Effect of Ethanol on Red Wine Tannin−Protein (BSA) Interactions

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The interactions between salivary proteins and tannins, responsible for the astringency of wine, may reduce phenolic bioavailability. The effect of ethanol on protein−tannins interaction and consequently on *in-vitro* total radical-trapping antioxidant potential (TRAP) of wine was investigated. Scalar amounts of ethanol proportionally reduce the extent of tannin precipitation with respect to alcohol-free red wine. This effect is parallel to and has a high correlation ($r = 0.978$) with the TRAP increase in wine fractions, partially restoring the antioxidant activity lost as a consequence of the interactions with proteins. We postulate that ethanol could reduce the chemical interactions between proteins and red wine tannins. The present work suggests that alcohol seems to contribute indirectly to the antioxidant capacity of wine by increasing the bioavailability of its phenolic compounds.

Keywords: *Tannins; wine; antioxidant; alcohol; proteins*

INTRODUCTION

Several epidemiological studies have reported an inverse correlation between moderate alcohol consumption and coronary heart disease (CHD) (Lazarus *et al.*, 1991; Rimm *et al.*, 1991; Friedman and Kimball, 1986). These studies have characterized light alcohol drinking as protective. Although the evidence is conflicting, the protective effect seems to be associated mainly with wine drinking (Gronbaek *et al.*, 1995). It is unclear which component of wine is responsible for its protective effect. Recently attention has been placed on the nonalcoholic fraction of wine, mainly represented by phenolic compounds. The latest evidence shows that the phenolic fraction of red wine increases antioxidant plasma capacity *in vivo* in man and is responsible for an improved resistance to oxidation in human LDL (Fuhrman *et al.*, 1995; Serafini *et al.*, submitted for publication).

When red wine is drunk its phenolic compounds produce a drying and puckering sensation in the mouth (Lea and Arnold, 1978) called astringency, which occurs over the whole surface of the tongue and the buccal mucosa. This has been shown to be caused by the interaction of tannins (water-soluble phenolic compounds having molecular weights between 500 and 3000 able to precipitate proteins) with salivary proteins rich in proline and hydroxyproline (PRPs). These interactions depend partly on the characteristics of the protein and pH and partly on the characteristics of the phenolic molecule (molecular size, flexibility, and water solubility) (Haslam and Lilley, 1988). Complexation takes place via the intermediation of hydrogen bonds between phenolic groups and peptide links and via hydrophobic interactions (Spencer *et al.*, 1988; Synge, 1978). The relative importance of these two types of interaction remains uncertain, but recent experimentation makes it possible to point out the importance of hydrophobic interactions (Spencer *et al.*, 1988).

The structure of salivary proteins has been shown to be a random coil or collagen-like helix (Muenzer *et al.*, 1979; Murray and Williamson, 1994). The carbonyl oxygens of the peptide bonds in either structure are more exposed and available for hydrogen bonding, conferring a high affinity with red wine phenolics such as proanthocyanidins (Hagerman and Butler, 1981; Ricardo-da-Silva *et al.*, 1991). Proline-rich proteins bind the polyphenols present in the diet, forming insoluble complexes (Mehansho *et al.*, 1987a; Warner and Azen, 1988) and thereby probably reducing their bioavailability. Previous studies have shown that interactions between proteins and phenolics are inhibited by the presence of a hydrogen bond acceptor or a nonpolar solvent like dioxane (Asano *et al.*, 1982; Siebert *et al.*, 1996).

The present investigation was undertaken to evaluate the effect of ethanol, a nonpolar solvent present in red wine, on protein−phenolics interaction and consequently upon its *in vitro* total radical-trapping antioxidant potential.

MATERIALS AND METHODS

Chemicals and Reagents. The HPLC grade ethanol was produced by Carlo Erba. All other chemicals came from Sigma Chemical Co., St Louis, MO, unless otherwise stated. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) came from Aldrich Chemical Co. (Milwaukee, MI). 2,2′-Diazobis(2-amidinopropane) dihydrochloride (ABAP) came from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Solutions and reagents for assays were made using Milli-Q (Millipore, Bedford, MA) double-distilled water (resistance > 18 mW/cm^2) and passed through Chelex 100 resin Na^+ form.

