pH-Dependent Forms of Red Wine Anthocyanins as Antioxidants†

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Anthocyanins are one of the main classes of flavonoids in red wines, and they appear to contribute significantly to the powerful antioxidant properties of the flavonoids. In grapes and wines the anthocyanins are in the flavylium form. However, during digestion they may reach higher pH values, forming the carbinol pseudo-base, quinoidal-base, or the chalcone, and these compounds appear to be absorbed from the gut into the blood system. The antioxidant activity of these compounds, in several metal-catalyzed lipid oxidation model systems, was evaluated in comparison with other antioxidants. The pseudo-base and quinoidal-base malvidin 3-glucoside significantly inhibited the peroxidation of linoleate by myoglobin. Both compounds were found to work better than catechin, a well-known antioxidant. In a membrane lipid peroxidation system, the effectiveness of the antioxidant was dependent on the catalyst: In the presence of H_2O_2-activated myoglobin, the inhibition efficiency of the antioxidant was malvidin 3-glucoside > catechin > malvidin > resveratrol. However, in the presence of an iron redox cycle catalyzer, the order of effectiveness was resveratrol > malvidin 3-glucoside = malvidin > catechin. The pH-transformed forms of the anthocyanins remained effective antioxidants in these systems, and their I_{50} values were between 0.5 and 6.2 μM.

Keywords: *Antioxidants; anthocyanins; flavonoids; resveratrol; red wine; grapes; lipid peroxidation*

INTRODUCTION

Anthocyanins possess the characteristic $C_6C_3C_6$ carbon skeleton of flavonoids and are generated from the same biosynthetic pathway in plants. They are glucosides of anthocyanidins, which are polyhydroxylated and polymethoxylated derivatives of flavylium salts (Figure 1).

The anthocyanins differ from other natural flavonoids by the range of colors that can be derived from them and by their ability to form resonance structures through variation of the pH (Mazza and Brouillard, 1987). The reported (Frankel et al., 1993; Kinsella et al., 1993; Kanner et al., 1994) beneficial effects of red wine were suggested by us to derive from phenolic compounds, mostly flavonoids, which demonstrated powerful antioxidant properties against low-density lipoprotein oxidation.

The general antioxidant effect of red wines correlates well with their total phenolic content (Kanner et al., 1994; Mayer et al., 1997). Anthocyanins are one of the main classes of flavonoids in red wines, and they appear to significantly contribute powerful antioxidant properties to them. The 3-glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin are present in red wines, but malvidin 3-glucoside, malvidin 3-glucoside acetate, and malvidin 3-glucoside coumarate are the most abundant pigments (Mazza, 1995).

The antioxidant activity of anthocyanins has been studied by several researchers, in systems that generate $O_2^{\bullet-}$, $HO^{\bullet}$, and lipid radicals (Igarashi et al., 1989; Meunier et al., 1989; Tamura and Yamagami, 1994; Tsuda et al., 1994a,b, 1996; Yamasaki et al., 1996; Satué-Gracia et al., 1997; Wang et al., 1997).

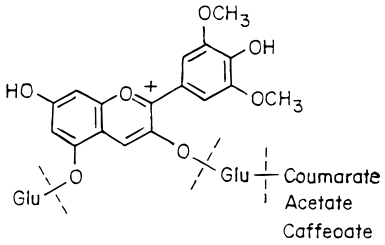

Figure 1. Structure of major anthocyanins in red grapes and wine.

Anthocyanins exist in an aqueous phase in a mixture of four molecular species, the concentrations of which depend on the pH (Mazza and Brouillard, 1987). At pH 1−3 the flavylium cation is red, at pH 4−5 the carbinol pseudo-base (pb) generated is colorless, and at pH 7−8 the quinoidal-base (qb) formed is blue-purple, which could turn to a chalcone.

In grapes and wines the anthocyanins are in the flavylium form. However, during digestion, they may reach higher pH values, forming the carbinol pseudo-base, quinoidal-base, or chalcone, and these compounds appear to be the potential forms to be absorbed from the gut into the blood system (Lapidot et al., 1988).

