JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Some Phenolic Compounds and Beverages on Pepsin Activity during Simulated Gastric Digestion

DAVIDE TAGLIAZUCCHI,* ELENA VERZELLONI, AND ANGELA CONTE

Department of Agricultural Science, University of Modena and Reggio Emilia, Via Kennedy 17, 42100 Reggio Emilia, Italy

The effect of some polyphenols (resveratrol, catechin, epigallocatechin-3-gallate, and quercetin) and beverages (red wine and green tea) on the enzymatic activity of pepsin during the digestion of three different substrates (pork meat, insoluble azocasein, and denatured hemoglobin) has been investigated. The tested polyphenols and beverages increase the initial velocity of the reaction, and the activating effect is concentration dependent. The order of effectiveness of polyphenols in increasing the initial velocity of the reaction is resveratrol ≥ quercetin > epigallocatechin-3-gallate > catechin. The kinetic data obtained with soluble denatured hemoglobin show that the K_m for the substrate is not changed, whereas the V_{max} of the reaction is increased. Pepsin activity follows a simple Michaelis−Menten kinetic suggesting that k_3 is increased by polyphenols. To the authors' knowledge, the present study is the first demonstration that some polyphenols and related beverages are able to enhance the enzymatic activity of pepsin.

KEYWORDS: Pepsin; polyphenols; red wine; green tea; digestion

INTRODUCTION

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants (*1*). These phenolic substances or polyphenols include many classes of compounds including phenolic acids, colored anthocyanins, simple flavonoids, and complex flavonoids (*1*). Many foods and food derivatives contain phenolic compounds, such as wine, tea, olive oil, cereals, legumes, fruits, and fruit juices (*2*). The most abundant phenolic compounds in red wine are phenolic acids, flavonoids, and non-flavonoid phenolic compounds. The most common phenolic acids in red wine include cinnamic acids (coumaric acid, ferulic acid, caffeic acid, and chlorogenic acid) and benzoic acids (*p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and gallic acid). Flavonoids include flavanols (catechin, epicatechin, and their ester forms), flavonols (quercetin), red and blue anthocyanins, and the polymeric tannins. The most abundant non-flavonoid phenolic compound in red wine is resveratrol (*3*, *4*). White wine contains more phenolic acids (mainly cinnamic acids) but fewer flavonoids and resveratrol with respect to red wine (*5*). The concentration of total phenols ranges from 800 to 4000 mg/L for red wines and from 200 to 1000 mg/L for white wines (*6*−*8*). Green tea contains many polyphenols known as catechins. They are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epicatechin, and catechin (*9*). In contrast, black tea contains fewer catechins but a larger proportion of other polymeric compounds, such as the dimeric theaflavins (10%) and the more extensively oxidized and polymerized thearubigins (50%) (*9*).

Plant phenolics present in fruits and vegetables and particularly those in green tea and red wine have received considerable attention for their antioxidant activity and potential health benefits (*10*, *11*). In recent years, various researchers have shown that polyphenols present in red wine and green tea show a protective effect on brain degenerative processes (*12*, *13*) and antiinflammatory (*14*), anticarcinogenic (*15*, *16*), antiatherogenic (*17*), and cardioprotective (*18*) effects.

The absorption of polyphenolic compounds in the gastrointestinal tract as a result of food intake has been found to be low (*19*); therefore, most of the consumed polyphenols remain in the gastrointestinal tract, where they may exert some of their biological actions. In particular, this could be true for the stomach because polyphenols concentration could quickly reach high levels directly following the consumption of red wine, tea, or other fruits and vegetables. Indeed, tannins have several effects on the digestive tract. In the mouth they can link human salivary proteins, especially proline-rich proteins, and this ability of tannins is believed to be involved in the astringency sensation perceived in the buccal cavity when wine is swallowed (*20*, *21*). It has been demonstrated that red wines decrease the formation of hydroperoxides during the digestion of meat by pepsin in vitro (*22*). Furthermore, wine and tea antioxidants prevent the postprandial rise of lipid hydroperoxides in plasma (*23*, *24*). Naturally occurring polyphenols, and in particular condensed tannins, can also inhibit in vitro a number of digestive enzymes including trypsin, lipase, and α-amylase. The inhibition of digestive enzymes may be explained with the ability of condensed tannins to form insoluble complexes with proteins

* Corresponding author (telephone +39-0522-522060; fax +39-0522-522053; e-mail tagliazucchi.davide@unimore.it).