Wine Samples. Red wine ("Chianti Classico Rocca Castagnoli" Farm, Siena Italy), was used in the study. Wine was dealcoholized, prior to the tests, in a rotary evaporator at 25 °C for 4 h. To avoid mechanical stress, the vacuum was applied progressively and gradually. The wine was processed in a large single batch sufficient for all the experiments.

Protein Precipitation Assay. The method to evaluate the interaction between polyphenols and proteins (Hagerman and Butler, 1978) involves the formation of a protein−tannin complex between the tannin-containing solution and the protein, bovine serum albumin (BSA). The protein solution, 1.0 mg/mL (Sigma fraction V), was prepared in 0.20 M acetate buffer, pH 5.0 containing 0.17 M sodium chloride. Wine samples (1 mL) were added to 2.0 mL of the protein solution (1 mg/mL) in a glass centrifuge tube. The solutions were mixed and allowed to stand at room temperature for about 15

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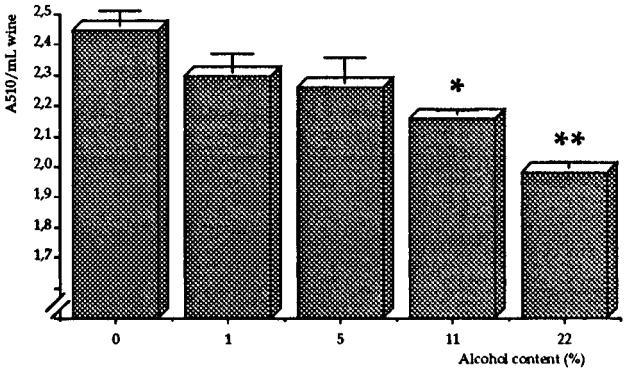

Figure 1. Effect of ethanol on red wine tannin precipitation (expressed as A_{510}/mL). Values are mean±SD of three separate experiments. * $p < 0.05$; ** $p < 0.01$ vs alcohol-free wine (Fisher's LSD).

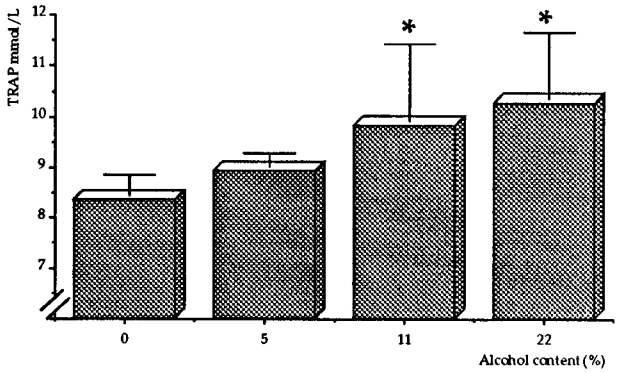

Figure 2. Effect of ethanol on total radical-trapping antioxidant parameter (TRAP) expressed as mmol/L, of supernatants containing phenolic fraction not linked to BSA. Values are mean ±SD of five separate experiments. * $p < 0.05$ vs alcohol-free wine (Fisher's LSD).

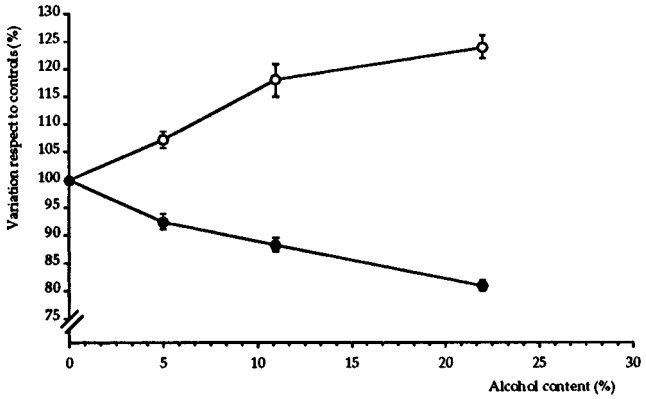

Figure 3. TRAP values (○) of supernatant and of tannin–protein precipitate (●) at different alcohol content. Values are expressed as mean ±SD respect to alcohol-free red wine (100%).