The aim of our study was to evaluate the antioxidant activity of these compounds in several model systems by which metals catalyzed the generation of reactive oxygen and lipid species.

MATERIALS AND METHODS

Myoglobin (type 1) from equine skeletal muscle (as metmyoglobin), cytochrome *c* (horse heart), ascorbic acid, linoleic acid, resveratrol, thiobarbituric acid, Tween 20, and catechin were obtained from Sigma Chemical Co. (St. Louis, MO). Ferric chloride was obtained from Riedel-de-Haen (Hannover, Germany). Hydrogen peroxide (30%) and trichloroacetic acid were

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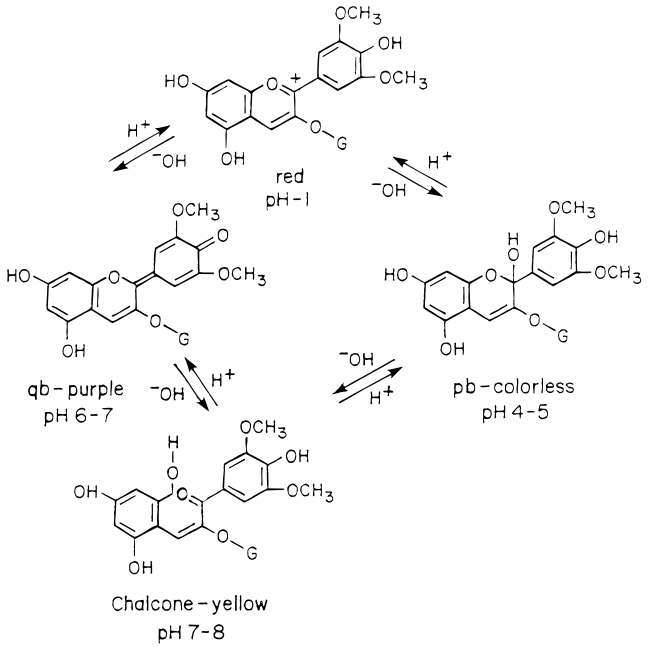

Figure 2. Anthocyanin structure transformation by pH.

obtained from Merck (Darmestadt, Germany). Malvidin and malvidin 3-glucoside were obtained from Roth (Karlsruhe, Germany), and malvidin 3-glucoside was also isolated and purified from red wine by an HPLC method described by us previously (Lapidot et al., 1998).

Pseudo-base (pb) was generated at pH 4 and tested as an antioxidant at pH 7. Once the pb is generated, the compound is stabled at pH 7. The quinoidal-base (qb) generated at pH 7 and tasted the same as an antioxidant in the same pH 7. Except for catechin, all other antioxidants were solubilized in ethanol at a concentration that will give in the assay not more than 1%.

The control contained the same concentration of ethanol.

Linoleate oxidizing activity was assayed spectrophotometrically (Kanner et al., 1994). The technique consists of following the increase in conjugated dienes and their absorbance at 240 nm. The test sample contained 1.5 mL of buffered linoleate at pH 7.0, 0.1−0.4 mL of active fraction, and distilled water in a mixture as follows: linoleate, 2 mM; linoleate hydroperoxide, 2−4 μM; Tween 20, 0.05%; phosphate buffer, pH 7.0, 0.05 M; diethylenetriaminepentaacetic acid (DETA), 0.5 mM. The blank sample contained all of the reagents except the catalyzers.

Isolation of the microsomal fraction from muscle tissues was done according to a procedure described previously (Kanner and Harel, 1985).

Microsomes for lipid peroxidation assays were incubated in air in a shaking water bath at 37 °C. The reaction mixture contained microsomes at 1 mg of proteins/mL and 4 mL of 50 mM acetate buffer, pH 7.0. The thiobarbituric acid reactive substances (TBARS) were determined according to a procedure of Bidlack et al. (1973).

Protein determination assay were conducted according to the modified Lowry procedure (Markwell et al., 1978), using bovine serum albumin as standard.

Results are the means of triplicates, and in the figures each error bar (I) denotes the standard deviation.