10.1021/jf058074n CCC: $30.25
Published on Web 09/30/2005

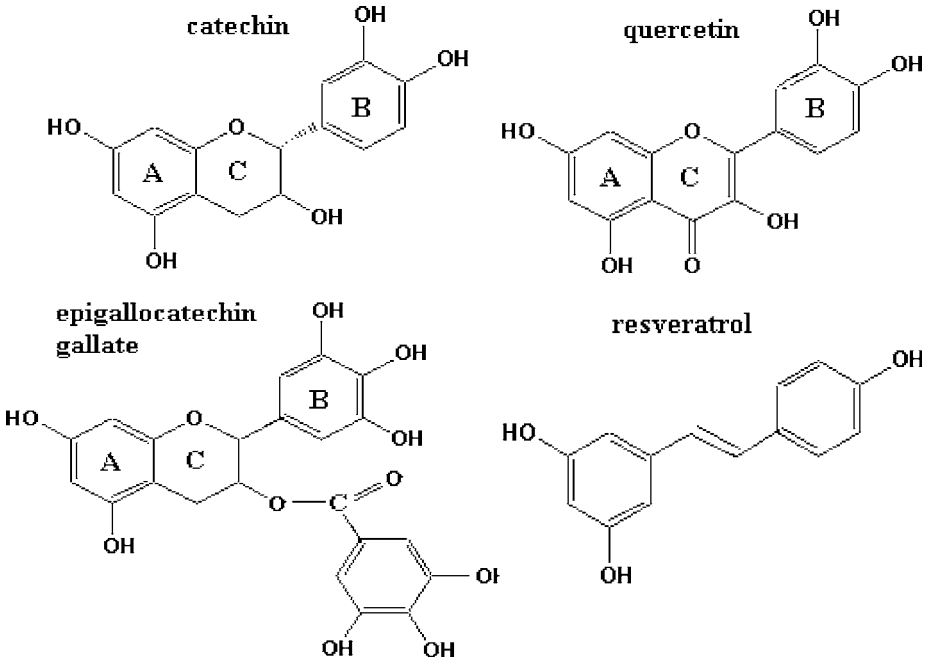

Figure 1. Chemical structures of catechin, epigallocatechin gallate (EGCG), quercetin, and resveratrol.

in the gastrointestinal tract (*25*). Recently, it has been demonstrated that also simple phenolic compounds and in particular phenolic acids are able to inhibit trypsin, α-chymotrypsin, and α-amylase activity, after the covalent attachment of the phenolic compounds to reactive nucleophilic sites in the enzymes (*26*). The ability of polyphenols to form insoluble complexes with nutritional macromolecules such as proteins has long been associated with the observed reduction in nutritive value resulting from their presence in diet. Furthermore, polyphenols are able to chelate several transition metals such as iron and copper, decreasing the bioavailability of these metals (*27*, *28*). In our recent work on the traditional choice of wines accompanying foods we observed a significant positive correlation between the phenols content of wines and the iron and proteins content of the combined foods (*6*). Plant phenols decrease the intestinal absorption of iron and have antioxidant activity in the intestinal tract. These positive effects compensate the antinutritional activity toward proteins digestion. The traditional combination of wines and foods appears to be very favorable because wines poor in phenols are combined with foods poor in iron and proteins to minimize their possible antinutritional effects, whereas phenol-rich wines are combined with foods rich in iron and proteins to decrease iron absorption and prandial peroxidative stress (*6*).

The aim of the present work was to investigate the effect of some phenolic compounds (catechin, EGCG, resveratrol, and quercetin, **Figure 1**) and beverages, which are rich in these compounds (red wine and green tea), on proteolytic activity of pepsin during a simulated gastric digestion of meat or of an insoluble single protein (insoluble azocasein) and a soluble protein (hemoglobin). The objective was to increase the knowledge about the complex effects that polyphenols and beverages rich in phenolic compounds show during the gastric digestion.

MATERIALS AND METHODS

Materials. Pepsin, catechin, resveratrol, quercetin, epigallocatechin gallate, insoluble azocasein, denatured hemoglobin, Bradford reagent, trichloroacetic acid (TCA), and polyvinylpyrrolidone (PVP-40) were supplied from Sigma (Milan, Italy). All other chemical reagents were from Carlo Erba (Milan, Italy). Red wine (Nero d'Avola), green tea, and pork meat were purchased from local supermarkets.

Pepsin Activity Assay. The pepsin from Sigma that we have used was from porcine gastric mucosa with 1500 units/mg of protein. One unit will produce a ΔA_{280} of 0.001 per minute at pH 2.0 at 37 °C, measured as TCA-soluble products using hemoglobin as substrate (final volume = 16 mL; light path = 1 cm) according to the method of Anson (*29*). In this method the hemoglobin concentration in the assay was 1.67% corresponding to 259 μM considering for hemoglobin a molecular mass of 64500 Da. We evaluated the effect of polyphenols on pepsin activity utilizing three different substrates—pork meat, insoluble azocasein, and denaturated hemoglobin—and the peptides obtained by the pepsin activity were detected by recording the increase of absorbance at 280 or 440 nm (insoluble azocasein) and with Bradford (*30*) and/or Lowry (*31*) methods. Pepsinogen content was determined by destroying pepsin at pH 8 and then converting the pepsinogen to pepsin by acidification to pH 2 (*32*). The pepsinogen content of the pepsin that we have used was 0.7% of total pepsin activity.

