Anthocyanins as Antioxidants on Human Low-Density Lipoprotein and Lecithin−Liposome Systems

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The antioxidant activity of several anthocyanins was tested *in vitro* on human low-density lipoproteins (LDL) and on a lecithin−liposome system. Samples were incubated at 37 °C, and the extent of oxidation was measured by determining the formation of conjugated diene and hexanal. The inhibition of oxidation increased with concentration of the antioxidant. In the LDL system, when the oxidation was catalyzed with 10 μM copper, malvidin was the best oxidation inhibitor, followed by delphinidin, cyanidin, and pelargonin. When the oxidation was catalyzed with 80 μM copper, the order of antioxidant activity changed and decreased in the following order at all concentrations tested: delphinidin, cyanidin, malvidin, and pelargonin. In the liposome system, catalyzed with either 3 or 10 μM copper, malvidin was the best inhibitor of both conjugated diene and hexanal formation. At 3 μM copper, delphinidin, cyanidin, and pelargonin showed prooxidant activity. At 10 μM copper, pelargonin followed malvidin in antioxidant potency, and cyanidin and delphinidin were prooxidants. Several antioxidant mechanisms may explain the effect of anthocyanins, including hydrogen donation, metal chelation, and protein binding.

Keywords: *Anthocyanin; antioxidant evaluation; LDL oxidation; liposome oxidation; natural antioxidants*

INTRODUCTION

The anthocyanidins are colored compounds occurring in the form of glycosides (anthocyanins) in many fruits and vegetables. A whole range of anthocyanidins are characterized by having the basic flavylium cation structure and different substituents on ring B (Figure 1). The electron deficiency of their structure makes anthocyanidins highly reactive, and their stability is pH and temperature dependent. The glycosides are much more stable than the aglycons. The sugar moiety can be located on carbons 3, 5, 7, 3′, and 5′, and the most common are glucose, rhamnose, galactose, xylose, and arabinose (Francis, 1989). These compounds belong to the general class of the flavonoids and can contribute, at least in part, to the antioxidant properties of many fruits and vegetables that owe their color to anthocyanins.

Many studies on the chemical structure and reactivity of anthocyanins report the use of these substances as food colorants (Markakis, 1982; Mazza and Bouillard, 1987; Francis, 1989). However, little is known about their biological activity. There are reports on the effect of anthocyanins on tumor cells (Kamei et al., 1995), antiinflammatory activity (Vlaskovska et al., 1990), anticonvulsant activity (Drenska et al., 1989), and antioxidant activity (Tamura and Yamagami, 1995; Wang et al., 1997). There is, however, a lack of information on the mechanisms by which these substances exert their antioxidant activity on biological systems. The antioxidant potency of anthocyanins can change depending on the substituents (Rice-Evans et al., 1996). As other polyphenols, anthocyanins can act as free radical scavengers, but other mechanisms, including metal chelation (Kühnau, 1976) or protein binding (Haslam, 1989), are important in biological systems.

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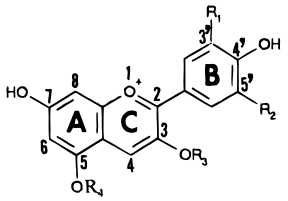

	R_1	R_2	R_3	R_4
DELPHINIDIN	OH	OH	H	H
CYANIDIN	OH	H	H	H
MALVIDIN	OCH_3	OCH_3	H	H
MALVIN	OCH_3	OCH_3	Glucose	Glucose
PELARGONIN	H	H	Glucose	Glucose

Figure 1. Structures of the different anthocyanins used in the study.

The literature on evaluation of antioxidant potency of anthocyanins and other naturally occurring phenolic compounds is confusing and difficult to interpret because different testing systems and different methods are used to determine oxidation (Frankel, 1993). In this study the antioxidant activity of several anthocyanins was evaluated on human low-density lipoprotein (LDL) and on a soybean lecithin−liposome system using two different levels of copper as oxidation initiator. The aim was to assess the contribution of anthocyanins to the possible health effect of foods in which they are present and to further elucidate the mechanisms of action of this widespread class of compounds.

