27, v/v/v). The extract was filtered through Whatman filter paper (No. 5, Whatman International Ltd., Maidstone, UK) and concentrated under vacuum at 40°C using a rotary evaporator (Eyela, Tokyo, Japan). The concentrated anthocyanin stock solution was distributed in eppendorf tubes and frozen until use. Total anthocyanin content was determined based on the absorption maximum at 520 nm and calculated as cyaniding-3-glucoside using an extinction coefficient (e) of 26,000 and a molecular weight of 445 (14). Prior to experiment, the stock solution was thawed in the refrigerator and filtered through a 0.45-μm syringe filter (Sartorius AG 37070; Goettingen, Germany) to exclude any particulate matter and risk of microbial growth.

Preparation of water-in-oil microemulsion containing anthocyanins and linolenic acids Solubilization of anthocyanins in reverse micellar organic phase was carried out by injecting buffered anthocyanin stock solution (McIlvaine buffer, 50 mM citric acid-Na_2HPO_4, pH 3.0) into an AOT/hexane solution using a syringe. The concentration of AOT and volume of buffered anthocyanins were appropriately adjusted to obtain the micellar organic solution at the desired Wo (molar ratio of water to surfactant). The solution (8 mL) was then stirred vigorously (about 40 min) until a transparent single phase was obtained. Finally, linolenic acid dissolved in hexane (1 g/2 mL) was mixed into the micellar solution.

Determination of lipid oxidation The samples were evenly distributed (10 mL of sample in a 25-mL vial) in air-tight clear vials (Wheaton, Millville, NJ, USA) and placed in a 20°C incubator (Vision Scientific, Seoul, Korea) for 10 days. Samples were taken every other day, and the peroxide value was determined to monitor the extent of lipid oxidation (15). All oxidation experiments and peroxide value measurements were carried out in triplicates.

Effect of anthocyanins on lipid oxidation in water-in-oil microemulsion Micellar solutions containing different concentrations of anthocyanins (5-20 μM) and linolenic acid (10%, w/v) were prepared at a fixed concentration of AOT (50 mM). All concentrations are given with respect to the total volume of the system, and total volume ratios of aqueous to organic phase were kept constant.

Effect of micelle size and number on lipid oxidation in water-in-oil microemulsion To examine the effect of micelle size on lipid oxidation, reverse micelles with different Wo values (5-20) were prepared. The Wo value is defined as the mole ratio of water to surfactant and is a key parameter in the description of microemulsions, because it relates to the size of the microemulsion droplets (16). For the preparation of a particular Wo value, the required aqueous volume was achieved by injection of the McIlvaine buffer. Under this condition all samples contained the same concentrations of anthocyanins (5 or 10 μM), AOT (50 mM), and linolenic acid (10%, w/v). The effect of micelle number was examined at a constant Wo of 10. To make water-in-oil emulsion micelles with different micelle numbers, the concentration of AOT (25-100 μM) and water (250-1,000 mM) were changed in equal proportions to insure that hydration degree remained unchanged.

Effect of AOT on antioxidative ability of anthocyanins Anthocyanins (10 μM), AOT (50 and 100 mM), and linolenic acid (10%, w/v) were solubilized in ethanol (99%), a common solvent for solubilizing anthocyanins, AOT, and linolenic acid, and the extent of lipid oxidation in the ethanol medium was compared to those of water-in-oil microemulsion (Wo = 10). The concentrations of all solutes dissolved in ethanol were equal to those used for water-in-oil microemulsion.

Synergistic effect between anthocyanins and α-tocopherol on lipid oxidation To evaluate any synergistic antioxidative effect between anthocyanins and α-tocopherol in water-in-oil microemulsion, α-tocopherol (100 ppm) was directly added into the organic phase, and water-in-oil emulsions containing anthocyanins (10 μM) were prepared. The changes in peroxide value during storage were determined. In all cases, Wo value of the micellar solution was fix as 10.

Statistical analyses Peroxide values at each storage period within the same treatment group were analyzed by analysis of variance (ANOVA) using Minitab Ver. 13.1 (Minitab Inc., State College, PA, USA). Tukey's test was used for multiple comparison of the treatment means at the significant level of $p<0.05$.