Simulated Gastric Digestion with Grilled Pork Meat and Insoluble Azocasein. The grilled pork meat, in the form of small slices, was divided into portions and kept at −80 °C pending use in the experiments. One part of this muscle tissue was ground with one part of digestive solution for 1 min in a laboratory blender and adjusted to pH 2.0 with HCl. When insoluble azocasein was used, 1 mg of insoluble azocasein was introduced in a tube with 3 mL of digestive solution. Insoluble azocasein ($E1\%_{440}$ in 0.1 N NaOH = 34) was washed two times with HCl to remove soluble substances if they were present. The digestive solution was simulated gastric fluid (SGF) freshly prepared according to the U.S. Pharmacopoeia (*33*). Briefly, 3.2 g of pepsin and 2.0 g of sodium chloride were dissolved in 7.0 mL of hydrochloric acid and sufficient water to make 1000 mL of SGF. SGF was prepared with or without added polyphenols, beverages, ethanol solution, or dimethyl sulfoxide (DMSO) solution. Catechin and EGCG were dissolved in water to have a final concentration in the assay of 0.05, 0.1, or 0.5 mM. Resveratrol and quercetin were dissolved in DMSO to have a final concentration in the assay of 0.05 or 0.1 mM. The final concentration of DMSO in the assay was 5%. The wine was added to have final polyphenols and ethanol concentrations in the assay of 2.3 mM and 3%, respectively. Freshly prepared green tea infusion was added to have a final polyphenols concentration in the assay of 0.9 mM. Careful measurements and corrections of the final pH of the assay were made with concentrated solutions of HCl and NaOH. The test tubes containing the reaction mixture were incubated in a shaking bath at 37 °C for 180 min. At different times three test tubes were removed,

and the reaction was stopped by cooling the test tubes in ice; the tubes were centrifuged at 17500*g* at 5 °C for 15 min, and the supernatants were treated with PVP-40 to precipitate the polyphenols (*34*). Briefly, to 0.5 mL of supernatant was added 0.5 mL of a solution of PVP-40 to have a final concentration ranging from 1 to 10% depending on whether wine, tea, or single polyphenols were used. After 40 min of incubation at room temperature, the samples were centrifuged at 17500*g* for 30 min, the supernatants were collected, and the peptides were detected by recording the increase of absorbance at 280 nm and with Bradford and/or Lowry methods. When insoluble azocasein was used, the absorbance at 440 nm was also recorded.

To test the possible interference of PVP with the different methods for peptides assay utilized and to test the possibility that PVP precipitated the digested peptides together with polyphenols, a number of control digestions were conducted.

Digestion of Hemoglobin. The pepsin activity was assayed by utilizing denatured hemoglobin as substrate (*29*). The incubation mixture, in a final volume of 1.6 mL, contained 30 units of pepsin, 10 mM HCl, and variable amounts of denaturated hemoglobin ranging from 1.25 to 15 μM corresponding to a range of 80.6−967.5 μg/mL as final concentration in the test tube. To evaluate the effect of polyphenols on the enzymatic activity, the assay was conducted in the presence of catechin, resveratrol, or quercetin at different concentrations. After 10 min of incubation at 37 °C, 0.70 mL of 10% TCA was added to precipitate hemoglobin. After centrifugation, 1 mL of supernatant was treated with 1 mL of PVP solution. The final PVP concentration ranged from 1 to 5% PVP depending on the polyphenol utilized. After centrifugation as described above, formed peptides were determined by recording the absorbance at 280 nm and with Bradford and Lowry assays. Control samples for pepsin autodigestion (pepsin without substrate) and substrate stability (reaction mixture with substrate but without pepsin) in the presence and absence of polyphenols were included. These control reactions were treated exactly as described above. With all tested compounds, measurements have been carried out to make sure that the reaction was linear up to 10 min of incubation.

Determination of Total Phenols. Total soluble phenolic compounds were determined with Folin−Ciocalteu reagent according to the method of Singleton (*35*), adapted to a microscale. In a 1.5 mL Eppendorf tube were added and mixed 790 μL of distilled water, 10 μL of sample appropriately diluted, and 50 μL of Folin−Ciocalteu reagent. After exactly 1 min, 150 μL of 20% aqueous sodium carbonate was added, the mixture was mixed and allowed to stand at room temperature in the dark for 120 min. Detection was achieved at 760 nm. Catechin was used as a standard. The measurements were done in triplicate, and results are expressed as the mean value.