min and then centrifuged for 15 min (Beckman TJ-6 Centrifuge). The supernatant was collected, and the surface of the pellet was washed with buffer. The precipitate was dissolved in a detergent solution consisting of 1% sodium dodecyl sulfate (SDS) and 5% (v/v) triethanolamine in double-distilled water. The tannins/phenolics present in the dissolved complex were measured spectrophotometrically (Beckman Spectrophotometer DU-7400) at 510 nm 15 min after the addition of ferric chloride (0.01 M in 0.01 M of HCl). The results are expressed as A_{510nm}/mL of wine. A reagent blank (deionized water) and a control (solution of SDS–triethanolamine plus ferric chloride) have been included as described in the original work (Hagerman and Butler, 1978). The average A_{510} of triplicate samples of the control reagent has been subtracted from the A_{510} of each sample to correct for background absorbance.

Total Radical-Trapping Antioxidant Parameter: TRAP Assay. The method employed to assess the *in vitro* total radical-trapping antioxidant parameter of wine was developed in our laboratory (Ghiselli *et al.*, 1995). It is based on the protection afforded by plasma, or any other substance, against the decay of a fluorescent target, (*R*)-Phycoerythrin (R-PE), during a controlled peroxidation reaction. The reaction mixture is 1.5×10^{-8} mol/L R-PE in 75 mM phosphate buffer, pH 7.0. In the present study, wine samples were added to the reaction mixture, made up to a 2.0 mL final volume, and preincubated at 37 °C for 5 min in 10 mm quartz fluorometer cells. In order to check for possible interferences from reagents used to precipitate protein, we tested antioxidant activity of BSA and triethanolamine. No antioxidant activity was found under the conditions of our assay (data not shown). The oxidation reaction was started by adding ABAP to a final concentration of 4.0 mM The decay of R-PE fluorescence was monitored every 5 min for 90 min on a Perkin-Elmer (Norwalk, CT) LS-5 luminescence spectrometer equipped with a thermostatically controlled cell-holder. The monochromators operated at an excitation wavelength of 495 nm/5 nm slit width and at an emission wavelength 575 nm/5 nm slit width. The results were standardized using Trolox, a water soluble analogue of α-tocopherol, and expressed as TRAP (mM).

Statistics. Data are expressed as means ± standard deviation (SD). Linear regression, analysis of variance, and Fisher's LSD were calculated using Statview II (Abacus Concepts Inc., Berkeley, CA) software for Macintosh.

RESULTS

The extent of the tannin precipitation of red wine at different ethanol percentages after adding BSA is illustrated in Figure 1. Analysis of variance shows that scalar amounts of ethanol proportionally reduce the extent of tannin precipitation with respect to alcohol-free red wine ($p < 0.05$). Post-hoc comparison (Fisher's LSD) between the treatments levels shows that the extent of tannin precipitation is significantly lower at 11% ($p < 0.05$) and 22% ($p < 0.01$) compared to alcohol free-wine (Figure 1). Correlation between wine alcohol content and tannin–protein precipitates expressed as $A_{510/mL}$ of wine is linear and significant ($r = 0.981$).

After precipitation, supernatants containing the phenolics fraction not linked to proteins, were assayed for TRAP. Figure 2 describes TRAP values of the wine supernatants. TRAP value of alcohol-free supernatant is 8.3 ± 0.48 mM and significantly rises proportionally with the increase in ethanol content: 9.0 ± 0.2 mM; 9.8 ± 1.5 mM ($p < 0.05$); 10.3 ± 1.2 mM ($p < 0.05$), respectively, for 5%, 11%, and 22% ethanol (Fisher's LSD). The increase in TRAP values is linearly correlated ($r = 0.964$) to alcohol content.

There is a negative ($b = -0.212$), linear ($r = 0.978$) correlation between TRAP values and the extent of tannin precipitation. The antioxidant capacity of red wine increases with the decrease in the interaction between tannin and BSA.

Figure 3 summarizes the percent of variation of supernatant TRAP and of tannin–protein precipitate relative to alcohol-free red wine (100%), at different alcohol contents. An inversely related trend is visible for the precipitates that decrease with the ethanol, while the antioxidant capacity of the supernatant fraction increases (Figure 3).