Results and Discussion

Effect of anthocyanins on lipid oxidation in water-in-oil microemulsion When anthocyanins were solubilized in water-in-oil microemulsion, the extent of lipid oxidation based on peroxide value significantly decreased in a dose-dependent manner (Fig. 1), which indicates that water-soluble anthocyanins can act effectively as antioxidants in the apolar medium. The formation of hydroperoxide in the control reached maximum after 6 days and decreased after prolonged storage due to the limited amount of lipid substrate (10%, w/v). In the presence of more than 10 μM anthocyanins, no appreciable oxidation occurred. The distinct red color of anthocyanins in water-in-oil microemulsion disappeared within 3 days of lipid oxidation (data not shown), while more than 90% of initial intensity was maintained after 10 days storage in the absence of lipid (13). In spite of the color loss, anthocyanins still retained antioxidative activity.

Generally, emulsion is considered to consist of three compartments, dispersed droplet, continuous phase, and interfacial region (17). Water-in-oil microemulsion system used in this study can be depicted similarly, that is, dispersed aqueous droplet containing anthocyanins, continuous hexane medium, and interface basically formed by the mixture of surfactant, aqueous phase, and oil.

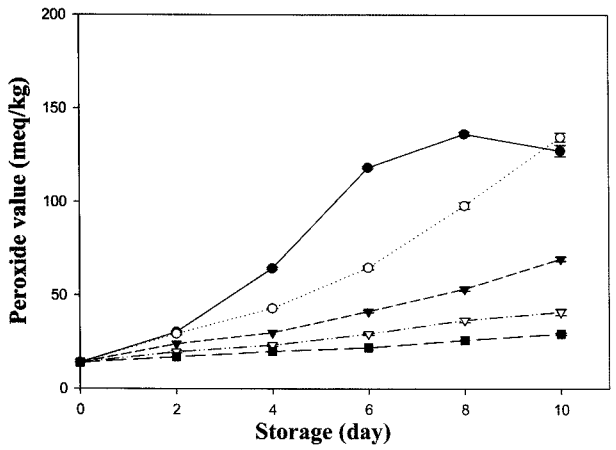

Fig. 1. Effect of anthocyanins on lipid oxidation in water-in-oil microemulsions during storage. The molar water to AOT ratio (Wo) was 10. ● : Control (buffer pH 3.0), ○ : 5 μM, ▼ : 10 μM, ▽ : 15 μM, ■ : 20 μM.

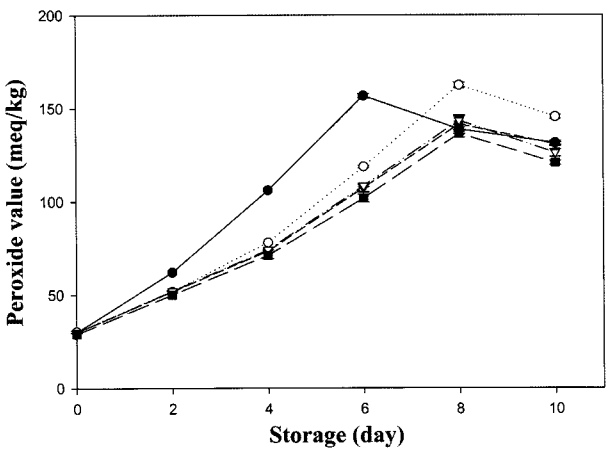

Fig. 2. Effect of molar water to AOT ratio (Wo) on antioxidative ability of anthocyanins in water-in-oil micro-emulsions. The concen-tration of anthocyanins was 5 μM. ● : Control (buffer, Wo = 10), ○ : Wo = 5, ▼ : Wo = 10, ▽ : Wo = 15, ■ : Wo = 20.

However, water-in-oil microemulsion has much larger interface area than that of classical emulsion, because the water pools in the reverse micelles are in the nanometer scale (18). In this context, lipid oxidation in the water-in-oil emulsion can be regarded as an interfacial phenomenon.