Wine Dealcoholization. Dealcoholized wine was prepared by removing ethanol by lyophilization. The resulting residue was dissolved in distilled water and the pH adjusted to the initial value. With this method other volatile compounds (for example, acetic acid) are partially or completely removed.

Statistical Analysis. The digestion assay were repeated 10 times. A maximum of ±5% from the averaged values was generally observed, and the standard deviation was calculated. The averaged values and the standard deviation are documented in the respective figures and tables.

RESULTS

Because the investigated polyphenols and beverages absorb at 280 nm and react with Folin reagent, they must be removed from the reaction mixture before the determination of the peptides at 280 nm or with Lowry reagent, or other methods must be used such as Bradford or biuret. We have evaluated three different methods to determine digested peptides: the absorbance at 280 nm and the Bradford and Lowry assays. We removed the polyphenols with PVP before the peptides were determined with the three methods. In **Figure 2** we report the absorbance at 280 nm of catechin, resveratrol, and wine with increasing concentrations of PVP to determine which is the PVP concentration necessary to remove polyphenols. With 1% PVP

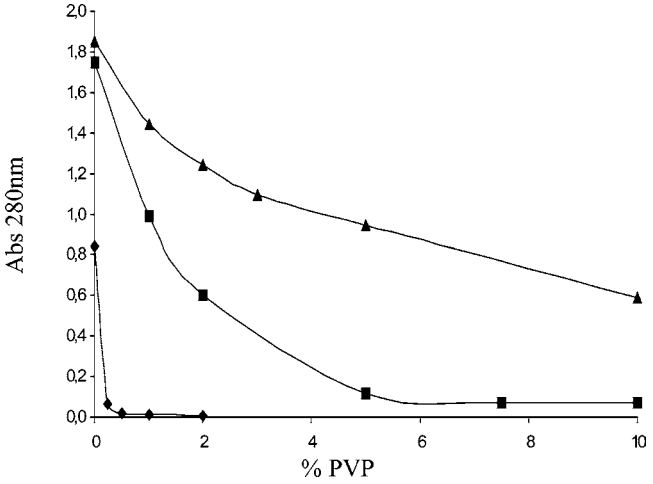

Figure 2. Effect of different PVP concentrations on polyphenols precipitation: (◆) 0.1 mM resveratrol; (■) 1 mM catechin; (▲) red wine containing 2.3 mM polyphenols. The polyphenols concentration was measured by recording the absorbance at 280 nm after precipitation of polyphenols with PVP as described under Materials and Methods. Data are means ± SD ($n = 10$).

0.1 mM resveratrol is completely removed, whereas 5% PVP is necessary to remove 1 mM catechin almost completely. The absorbance at 280 nm of red wine is reduced to 30% with 10% PVP. Similar tests have been made for different concentrations of resveratrol and catechin as well as for the other polyphenols tested and for different dilutions of beverages (data not shown). The method chosen for the determination of formed peptides depends on the type of polyphenol and the substrate utilized. In each assay we have utilized the method more suitable and appropriate controls.

We have tested the possible interference of polyphenols and PVP with the three assays utilized for peptides determination. **Table 1** reports the time course of the pork meat digestion by pepsin followed with three different methods of peptides determination in the presence and in the absence of 1% PVP and in the presence of 1% PVP with and without the addition of 0.1 mM resveratrol after the stopping of the reaction. The amount of the formed peptides at the different times of digestion, determined with the Lowry and Bradford assays, is similar until 90 min. At 180 min the Bradford assay underestimates the formed peptides by ∼15% respect to the Lowry assay. These differences, however, are not significant and could be due to the presence of small peptides that are not detected by the Bradford reagent (*36*). When the absorbance at 280 was utilized at each time of digestion, we always measured a higher peptides formation. It is possible that the different results obtained with the absorbance at 280 nm can be due to the different sensibilities of the three methods toward the amino acidic composition of the BSA used as standard. The initial velocity of the pepsin reaction is linear until 30 min of digestion. Similar results have been obtained with 0.5 mM catechin, 0.1 mM EGCG, and 0.1 mM quercetin and with higher PVP concentration until 10% (data not shown).

Effect of Single Polyphenols, Green Tea, and Red Wine on Pepsin Activity with Meat. Both alcoholic and dealcoholized red wines were tested to evaluate pepsin activity in the presence of red wine polyphenols with or without ethanol. The final polyphenols concentration in the assay of alcoholic and dealcoholized red wine was 2.30 mM. When alcoholic red wine was utilized, the final ethanol concentration was 3%. Green tea polyphenols concentration in the assay was 0.9 mM.