RESULTS

The structures of the three main forms of anthocyanins are presented in Figure 1. The main anthocyanin is malvidin 3-glucoside, followed by malvidin 3-glucoside acetate, and malvidin 3-coumarate. Anthocyanin structure transformation by pH is demonstrated in Figure 2 (Belitz and Grosh, 1987). The incubation of malvidin

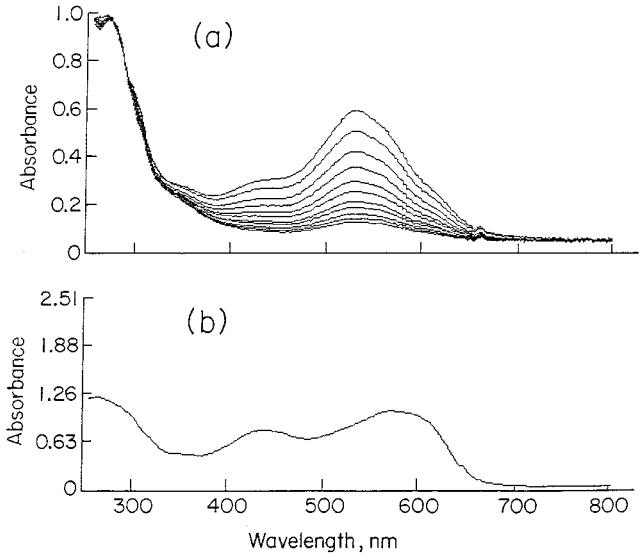

Figure 3. (a) Generation of malvidin 3-glucoside pb at pH 4; each line denotes 30 s. (b) Generation of malvidin 3-glucoside qb at pH 7.0. Structure transformation from pH 1.0 to 7.0 after 1 min.

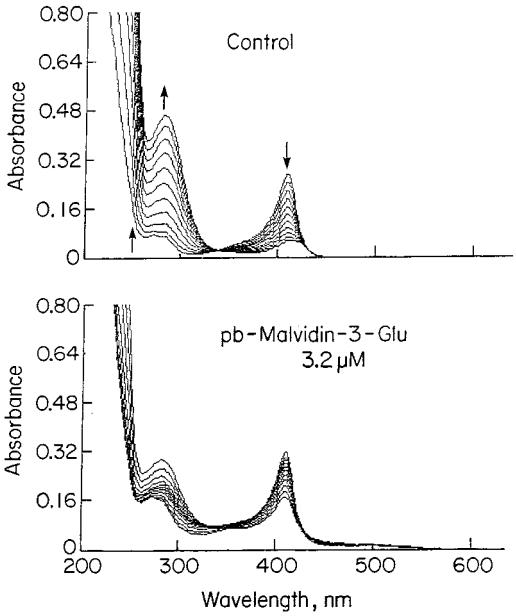

Figure 4. Spectral changes during linoleic acid (2 mM) peroxidation induced by myoglobin (2.5 μM), (a) without or (b) with 3.2 μM pb malvidin 3-glucoside, at pH 7.0 and 23 °C.

3-glucoside at pH 4 brings the pigment by a rapid transformation to a colorless pigment, the pb. The pb is relatively stable at pH 7.

If the flavylium red pigment is brought to pH 7, instantly the compound formed is a qb, a purple pigment (Figure 3). Both compounds, during a long incubation period of hours at pH 7−8, could form a chalcone, which is yellow.

Figure 4 demonstrates the catalytic oxidation effect of myoglobin in the presence of linoleate dispersed in a microemulsion at pH 7.0 and 25 °C. During this reaction myoglobin catalyzed the oxidation of linoleate via generation of free radicals, which break down the heme ring, producing bleaching of the heme and a rapid decrease in the Raman spectra at 408 nm. The radicals generated in this reaction oxidized linoleate, forming conjugated dienes, which increased the spectra at 234−