In relation to lipid oxidation in the emulsion system, McClements and Decker (17) indicated that hydroperoxides and their decomposed radical products are relatively polar and are likely to accumulate at the interface. Once free radicals form, they can interact with lipids in the vicinity. The types and concentration of molecules located at the interface influence lipid oxidation by attracting (or repelling) prooxidants and antioxidants or altering the interaction between lipids and water-soluble antioxidants (19). The lipid oxidation in water-in-oil emulsion used in this study is also dictated by characteristics and concentration of AOT (surfactant). AOT determines the size of aqueous droplet, charge distribution, and interactions between AOT and anthocyanins at the interface, which, in turn, affect the antioxidant ability of anthocyanins. Although the effect of antioxidant in the emulsified system is dependent upon the partitioning behavior of antioxidants, the partitioning of anthocyanins into organic solvent (hexane) was negligible in this study (20).

Effect of micelle size on antioxidant activity of anthocyanins in water-in-oil microemulsion To examine the effect of micelle size on the antioxidative activity of anthocyanins, the extent of lipid oxidation at different Wo values were compared, because Wo is directly proportional to the micellar radius (21). Lipid oxidation decreased as Wo increased at constant anthocyanin concentration (Fig. 2). The microemulsion with the smallest micelle size (Wo =5) showed significantly higher ($p<0.05$) peroxide value than those of other microemulsions after 4 days of storage. However, no significant differences in peroxide values were observed for other microemulsions with Wo of 10 to 20.

This result could be partly attributed to the possible changes in mobility of the solubilized anthocyanins. The bulk properties of water (viscosity, hydrogen-bonding ability) either inside the water pool (free) or at the interface (bound) change with Wo. According to Silber *et al.* (22), water is highly structured up to approximately Wo = 10 and is essentially frozen at Wo<5. The free water is dominant form for Wo>20, while the intermediate situations are present for 10<Wo<20 when water-in-oil microemulsion is prepared using AOT. This means larger micelles posses more free water to permit greater mobility of anthocyanins. The observed trend is quite reasonable based on the hypothesis that significant proportion of total water pool is involved in hydrating the high amounts of counter sodium ions and surfactant head groups in microemulsions with lower Wo (such as Wo=5). The increased free water content in larger micelles could be responsible for loosening the firmness of micellar interfacial structure, thus increasing the chances for anthocyanins to access peroxide near the interface, which, in turn, increases the antioxidative ability of anthocyanins in larger micelles.

Effect of micelle number on antioxidative activity of anthocyanins in water-in-oil microemulsion Mulinacci *et al.* (23) reported that surface charge, micelle, and water pool size critically influenced the biological activities of organic molecules in reverse micelles. To examine the effect of micelle number on lipid oxidation, the microemulsions, which have equal micelle size but different micelle concentrations, were prepared. As AOT concentration increased, the extent of lipid oxidation significantly increased at each storage time (Fig. 3), probably due to the reduced number of available anthocyanins at the interfaces. The concentration of anthocyanins contained in each micelle droplet decreased as the concentration of micelles increased, while the chances for interaction between AOT head and anthocyanins at the interfaces increased in high micelle concentrations (Fig. 4). The interactions between sulfonate head of AOT and flavylium cation of anthocyanins via

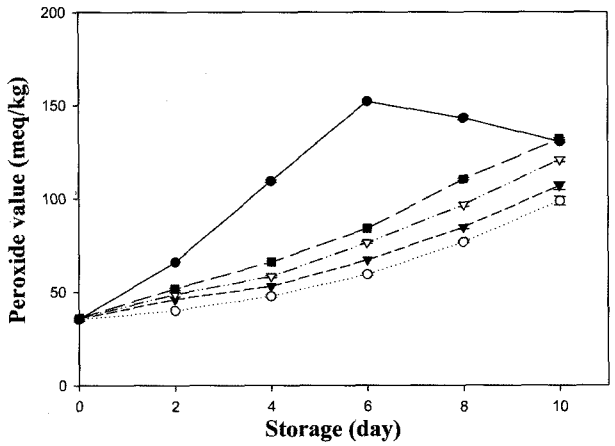

Fig. 3. Effect of micelle numbers on antioxidative activity of anthocyanins in water-in-oil microemulsions. ● : Control (buffer / AOT 50 mM, Wo=10), ○ : Anthocyanins 10 μM/AOT 25 mM, Wo = 10, ▼ : Anthocyanins 10 μM/AOT 50 mM, Wo=10, ▽ : Anthocyanins 10 μM/AOT 75 mM, Wo=10, ■ Anthocyanins 10 μM/AOT 100 mM, Wo=10.

their ionic nature might cause a reduction of available anthocyanins for reacting with hydroperoxides as radical scavengers. Based on the results of micelle size and number, the antioxidative activity can be modulated by localization of anthocyanins in the microemulsion system, such as free water, bound water, and interactions with surfactant head portions.