MATERIALS AND METHODS

Chemicals. Delphinidin, cyanidin, and malvidin were all from Extrasynthèse (Genay, France). Pelargonin and malvin were from Aldrich Co. (Milwaukee, WI). Hexanal, bovine serum albumin standard, Lowry modified reagent, Folin−Ciocalteu's phenol reagent, and lecithin from soybean (40% phosphatidylcholine) were purchased from Sigma Chemical Co.

S0021-8561(97)00234-3 CCC: $14.00

(St. Louis, MO). Copper sulfate and acetate were obtained from Fisher Scientific (Fair Lawn, NJ). Absolute ethanol was purchased from Quantum Chemical Co. (Anaheim, CA).

LDL Isolation. Blood was drawn from five normolipidemic healthy volunteers into sterile blood collection tubes containing 15.55 mg of K_3EDTA (Vacutainer, Becton Dickinson and Co., Franklin Lakes, NJ). The recovered plasma was pooled, and LDL particles were isolated by density gradient ultracentrifugation (Orr et al., 1991).

LDL Oxidation *in Vitro*. Before the oxidation experiments, the LDL samples were dialyzed overnight with deoxygenated phosphate saline buffer (PBS, pH 7.4) at 4 °C. Protein content in LDL was measured according to the Lowry method, and concentration was adjusted with PBS to 1 mg of protein/mL. LDL samples (250 μL) were incubated at 37 °C with either 10 or 80 μM copper sulfate solution and various concentrations of the anthocyanins, in sealed 6 mL headspace vials. The anthocyanins were added in ethanolic solution, and ethanol was removed by nitrogen flushing prior to the addition of LDL. After incubation, the extent of oxidation was determined by measuring the formation of hexanal and conjugated dienes.

Phosphatidylcholine Liposome Oxidation. Lecithin−liposomes were prepared as previously described (Huang and Frankel, 1996) using a Sonicair cell disruptor (Ultrasonics, Inc., Plainview, NY). For the antioxidant assay, ethanolic solutions of anthocyanins were added into screw-capped 50 mL Erlenmeyer flasks to reach a final concentration of either 10, 20, or 40 μM. Ethanol was evaporated by flushing with nitrogen. Liposome samples were then weighed into the flasks and diluted with doubly distilled water to a final lecithin concentration of 0.8% (by weight). The samples were oxidized by adding cupric acetate (3 or 10 μM) and shaking at 37 °C in the dark. The initial pH of the unbuffered liposome was 4.6, and it decreased to 2.9−4.0 at the end of the oxidation. The decrease of pH is due to the formation of organic acids during oxidation, and therefore, at the end of the oxidation assay pH will be lower in the most oxidized samples. Liposome oxidation was monitored by determining hexanal and conjugated diene formation. Antioxidant activity of anthocyanins was calculated both as percent inhibition of conjugated diene and hexanal production and expressed as

$$\% \text{ inhibition} = [(C - S)/C] \times 100$$

where C is the amount of hexanal or conjugated dienes formed in the control sample and S is the amount of hexanal or conjugated dienes formed in the sample containing anthocyanin.

Analysis of Hexanal and Conjugated Diene. In the LDL system, hexanal was determined by static headspace gas chromatography (GC) as described by Frankel (1992). After GC, the vials were opened and oxidation was stopped by adding 1 μL of a 25 mM ethylenediaminetetraacetic acid (EDTA) solution. An aliquot (100 μL) of the sample was diluted to 5 mL with absolute methanol, and conjugated dienes were measured by UV spectrophotometry at 234 nm. In the liposome system, aliquot samples (1.0 g and 100 μL) from the oxidation flask were taken at the same time for hexanal and conjugated diene analyses. Hexanal was measured as described previously (Frankel et al., 1994) and conjugated dienes as described above for LDL samples.

Copper Chelation. The complexing abilities of delphinidin and malvidin with copper ions were compared spectrophotometrically, as described by Nardini et al. (1995) for caffeic acid. Spectra of malvidin and delphinidin solutions before and after addition of equimolar concentration of copper solution (510 μM) were recorded in a Beckman DU7400 (Beckman Instruments Inc., Fullerton, CA) spectrophotometer. Because the aqueous solution acidified with hydrochloric acid to pH 2 showed no complex formation, it was treated with sodium hydroxide to pH 10.