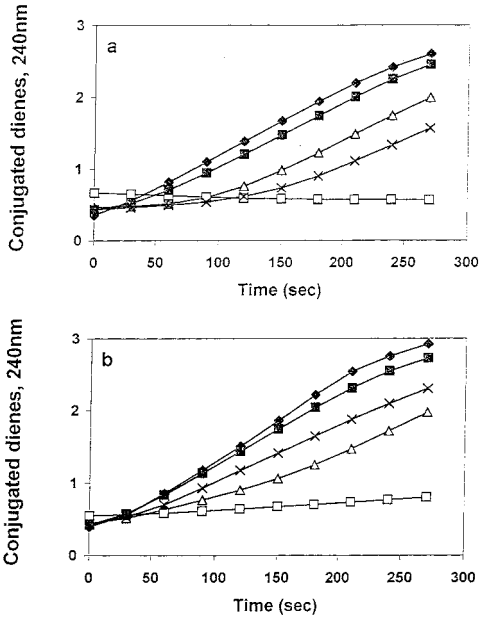

Figure 5. Inhibition of lipid peroxidation (conjugated dienes) by wine anthocyanins, induced by myoglobin (2.5 μM) at pH 7.0 and 23 °C. (a) Pseudo-base generated at pH 4.0: control, ◆; 1 μM catechin, ■; 5 μM catechin, ×; 1.6 μM pb malvidin 3-glucoside, △; 6.5 μM pb malvidin 3-glucoside, □. (b) quinonidal base generated at pH 7.0: control, ◆; 1 μM catechin, ■; 4 μM catechin, ×; 1 μM qb malvidin 3-glucoside, △; 4 μM qb malvidin 3-glucoside, □.

Table 1. Inhibition of Membrane Lipid Peroxidation by Wine Polyphenols, Catalyzed by Different Catalyzers

	I_{50}, μM		
polyphenol	Mb-H_2O_2[a]	AA-Fe[a]	AA-Fe-Mb[a]
catechin	0.7 ± 0.04 (1.00)[b]	30.0 ± 1.45 (1.00)	24.0 ± 1.15 (1.00)
pb-M-3glu	0.5 ± 0.02 (1.40)	6.2 ± 0.30 (4.84)	2.0 ± 0.09 (12.00)
pb malvidin	1.7 ± 0.08 (0.41)	6.2 ± 0.35 (4.84)	6.2 ± 0.32 (3.87)
resveratrol	4.7 ± 0.20 (0.15)	3.5 ± 0.15 (8.57)	2.0 ± 0.12 (12.00)

[a] Catalyst concentration: Mb-H_2O_2 (Mb 30 μM, H_2O_2 10 μM); AA-Fe (ascorbic acid 200 μM, $FeCl_3$ 5 μM); AA-Fe-Mb (200 μM, 5 μM, 30 μM). [b] Index of antioxidation.

240 nm. This reaction also generates carbonyls, which had absorption peaks at 280 nm (Kanner et al., 1994). Addition of pb malvidin 3-glucoside to this microemulsion at a concentration of 3.2 μM significantly inhibits peroxidation. Figure 5 presents data on the effectiveness of the pb- and qb malvidin 3-glucoside in inhibiting peroxidation of linoleate by myoglobin. Both compounds were found to work better than catechin, a well-known antioxidant.

We compared malvidin pb with catechin and resveratrol in a model system of membrane lipid peroxidation. The results shown in Table 1 demonstrate that the effectiveness of the antioxidant toward membrane lipid peroxidation depends on the catalyst. In the presence of H_2O_2-activated myoglobin, the best antioxidant found was malvidin 3-glucoside > catechin > malvidin > resveratrol. However, in the presence of an iron redox cycle catalyzer, the order of effectiveness was resveratrol > malvidin 3-glucoside = malvidin > catechin. We also added myoglobin to an iron redox cycle catalyst to simulate a possible catalysis by both compounds, such as could happen in biological systems. In this system the antioxidant effectiveness order was resveratrol = malvidin 3-glucoside > malvidin > catechin.

Figure 6 demonstrates the effect of antioxidant concentration on the inhibition of membrane lipid peroxidation catalyzed by H_2O_2-activated myoglobin.