Effect of AOT on antioxidative ability of anthocyanins Ethanol was chosen to examine the effect of AOT on antioxidant activity of anthocyanins, because it can solubilize anthocyanins, AOT, and linolenic acid. Anthocyanins (10 μM) completely prevented the oxidation of linolenic acid in ethanol medium during 10 days storage, while AOT (100 mM) did not show any antioxidative effect (Fig. 5). This indicated that the presence of AOT did not critically affect process of oxidation when anthocyanins were not present in the system. When AOT was supplied with anthcyanins into the medium, the lipid oxidation increased. The antioxidative ability of anthocyanins decreased significantly at higher concentrations of AOT present in the medium. This result confirms that interaction between anthocyanins and AOT weakens the antioxidative activity of anthocyanins.

The extent of lipid oxidation in AOT/hexane was significantly higher than that of AOT/ethanol system, and the effect of AOT concentration on lipid oxidation was more clearly observed. By taking into account the high solubility of anthocyanins in ethanol, AOT did associate and form aggregate in polar solvent such as ethanol, but likely did not make well organized interface that can be form in AOT/hexane system. Therefore, less ordered interface created for polar solvent and the propensity of anthocyanins to partition to the surfactant interface would be lower in ethanol. In addition, the difference in transferring efficiency of a phenolic H-atom to peroxyl radicals in different medium cannot be ruled out. Foti and Ruberto (24) reported that electron-transfer reaction easily occurred in polar solvents due to the stabilizing effect on

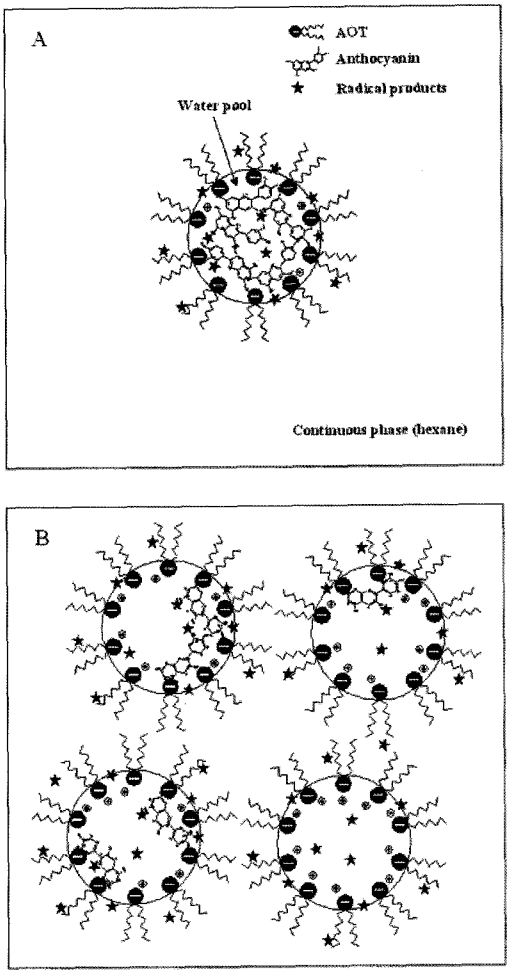

Fig. 4. Possible schematic distribution of anthocyanins, AOT and radical products at different micelle concentrations. A: low concentration of micelles, B: high concentration of micelles.