Statistics. Significance within sets of data was determined by one-way analysis of variance (Wagner, 1992) using Minitab Statistical Software (Addison-Wesley, Reading, MA). Significance level was in all cases $p < 0.001$.

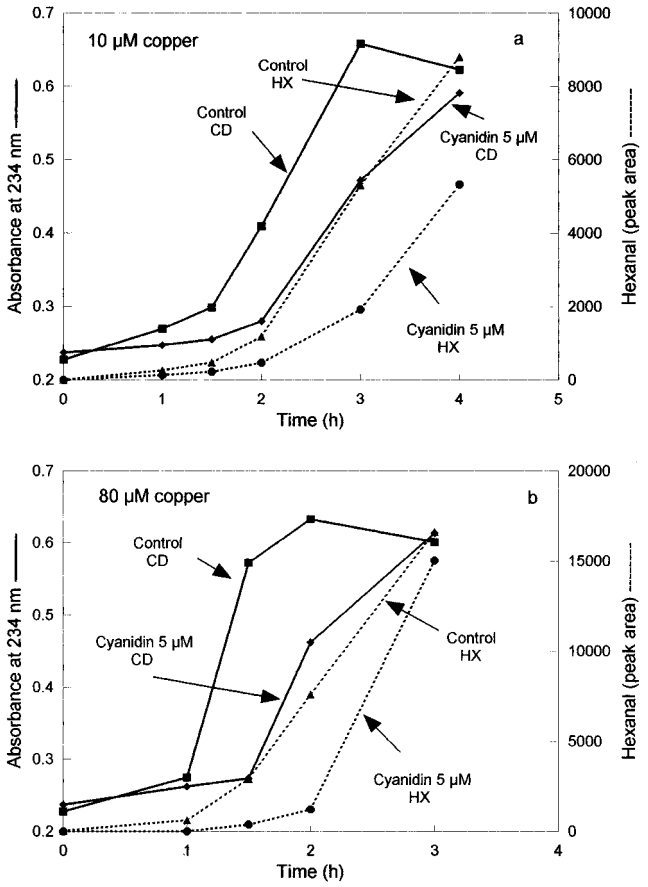

Figure 2. Oxidation curve for (a) LDL oxidized with 10 μM copper and (b) LDL oxidized with 80 μM copper. Solid lines represent conjugated dienes (CD); dotted lines represent hexanal (HX).

RESULTS

Typical oxidation curves for LDL and liposome show three phases of oxidation, including induction period, propagation phase, and termination phase (Figures 2 and 3). During the induction period, the rate of oxidation is very slow. The induction period is followed by the propagation phase during which oxidation increases at different rates. The rate of oxidation decreases during the termination phase, which follows the propagation phase. The percent inhibition was, thus, calculated at different time points during the propagation phase for the formation of both conjugated dienes and hexanal.

LDL Oxidation *in Vitro*. When LDL was oxidized with 10 μM copper, the rate curves showed induction periods in the control sample of 1.5 h for conjugated diene formation and 2 h for hexanal formation (Figure 2a). The induction periods were followed by propagation phases ending after 3 h for conjugated diene formation and continuing for up to 4 h of oxidation for hexanal. When LDL was oxidized with 80 μM copper, the induction period was 1 h for conjugated diene and hexanal formation, followed by a propagation phase ending after 2 h for conjugated diene and continuing for up to 3 h for hexanal (Figure 2b). Inhibition of both conjugated dienes and hexanal by anthocyanins was determined at the propagation phase, after 3 h for 10 μM copper and after 2 h for 80 μM copper (Table 1).

The antioxidant activity of the anthocyanins tested generally increased with concentration. Inhibition of conjugated diene and hexanal followed the same trend, but inhibition of hexanal was usually higher (Table 1).