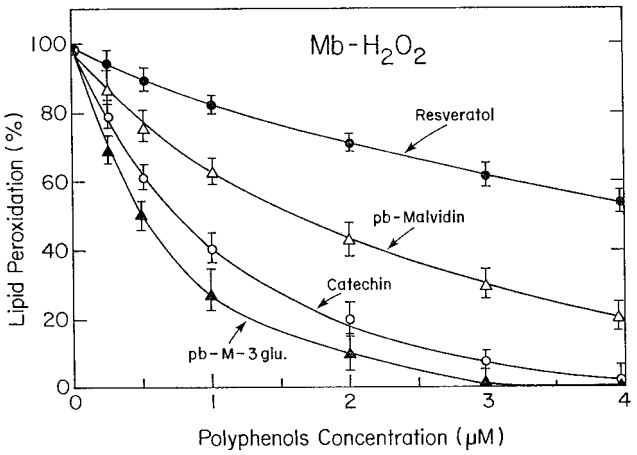

Figure 6. Membrane lipid peroxidation catalyzed by Mb-H_2O_2 (30−10 μM) as affected by polyphenol concentration.

DISCUSSION

Anthocyanins, which are natural plant pigments of the flavonoid family, are found in many components of the human diet. Many foods, especially red grapes and wines, contain large amounts of anthocyanins. The antioxidative effects of those compounds have been described by several researchers (Igarashi et al., 1989; Meunier et al., 1989; Tamura and Yamagami, 1994; Tsuda et al., 1994a,b, 1996; Yamasaki et al., 1996; Satué-Gracia et al., 1997; Wang et al., 1997). However, information is lacking on the mechanism by which these compounds exert their antioxidative activity in biological systems.

The pH-transformed forms of malvidin and malvidin 3-glucoside, the pseudo-base and quinoidal base, remained active as very effective antioxidants in both model systems of lipid peroxidation determined by us. These results are important as most probably during ingestion of those compounds they may undergo transformation at the high pH of the intestine and blood system.

The pb and qb of malvidin 3-glucoside were found to act as antioxidants better than the aglycons, in the linoleate model system activated by myoglobin. It may be possible that this effect was achieved due to a better interaction of the glucoside with proteins. It is well-known that polyphenols have the capability to bind proteins. However, it may be that the anthocyanidin 3-glucoside has a better capability to interact with the heme, which is located in a crevice of the myoglobin molecule. Data from several authors on the antioxidant activity of anthocyanins and their aglycons are conflicting. Tsuda et al. (1994a) found that the aglycons act better than glucosides, but Wang et al. (1997) reported that some glucosides act better than the aglycons. The antioxidant activity of anthocyanins and other polyphenolics is strongly affected by the system and catalyzers used. In the system catalyzed by H_2O_2-activated myoglobin, malvidin 3-glucoside was the best antioxidant, whereas in the system catalyzed by AA-Fe, an iron redox cycle, resveratrol was the most efficient antioxidant. Catechin, which was found to be a relatively effective antioxidant in the system oxidized by H_2O_2−myoglobin (I_{50} = 0.7 μM), lost its effect in the system oxidized by AA-Fe (I_{50} = 30 μM). This dramatic change in its

antioxidant activity could be explained only by its reactivity being lost during autoxidation in the presence of iron ions. Our results and those published most recently by Satué-Gracia et al. (1997) demonstrated that the use of radical assays which are not relevant to oxidizable substrates or catalyzers (Rice-Evans et al., 1994; Wang et al., 1997) may not provide data which are relevant to the mechanism of action of these antioxidants in biological systems. We should take into consideration that the catalysts, the substrates to be oxidized, and the dispersion medium (cytosol, liposomes, membranes, LDL) all may affect the effectiveness of the antioxidants. In addition, the present results are of only very limited relevance to human nutrition if the bioavailability of each of these antioxidants is different, very low, or does not exist at all. More important, a good antioxidant is not only a compound that donates electrons but one of high bioavailability which could reach the target of oxidation in vivo and at a critical concentration to provide protection.

ABBREVIATIONS USED

pb-M-3glu, pseudo-base malvidin 3-glucoside; qb-M-3glu, quinoidal-base malvidin 3-glucoside; DETA, diethylenetriaminepentaacetic acid; TBARS, thiobarbituric acid reactive substances.

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