Table 1. Peptide Determination after Pepsin Digestion of Pork Meat[a]

	peptides[b] (μg/mL)								
	Abs 280 nm			Lowry assay			Bradford assay		
time (min)	no PVP	PVP	PVP + resveratrol	no PVP	PVP	PVP + resveratrol	no PVP	PVP	PVP + resveratrol
15	34.1 ± 4.7	32.2 ± 3.5	35.3 ± 4.3	23.5 ± 2.7	22.8 ± 2.1	21.2 ± 2.3	21.2 ± 3.1	22.8 ± 2.8	23.1 ± 3.7
30	67.9 ± 8.7	71.6 ± 9.0	72.1 ± 9.0	50.7 ± 8.6	47.9 ± 8.0	53.5 ± 9.6	47.4 ± 5.5	54.0 ± 7.1	53.5 ± 8.1
60	112.2 ± 14.4	118.5 ± 15.3	104.4 ± 11.5	82.4 ± 12.4	78.5 ± 10.1	75.1 ± 11.1	80.5 ± 8.2	76.6 ± 7.6	82.9 ± 9.0
90	147.9 ± 19.8	159.6 ± 18.3	142.9 ± 15.2	108.4 ± 17.5	111.6 ± 19.9	107.1 ± 10.9	110.5 ± 13.1	100.5 ± 13.0	109.2 ± 16.0
180	214.3 ± 25.6	204.4 ± 22.4	213.7 ± 23.4	147.8 ± 22.0	160.9 ± 21.0	160.3 ± 24.1	136.7 ± 18.8	144.7 ± 17.2	129.2 ± 20.0

[a] Comparison of three different assays in the absence of PVP, in the presence of 1% PVP, or in the presence of 1% PVP and 0.1 mM resveratrol. [b] The peptide concentration was measured by recording the absorbance at 280 nm and by the Lowry and Bradford methods as described under Materials and Methods. At 0 time of digestion no measurable amount of peptides was observed. Data are expressed as means ± SD ($n = 10$).

Table 2. Effect of Beverages and Polyphenols on Peptic Digestion of Pork Meat

	peptides[a] (μg/mL)		
added compound	after 15 min of digestion	after 30 min of digestion	after 120 min of digestion
no addition	19.8 ± 3.1	43.6 ± 5.6	120.3 ± 10.8
5% DMSO	10.9 ± 1.4*	21.4 ± 2.9*	73.2 ± 7.5*
green tea[b]	30.1 ± 3.1*	66.8 ± 6.8*	133.5 ± 10.6*
dealcoholized red wine[b]	42.4 ± 0.4*	57.7 ± 0.8*	133.3 ± 1.1*
alcoholic red wine[b]	24.5 ± 4.4*	24.0 ± 3.3	66.0 ± 11.8
0.5 mM catechin	24.3 ± 2.5*	54.3 ± 5.5*	132.8 ± 4.9*
0.1 mM EGCG	58.2 ± 5.5*	94.4 ± 11.3*	165.4 ± 1.6*
0.1 mM resveratrol in 5% DMSO	57.8 ± 7.8^	123.5 ± 8.2^	247.8 ± 14.4^
0.05 mM quercetin in 5% DMSO	64.0 ± 5.8^	132.0 ± 10.2^	212.6 ± 13.3^

[a] The peptide concentration was measured by the Bradford method after precipitation of polyphenols with 10% PVP for red wine and green tea, 5% PVP for 0.5 mM catechin, 1% PVP for 0.1 mM resveratrol, 0.05 mM quercetin, 0.1 mM EGCG as described under Materials and Methods. At 0 time of digestion no measurable amount of peptides was observed. Data are expressed as means ± SD ($n = 10$). * denotes $P < 0.05$ with respect to no addition. ^ denotes $P < 0.05$ with respect to 5% DMSO. [b] Green tea contained 0.90 mM polyphenols. Red wine contained 2.3 mM polyphenols.

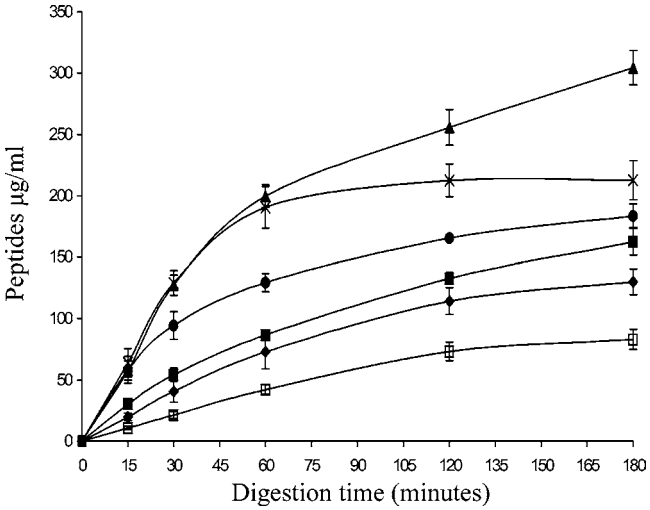

Figure 3. Effect of polyphenols on pepsin enzymatic activity in SGF with pork meat: (◆) no addition; (□) 5% DMSO; (■) 0.5 mM catechin; (●) 0.1 mM EGCG; (×) 0.05 mM quercetin; (▲) 0.1 mM resveratrol. The peptides concentration was measured by the Bradford method after precipitation of polyphenols with 5% PVP for catechin and 1% PVP for resveratrol, quercetin, and EGCG as described under Materials and Methods. Data are means ± SD ($n = 10$).