Table 1. Inhibition of Conjugated Diene and Hexanal Formation by Anthocyanins Tested on Human LDL (Percent Inhibition, Mean ± SD)[a]

	conjugated dienes		hexanal	
sample	10 μM Cu, 3 h	80 μM Cu, 2 h	10 μM Cu, 3 h	80 μM Cu, 2 h
delphinidin, 2.5 μM	9.4 ± 6.8 ab	−4.7 ± 4.4 a,b	36.9 ± 13.5 b	59.2 ± 3.2 f
delphinidin, 5 μM	22.5 ± 1.3 bc	−0.2 ± 5.6 ab	65.3 ± 1.8 de	71.8 ± 1.2 g
delphinidin, 10 μM		50.3 ± 3.5 f		94.7 ± 0.6 hi
delphinidin, 20 μM		60.1 ± 4.0 f		99.3 ± 0.0 i
cyanidin, 2.5 μM	13.0 ± 5.5 ab	−2.8 ± 1.2 ab	56.6 ± 0.7 cd	33.3 ± 4.0 cd
cyanidin, 5 μM	31.7 ± 4.0 cd	28.4 ± 0.1 de	60.9 ± 0.7 cd	79.4 ± 0.1 g
cyanidin, 10 μM		54.4 ± 0.7 f		97.8 ± 1.3 i
cyanidin, 20 μM		52.0 ± 0.5 f		97.6 ± 0.2 i
malvidin, 2.5 μM	30.8 ± 5.4 cd	−1.5 ± 2.9 ab	59.0 ± 1.8 cd	19.4 ± 4.1 ab
malvidin, 5 μM	43.9 ± 2.4 d	4.4 ± 0.1 bc	78.8 ± 3.7 e	59.3 ± 0.0 f
malvidin, 10 μM		5.4 ± 2.9 bc		61.3 ± 1.0 f
malvidin, 20 μM		28.3 ± 3.2 de		87.3 ± 4.2 h
malvin, 2.5 μM		0.0 ± 0.2 ab		21.2 ± 1.1 ab
malvin, 5 μM		−15.1 ± 6.9 ab		16.1 ± 1.2 a
malvin, 10 μM		−4.8 ± 0.2 ab		41.6 ± 2.5 de
malvin, 20 μM		42.4 ± 4.7ef		88.2 ± 0.9 h
pelargonin, 2.5 μM	-1.6 ± 1.2 a	−1.2 ± 0.7 ab	15.8 ± 1.9 a	27.1 ± 0.1 bc
pelargonin, 5 μM	12.5 ± 2.6 ab	−1.0 ± 0.7 ab	44.4 ± 2.9 bc	34.0 ± 9.0 cd
pelargonin, 10 μM		−17.2 ± 3.7 a		47.0 ± 0.9 e
pelargonin, 20 μM		21.4 ± 1.7 cd		75.1 ± 8.0 g

[a] SD, standard deviation. Negative values indicate prooxidant activity. Values within each column followed by the same letter are not significantly different ($p > 0.001$).

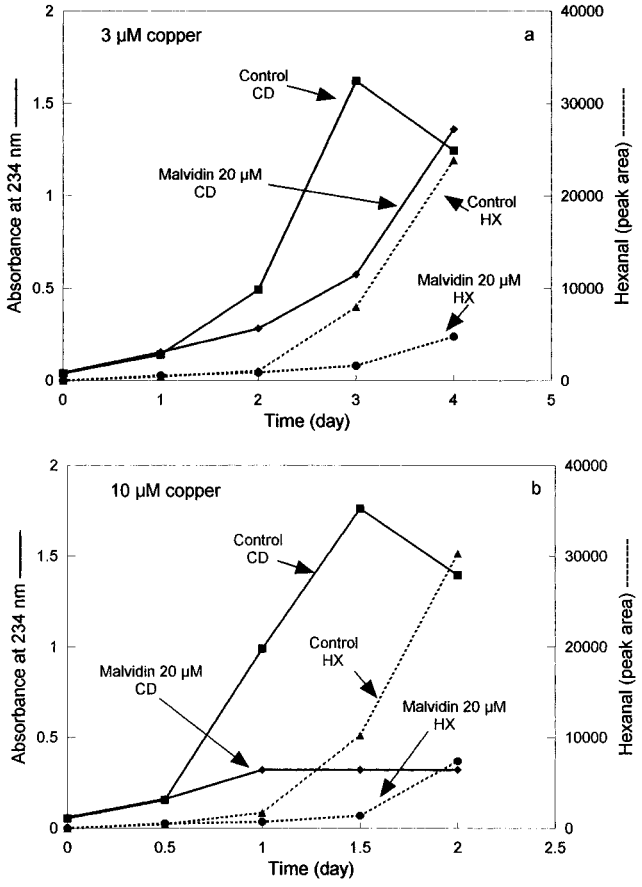

Figure 3. Oxidation curve for (a) liposomes oxidized with 3 μM copper and (b) liposomes oxidized with 10 μM copper. Solid lines represent conjugated dienes (CD), dotted lines represent hexanal (HX).