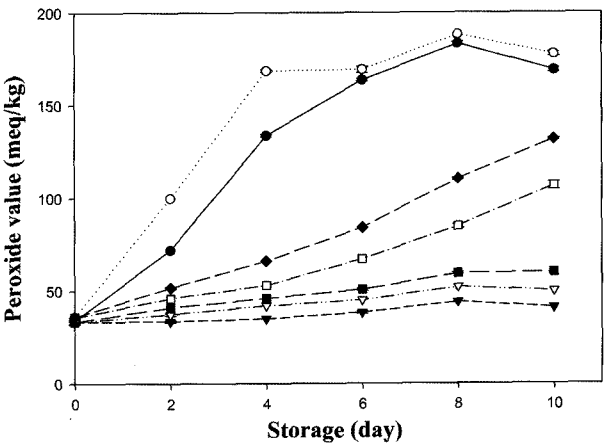

Fig. 5. Effect of medium and AOT on antioxidative ability of anthocyanins. ● : Ethanol (buffer), ○ : Ethanol (buffer/AOT 100 mM), ▼ : Ethanol (anthocyanins 10 μM), ▽ : Ethanol (anthocyanins 10 μM/AOT 50 mM), ■ : Ethanol (anthocyanins 10 μM / AOT 100 mM), □ : Hexane (anthocyanins 10 μM/AOT 50 mM), ◆ : Hexane (anthocyanins 10 μM/AOT 100 mM).

the ionic pair.

Synergistic antioxidative effect between anthocyanins and α-tocopherol The ability of antioxidants to retard lipid oxidation varies depending on the chemical properties of antioxidants and their physical location within a system. Surfactants strongly influence the physical location of antioxidants in emulsion system by affecting the partitioning of solutes including antioxidants (19). Decker (25) pointed out that the use of multicomponent antioxidant system could be an effective way to maximize oxidative stability in the polyphasic system. In this regard, the combined effect of anthocyanins and α-tocopherol was examined.

When α-tocopherol (100 ppm) was solely present in the microemulsion, its antioxidative activity was not enough to delay the oxidation of linolenic acid, and the concentration of hydroperoxide after 6 days storage was similar to that of the control (Fig. 6). The presence of anthocyanins (10 μM) resulted in better antioxidative activity than that of α-tocopherol; however, the concentration of hydroperoxide after 10 days storage did not have significant effect. On the other hand, simultaneous addition of α-tocoperol with anthocyanins efficiently retarded the oxidation during the test period. This result was consistent with the previous reports of Yi and Cho (26), and Han *et al.* (27), who reported that induction periods of a variety of oils containing ascorbic acid in the form of reverse micelles were significantly increased by the incorporation of δ-tocopherol.

The antioxidative synergism found in this study can be postulated in two ways. Firstly, antioxidants can be effectively regenerated by hydrogen transfer between anthocyanins and α-tocopherol at the interface. Secondly, α-tocopherol might loosen the rigidity of micellar interface through the interaction with hydrophobic tail of AOT, which facilitates the contact between anthocyanins and hydroperoxides at the interface and also contributes to the regeneration of antioxidants.

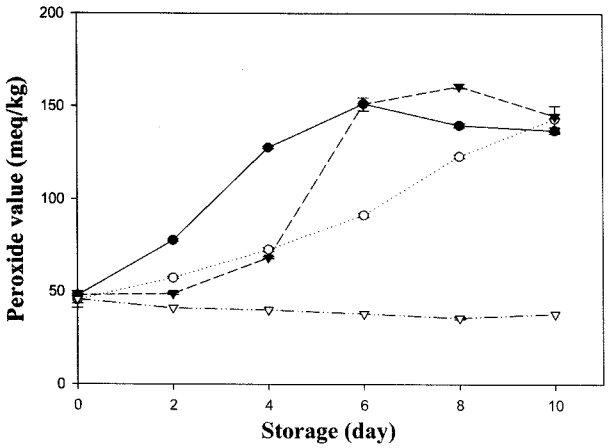

Fig. 6. Synergistic effect of α-tocopherol and anthocyanins on the lipid oxidation of water-in-oil micromeulsion during storage. ● : Control (buffer, pH 3.0), ○ : Anthocyanins 10 μM, ▼ : α-tocopherol 100 ppm, ▽ : Anthocyanins 10 μM + α-tocopherol 100 ppm.

In this paper we demonstrated the antioxidative behaviors of anthocyanins in water-in-oil microemulsion system. The antioxidative activity of anthocyanins was critically influenced by structural parameters such as size and concentration of micelles. The interaction between negatively charged AOT head and anthocyanins significantly decreased the antioxidative activity. The oxidation of water-in-oil microemulsion was effectively inhibited by the combination of anthocyanins and α-tocopherol.

Acknowledgments

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