Green tea and both alcoholic and dealcoholized red wines are able to enhance the concentration of digested peptides measured with Bradford reagent during a 120 min digestion (**Table 2**). When the single polyphenols catechin, EGCG, resveratrol, and quercetin were tested, a significant increase of pepsin activity was also observed (**Table 2**). Similar results were obtained when the peptides were measured with the Lowry assay (data not shown). All of the polyphenols tested are able to increase the initial velocity of the reaction with resveratrol and quercetin, which are more active with respect to EGCG and catechin. **Figure 3** reports some examples of the time course of peptide formation. In all of the experiments in the absence of pepsin no peptides are found during 180 min of digestion (data not shown).

Considering that polyphenols and beverages activate pepsin digestion of a complex substrate constituted by the pork meat suspension, we have carried out a simplified digestion assay with single proteins as substrate utilizing both a suspension of the insoluble protein insoluble azocasein and the soluble hemoglobin.

Effect of Polyphenols and Beverages on Pepsin Activity with Insoluble Azocasein. Insoluble azocasein is an insoluble protein that can simulate proteinaceous material in suspension during digestion and, moreover, the soluble peptides from digestion are directly determined by a photometric method because they are colored. At 440 nm the interference of polyphenols is lower than in the ultraviolet region.

The concentration of the digested peptides in solution was measured by recording the absorbance at 440 nm and/or at 280 nm and/or with Lowry assay.

Because ethanol is a well-known inhibitor of pepsin, we have investigated the behavior of pepsin in the digestion assay conducted with insoluble azocasein in the presence of different concentrations of ethanol. Ethanol shows a concentration-dependent inhibition of pepsin. At a concentration of 12% ethanol, the enzymatic activity of pepsin is greatly decreased (**Table 3**).

Resveratrol and quercetin are not freely soluble in water, so we needed to dissolve these phenolic compounds in DMSO. For this reason, we tested the effect of DMSO on pepsin activity. Like ethanol, also DMSO at a concentration of 5% (final concentration in the assay) shows an inhibitory effect on pepsin activity (**Table 3**). The inhibitory effect of DMSO is similar to that of ethanol. In the absence of pepsin the quantity of peptides released from insoluble azocasein during 180 min of incubation is negligible.

In **Table 4** is shown the effect of different concentrations of catechin, EGCG, resveratrol, and quercetin on pepsin activity in SGF with insoluble azocasein as substrate. At 0.1 mM

Table 3. Effect of Ethanol and DMSO on Peptic Digestion of Insoluble Azocasein

	peptides[a] (μg/mL)		
added compound	after 15 min of digestion	after 30 min of digestion	after 120 min of digestion
no addition	1.1 ± 0.2	2.2 ± 0.2	6.9 ± 0.5
3% ethanol	0.5 ± 0.1*	1.4 ± 0.3*	5.9 ± 0.1*
6% ethanol	0.2 ± 0.1*	0.4 ± 0.1*	4.1 ± 0.5*
12% ethanol	0.1 ± 0.1*	0.2 ± 0.2*	0.8 ± 0.3*
5% DMSO	0.5 ± 0.1*	1.4 ± 0.2*	3.8 ± 0.8*

[a] The peptide concentration was measured by the Lowry method as described under Materials and Methods. At 0 time of digestion no measurable amount of peptides was observed. Data are expressed as means ± SD ($n = 10$). * denotes $P < 0.05$ with respect to no addition.

Table 4. Effect of Polyphenols and Beverages on Peptic Digestion of Insoluble Azocasein