In the LDL system, all of the compounds were more efficient in inhibiting hexanal (15.8−99.3%) than conjugated diene (−17.2% to 60.1%) formation. At 10 μM copper used as catalyst, on the basis of conjugated diene formation, the activities of cyanidin and malvidin at 5 μM (31.7% and 43.9%) and cyanidin and delphinidin at 2.5 μM (13.0% and 9.4%) and 5 μM (31.7% and 22.5%) were not significantly different ($p > 0.001$). Malvidin showed the highest antioxidant activity in inhibiting hexanal formation at all of the concentrations tested (59.0% at 2.5 μM and 78.8% at 5 μM), followed by cyanidin (56.6% and 60.9%), delphinidin (36.9% and 65.3%), and pelargonin (15.8% and 44.4%). On the basis of hexanal formation, the antioxidant activities were not significantly different ($p > 0.001$) for cyanidin and malvidin at 2.5 μM (56.6% and 59.0%) and for delphinidin and malvidin at 5 μM (65.3% and 78.8%).

At 80 μM copper, the order of antioxidant potency toward LDL oxidation changed on the basis of both conjugated diene and hexanal inhibition. Delphinidin and cyanidin were the most efficient inhibitors of both conjugated diene and hexanal formation, at all of the concentrations tested. Conjugated diene formation was not inhibited by delphinidin, malvidin, malvin, or pelargonin at 2.5 or 5 μM. Cyanidin was not active at 2.5 μM, but at 5 μM it inhibited conjugated diene formation by 28.4%. At 10 μM, cyanidin and delphinidin were the most active with 54.4% and 50.3% inhibition, respectively, followed by malvidin (5.4%). Malvin and pelargonin at 10 μM were not different from the control. At 20 μM, delphinidin (60.1%), cyanidin (52.0%), and malvin (42.4%) were the most active, followed by malvidin (28.3%) and pelargonin (21.4%). In inhibiting hexanal formation, delphinidin and cyanidin had the same activity followed by malvidin, malvin, and pelargonin. According to the antioxidant concentration, the order changed; at 5 μM, pelargonin was more active than malvin (34.0% and 16.1%) and at 2.5 μM, it was more active than malvidin and malvin (27.1%, 19.4%, and 21.2%).

Lecithin−Liposome Oxidation. When the liposome system was oxidized with 3 μM copper, conjugated diene formation in the control sample showed an induction period of 1 day and hexanal formation showed an induction period of 2 days (Figure 3a). The induction periods were followed by propagation phases ending after 3 days for conjugated diene formation and continuing for up to 4 days for hexanal. When the liposome was oxidized with 10 μM copper, the corresponding induction periods were 0.5 day for conjugated diene and

Table 2. Inhibition of Conjugated Diene and Hexanal Formation by Anthocyanins Tested in a Lecithin−Liposome System (Percent Inhibition, Mean ± SD)[a]