In order to establish the proportion of TRAP that may be due to the antioxidant effect of ethanol, we measured wine TRAP values at different ethanol contents. There was no change in the TRAP values of the red wine over the whole range of ethanol percentages, from 0% (38.2

± 6.1 mM), 5% (38.2 ± 3.3 mM), 11% (38.2 ± 4.0 mM) to 22% (38.4 ± 5.3 mM).

DISCUSSION

Tannins, defined as "water-soluble phenolic compounds having molecular weights between 500 and 3000 able to precipitate proteins" (Gupta and Haslam, 1980), are responsible for the astringent taste of wine. Protein with high proline content has a very strong binding action toward phenolic compounds (Hagerman and Butler, 1981; Ricardo-da-Silva *et al*., 1991). The chemical reactions form the basis of astringency and consist of links between tannins and salivary proteins rich in proline and hydroxyproline (PRPs). These represent up to 70% of the protein in parotid saliva (Kauffman and Keller, 1979). It has been hypothesized that salivary proline-rich proteins improve the bioavailability of the dietary proteins by protecting them from interactions with tannins (Warner and Azen, 1988; Mehansho *et al.*, 1987a). Feeding tannin-rich diets to rats and mice led to an initial weight loss, resumed after about 3 days which correlated well with the induction of salivary PRP synthesis (Mehansho *et al.*, 1987b).

The effect of dietary tannin consumption on the nutrient status of humans has been debated for many years, but without any clear conclusion. For a long time, wine tannins have been considered as factors which interfere with nutrient bioavailability (Bezwoda *et al.*, 1985). By virtue of recent evidence describing their in-vivo antioxidant activity (Whithead *et al.*, 1995; Fuhrman *et al.*, 1995), interest in wine tannins has been renewed as protective compounds against free-radical-mediated diseases. The interactions between proline-rich protein and diet polyphenols, and the formation of an insoluble complex (Mehansho *et al.*, 1987a; Warner and Azen, 1988), may reduce polyphenol bioavailability.

In this paper we have established that the alcohol content of wine produces a linear reduction in BSA−tannins interaction. This effect is paralleled by, and highly correlated with, a concomitant increase in the antioxidant capacity of the fraction of wine containing phenol not linked to protein. Thereafter, there is a partial restoration of the antioxidant activity lost as a result of the interactions with proteins.

The concentration of BSA that we used in our study is comparable to the concentration of salivary total proteins in human subjects (about 1.5 mg/mL) (Yavuzyilmaz *et al.*, 1996; Zipkin, 1967). Furthermore, the binding curves for proline-rich proteins to tannins have affinities 10 fold greater than BSA and tannins (Mehansho *et al.*, 1983), therefore we can reasonably speculate that the reduction in BSA−tannins interaction exerted in vitro by the alcohol may occur also in vivo with PRPs.

The high affinity of salivary proteins for polyphenols has been attributed to their open, extended structure and high proline content. PRPs are strong hydrogen bond acceptors, and their tertiary structures contain a large region of random coil or collagen-like helices (Muenzer *et al.*, 1979). The carbonyl oxygens in both structures are exposed and thus available for hydrogen bonding to a degree that is not found in a compactly folded protein. This structure leads to the formation of bonds of phenolics with the carbonyl group of the protein. Previous studies showed that the interactions between proteins and phenolics are inhibited by the presence of hydrophobic solvents or of hydrogen bond acceptor solvents (Asano *et al.*, 1982; Siebert *et al.*, 1996, Spencer, 1988).

On this basis and in the light of our results, bearing in mind that we used BSA and not purified PRPs, we postulate that chemical interactions between PRPs and polyphenols could be reduced in the presence of ethanol. Ethanol could disrupt the hydrophobic interactions or modify the random coil or collagen-like helices region of the salivary proteins. As a consequence their carbonyl groups would be hidden, and the affinity or capacity of protein to bind tannins would be reduced. The practical outcome would be that alcohol would contribute indirectly to the antioxidant capacity of wine through the increased bioavailability of its phenolics.

Our findings suggest a specific and separate role for the alcohol moiety of wine. It also highlights the complexity of the factors that can interfere with or promote dietary phenol bioavailability.

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