	peptides[a] (μg/mL)		
added compound	after 15 min of digestion	after 30 min of digestion	after 120 min of digestion
no addition	1.1 ± 0.2	2.2 ± 0.2	6.9 ± 0.5
5% DMSO	0.5 ± 0.1*	1.4 ± 0.2*	3.8 ± 0.8*
0.05 mM catechin	1.0 ± 0.2	2.3 ± 0.5	7.3 ± 1.2
0.1 mM catechin	1.7 ± 0.3*	3.2 ± 0.6*	9.4 ± 0.9*
0.5 mM catechin	1.9 ± 0.3*	4.1 ± 0.6*	10.8 ± 0.8*
0.05 mM EGCG	0.7 ± 0.2	2.2 ± 0.3	17.9 ± 1.3*
0.1 mM EGCG	1.5 ± 0.3*	4.1 ± 0.2*	27.8 ± 3.5*
0.05 mM resveratrol in 5% DMSO	1.0 ± 0.2^	2.0 ± 0.3^	6.4 ± 0.5^
0.1 mM resveratrol in 5% DMSO	1.1 ± 0.1^	2.8 ± 0.3^	17.2 ± 2.0^
0.05 mM quercetin in 5% DMSO	0.8 ± 0.1^	2.0 ± 0.2^	10.3 ± 0.9^
green tea[b]	1.9 ± 0.5*	3.9 ± 0.6*	8.4 ± 0.7*
dealcoholized red wine[b]	1.7 ± 0.4*	3.7 ± 0.8*	7.5 ± 0.1*
alcoholic red wine[b]	1.8 ± 0.5*	3.2 ± 0.7*	7.2 ± 0.4

[a] The peptide concentration was measured by the Lowry method after precipitation of polyphenols with 10% PVP for green tea and red wine, 5% PVP for 0.5 mM catechin, 1% PVP for 0.05 and 0.1 mM catechin, 0.05 and 0.1 mM EGCG, 0.05 and 0.1 mM resveratrol, and 0.05 mM quercetin as described under Materials and Methods. At 0 time of digestion no measurable amount of peptides was observed. Data are expressed as means ± SD ($n = 10$). * denotes $P < 0.05$ with respect to no addition. ^ denotes $P < 0.05$ with respect to 5% DMSO. [b] Green tea contained 0.90 mM polyphenols. Red wine contained 2.3 mM polyphenols.

concentration, catechin is an activator of pepsin. The effect is concentration-dependent because at 0.5 mM concentration, catechin shows a greater activating effect than at 0.1 mM. The effect on pepsin activity observed at 0.05 mM catechin is small and not statistically significant.

To test the influence of the galloyl moiety on pepsin activity, we have investigated the effect of EGCG at concentrations of 0.05 and 0.1 mM. Our data indicate that EGCG determines a greater activation of pepsin than catechin at the same concentrations. The effect of EGCG on pepsin activity is concentration-dependent (**Table 4**).

Resveratrol exhibits a positive and concentration-dependent effect on pepsin activity. With respect to catechin, resveratrol is active at lower concentration, showing a significant effect of activation on pepsin at a concentration of 0.05 mM (**Table 4**).

Quercetin, like the other polyphenols tested, shows a significant effect of activation on pepsin activity during insoluble azocasein digestion. Like resveratrol, the quercetin activation is significant at a concentration of 0.05 mM (**Table 4**).

Catechin, EGCG, resveratrol, and quercetin are able to increase the initial velocity of the reaction as well the concentration of formed peptides during the entire time course of digestion. As an example we report the time course of peptide formation in the presence of different concentrations of catechin measured with the Lowry assay (**Figure 4**). Similar results were obtained when the peptides were measured by recording the absorbance at 440 or 280 nm.

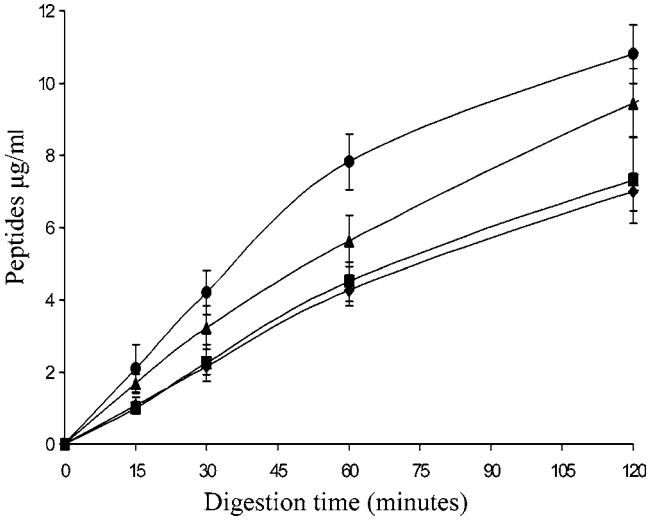

Figure 4. Effect of catechin on pepsin enzymatic activity in SGF with insoluble azocasein: (◆) no addition; (■) 0.05 mM catechin; (▲) 0.1 mM catechin; (●) 0.5 mM catechin. The peptides concentration was measured by the Lowry method after precipitation of polyphenols with 5% PVP for 0.5 mM catechin and 1% PVP for 0.1 and 0.05 mM catechin as described under Materials and Methods. Data are means ± SD ($n = 10$).