	conjugated dienes		hexanal	
sample	3 μM Cu, 2 days	10 μM Cu, 1 day	3 μM Cu, 3 days	10 μM Cu, 1.5 days
delphinidin 10 μM	−29.3 ± 1.8 f	−44.4 ± 1.7 c	−12.5 ± 0.7 g	−7.3 ± 1.2 d
delphinidin 20 μM	−40.8 ± 3.9 e	−69.1 ± 8.8 b	−26.6 ± 0.1 f	−24.0 ± 3.5 c
delphinidin 40 μM	−14.9 ± 0.6 g	−79.7 ± 0.2 a	−21.2 ± 2.1 fg	−34.9 ± 0.8 b
cyanidin 10 μM	−135.0 ± 10.1 b	−11.1 ± 1.3 d	−142.3 ± 2.1 c	−7.0 ± 0.2 d
cyanidin 20 μM	−118.2 ± 4.2 c	−4.4 ± 1.6 d	−93.8 ± 0.9 d	−4.1 ± 2.3 d
cyanidin 40 μM	−50.1 ± 2.9 e	−70.6 ± 0.4 b	−19.6 ± 1.1 fg	−77.2 ± 1.4 a
malvidin 10 μM	9.4 ± 3.9 h	55.5 ± 0.6 g	23.9 ± 0.4 h	49.2 ± 1.6 g
malvidin 20 μM	42.7 ± 0.4 i	67.6 ± 0.6 h	79.8 ± 0.4 i	86.5 ± 0.3 h
malvidin 40 μM	53.3 ± 0.4 j	71.6 ± 0.3 h	95.4 ± 0.0 j	90.3 ± 0.0 h
pelargonin 10 μM	−161.0 ± 3.1 a	25.6 ± 1.8 e	−235.7 ± 2.7 a	10.8 ± 3.2 e
pelargonin 20 μM	−143.0 ± 0.0 b	30.2 ± 3.1 ef	−216.1 ± 7.2 b	13.9 ± 0.9 e
pelargonin 40 μM	−84.2 ± 6.5 d	37.4 ± 0.6 f	−66.1 ± 2.1 e	27.9 ± 2.2 f

[a] SD, standard deviation. Negative values indicate prooxidant activity. Values within each column followed by the same letter are not significantly different ($p > 0.001$).

1 day for hexanal, followed by propagation phases ending after 1.5 days for conjugated diene formation and continuing after 2 days for hexanal (Figure 3b). Inhibition of conjugated dienes by anthocyanins was determined at the propagation phase, after 2 days for 3 μM copper and after 1 day for 10 μM copper (Table 2). Inhibition of hexanal was determined after 3 days for 3 μM copper and after 1.5 days for 10 μM copper.

At 3 μM copper, the potency in inhibiting conjugated diene and hexanal formation, at all the antioxidant concentrations tested, decreased in the following order: malvidin, delphinidin, cyanidin, and pelargonin. The only compound that showed antioxidant activity was malvidin. The other compounds were either inactive or prooxidants.

At 10 μM copper, malvidin was a particularly good inhibitor of conjugated dienes (55.5−71.6%) and hexanal (49.2−90.3%) (Table 2). Pelargonin was more active in inhibiting conjugated diene (25.6−37.4%) than hexanal (10.8−27.9%) formation. Cyanidin at 20 μM was inactive in inhibiting the formation of conjugated dienes. Cyanidin and delphinidin were prooxidant at the other concentrations tested, on the basis of both conjugated diene and hexanal formation. The activity of the anthocyanins in inhibiting hexanal formation decreased in the following order: malvidin, pelargonin, cyanidin, and delphinidin, at all concentrations tested. Cyanidin at 10 and 20 μM and delphinidin at 10 μM were both inactive in inhibiting the formation of hexanal.

Copper Chelation. Malvidin and delphinidin in acidic solution showed no complex formation with Cu^{2+} spectrophotometrically at pH 2. At pH 10, the addition of copper to a delphinidin solution produced an immediate shift to a higher wavelength (maximum from 532 to 586 nm) and a change in the shape of the spectrum (Figure 4). However, a malvidin solution showed only a decrease in the absorbance due to its apparent susceptibility to oxidation at high pH.

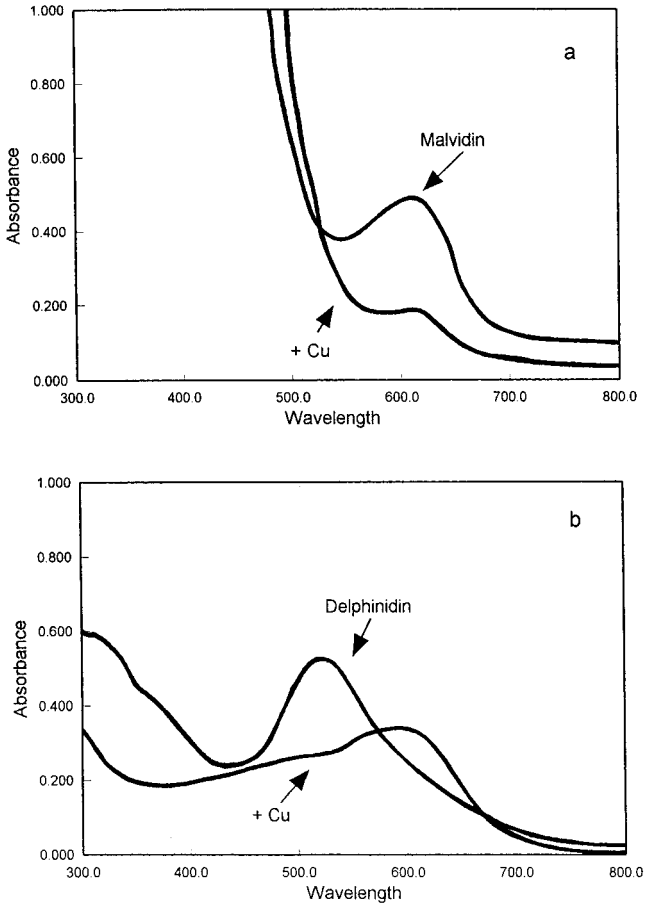

Figure 4. Spectra of malvidin (a) and delphinidin (b), before and after addition of copper.

DISCUSSION

The antioxidant effect of anthocyanins can be explained by several mechanisms. Flavonoids are well-known for their ability to scavenge peroxyl and alkoxyl radicals (Chimi et al., 1991). Additional antioxidant action of anthocyanins may be due to their metal chelation properties (Kühnau, 1976). The protein binding of flavonoids was also suggested as a mechanism for their antioxidant activity (Teissedre et al., 1996). Another mechanism to explain differences in activity of various phenolic compounds is based on the interfacial phenomena (Frankel et al., 1994) and phase distribution (Huang et al., 1996) observed in emulsion systems.

The *o*-diphenol substitution in ring B of anthocyanins (Figure 1) and the conjugated double-bond system are related to their radical scavenging activity, due to hydrogen donation and subsequent radical stabilization (Heimann and Reiff, 1953). This structure explains the higher activity of cyanidin compared with pelargonin observed in inhibiting hexanal and conjugated diene formation. The presence of additional hydroxyl groups in ring B in delphinidin did not enhance the antioxidant activity in the LDL system, as shown by the similar inhibitions observed with delphinidin and cyanidin. The

methoxy groups ortho in relation to position 4′ in malvidin lowered the antioxidant activity compared to cyanidin, in LDL oxidized with 80 μM copper. However, in LDL oxidized with 10 μM copper, malvidin had the highest activity in inhibiting the formation of both hexanal (59.0−78.8%) and conjugated dienes (30.8−43.9%).

In the LDL system, the antioxidant properties of anthocyanins may also be attributed to metal chelation. Cyanidin and delphinidin, with two ortho hydroxyl groups, form complexes with copper, while malvidin does not (Francis, 1989). In an experiment comparing delphinidin with malvidin, we found that the chelation ability of these compounds was not only dependent on the structure (hydroxyl groups in ortho position) but also on the pH. Complex formation is an equilibrium reaction favored by ionization of hydroxyl groups. At low pH, the hydroxyl groups on ring B of the anthocyanin cannot ionize and, therefore, do not form complexes. At high pH (pH 10), the hydroxyl groups on ring B can ionize and compounds with an *o*-diphenol structure, such as delphinidin, formed complexes with copper (Figure 4). At pH 7.4 of the LDL system, hydroxyl groups in the anthocyanins would be partly ionized and compounds such as cyanidin and delphinidin may act as metal chelators as well as radical scavengers. On the other hand, malvidin may be a strong hydrogen donor due to its higher negative charge caused by the methoxy substituents compared to the hydroxyl group of delphinidin. At lower copper concentration, when the hydrogen donation ability is not overwhelmed by excessive copper, the activity of malvidin in inhibiting oxidation was comparable to that of delphinidin and cyanidin, which can act as both hydrogen donors and metal chelators. Furthermore, the methoxy groups in anthocyanins increase their stability and that of the resulting radical (Francis, 1989).

In contrast to the LDL system, in the liposome system, malvidin was the best antioxidant at all concentrations of copper and antioxidant used. Pelargonin was the only compound used that changed its antioxidant activity with the concentration of copper in the liposome system. At the low concentrations of copper used (3 and 10 μM), the chelation ability of anthocyanins may be less relevant than that observed in the LDL system. Moreover, the lower pH of the liposome system (2.9−4.6) reduces the ability of hydroxyl groups to ionize and may impair copper chelation. On the other hand, because of its methoxy groups, the lower polarity of malvidin may impart a better affinity for the interface of the liposome particles and better prevent oxidation of the lecithin fatty acids. In contrast, cyanidin and delphinidin are more polar than malvidin and become inactive in the liposome system because they are more solube in the water phase and cannot protect the phospholipid against oxidation. Alternatively, at high concentrations of Cu^{2+}, these antioxidants may produce radicals that can promote lipid oxidation.

Although glycosylation in the 3-position stabilizes anthocyanins (Francis, 1989), it diminishes their antioxidant activity as measured by aqueous radical trapping capacity (Rice-Evans et al., 1996). Our results showed that malvidin is either more active than or as active as malvin in LDL. Further research is needed with more glycosides to clarify the antioxidant mechanisms of anthocyanins.

In marked contrast to LDL, in the liposome system, pelargonin showed antioxidant activity at 10 μM copper, while cyanidin and delphinidin were inactive or prooxidant. However, at 3 μM copper, pelargonin also showed prooxidant activity. Pelargonin would be expected to have low antioxidant activity because it has only one hydroxyl group in the B-ring and is more stable than anthocyanins with higher degree of hydroxylation. As a glucoside, pelargonin is also more stable than the correspondent aglycon. More work is needed to clarify the activity change of pelargonin with different copper concentrations.

Protein binding of anthocyanidins may provide another mechanism to explain their antioxidant activity. The apo B moiety of LDL is known to have specific sites for copper binding (Gieseg and Esterbauer, 1994), a step required for initiating copper-mediated oxidation in these particles. The polyphenols can act as multidentate ligands to bind with protein surfaces (Haslam, 1989). Wada et al. (1969) found that hydrogen bonds from phenol to protein were stronger for the more acidic phenols. However, complex formation is favored in neutral and basic pH conditions (Smyk and Drabent, 1989). The configuration and the number of hydroxy groups on the B-ring may affect the ability of polyphenolic compounds to complex with protein. Prodelphinidins bind proteins more tightly than procyanidins (Asano et al., 1984). Electron spin resonance (ESR) techniques showed that phenolic compounds found in wine produce super-hyperfine signals due to Cu^{2+} binding with amines, as well as a free radical signal attributed to anthocyanins (Troup et al., 1996). The formation of anthocyanin−protein cross-links in red wines was also reported (Waters et al., 1994). Therefore, anthocyanin−protein binding could provide a mechanism for the antioxidant activity of anthocyanins in two ways: (1) by blocking copper catalyst to the binding sites of LDL and (2) by favoring the access of the antioxidant to the lipids and improving their protection against oxidation.

In this paper we were able to show that the antioxidant activity of anthocyanins is greatly affected by the system used as substrate and the conditions used to catalyze oxidation (copper level, pH, and interfacial factors). Therefore, the use of artificial radical trapping assays without an oxidizable substrate or with artificial radical generators (Rice-Evans and Miller, 1994; Wang et al., 1997) may not provide valid evaluations of the activity and mechanisms of action of these natural antioxidants.

ABBREVIATIONS USED

LDL, low-density lipoprotein; GC, gas chromatography; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid.

ACKNOWLEDGMENT

M.T.S.G. was supported by the Spanish Ministry of Education and Culture (Subprograma de Perfeccionamiento para Doctores y Tecnólogos). M.H. was supported by Yrjö Jahnsson, Ella and Georg Erhnroot, Food Research, and Juho Vainio Foundations.

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Received for review March 24, 1997. Revised manuscript received June 24, 1997. Accepted July 2, 1997.[⊗]

JF970234A

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1997.