Green tea acts as an activator of pepsin enzymatic activity, increasing both the initial velocity of the reaction and the concentration of digested peptides measured with the Lowry assay (**Table 4**). Both alcoholic and dealcoholized red wines were able to activate significantly pepsin in the assay. We observed a difference between alcoholic and dealcoholized red wine, but it is not statistically significant. As observed in the digestion assay conducted with meat, also in the assay conducted with a simple protein both alcoholic and dealcoholized red wines are able to enhance the initial velocity of reaction. Similar resultswere obtained when the peptides were measured by recording the absorbance at 440 nm.

Polyphenols Effect on Pepsin Activity with Hemoglobin. All of the polyphenols tested (catechin, resveratrol, and quercetin) are able to significantly increase the pepsin activity also in the assay with denaturated hemoglobin (**Table 5**) as observed in the digestion assay with meat or insoluble azocasein. Resveratrol and quercetin are active at lower concentration with respect to catechin, showing a significant effect of activation on pepsin at 0.1 mM concentration. Similar results were obtained when the peptides were measured with Lowry and Bradford reagents. **Figure 5** reports the enzymatic activity as a function of hemoglobin concentration. In the same figure pepsin activity in the presence of two different concentrations of catechin (0.5 and 1 mM) is also shown. Catechin determines an activation of pepsin activity in a concentration-dependent manner, increasing the value of the V_{max} with respect to the control without changing the K_m. Resveratrol at 0.1 mM and quercetin at 0.1 mM increase the pepsin activity also, determining the increase of the V_{max}. The K_m value is not changed (**Figure 6**). The same results reported in **Figures 5** and **6** were obtained when the formed peptides were measured with Lowry or Bradford reagent.

Table 5. Effect of Some Phenolic Compounds on Pepsin Activity Assayed with Denatured Hemoglobin

added compound	pepsin units[a]
no addition	5.8 ± 0.2
5% DMSO	4.9 ± 0.2*
0.5 mM catechin	6.2 ± 0.1*
1 mM catechin	7.6 ± 0.3*
0.1 mM resveratrol in 5% DMSO	7.3 ± 0.2^
0.1 mM quercetin in 5% DMSO	6.5 ± 0.1^

[a] The pepsin activity was measured by recording the absorbance at 280 nm after precipitation of polyphenols with 5% PVP for 0.5 and 1 mM catechin and 1% PVP for 0.1 mM resveratrol and 0.1 mM quercetin as described under Materials and Methods. Denatured hemoglobin was used at 2.5 μM concentration. Data are expressed as means ± SD ($n = 10$). * denotes $P < 0.05$ with respect to no addition. ^ denotes $P < 0.05$ with respect to 5% DMSO.

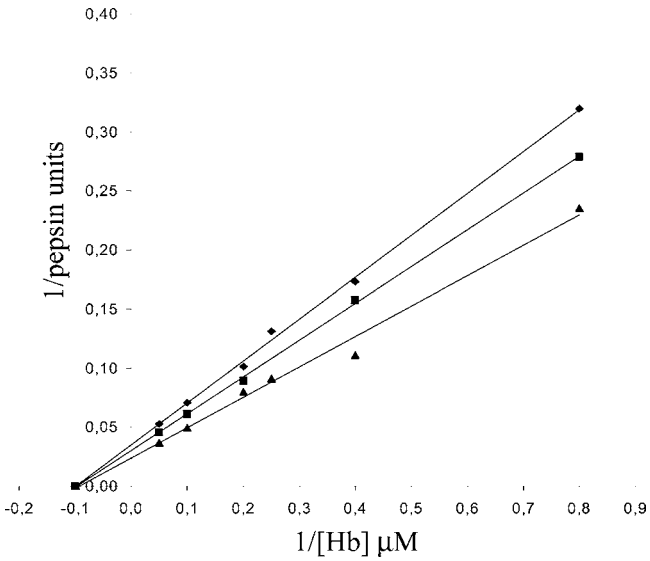

Figure 5. Effect of catechin on pepsin enzymatic activity. The pepsin activity was measured at different hemoglobin concentrations as substrate and by recording the absorbance at 280 nm after precipitation of polyphenols with 5% PVP as described under Materials and Methods: (◆) no addition; (■) 0.5 mM catechin; (▲) 1 mM catechin. Data are means ± SD ($n = 10$).

DISCUSSION

To our knowledge, the present study is the first demonstration that some polyphenolic compounds contained in beverages such as wine and tea that are widely utilized during meals are able to enhance the enzymatic activity of pepsin. Because the presence of polyphenols gives rise to some problems in the peptide determination, we have used three different assays to evaluate the digested peptides. With the three different methods of peptide determination utilized, we always observed an activation of the peptic digestion both by single polyphenols and by beverages. The activating effect is observed with three very different substrates: pork meat suspension, in which soluble and insoluble proteins are present; insoluble azocasein; and soluble denatured hemoglobin. The results that we have obtained clearly demonstrate that the activation of pepsin by polyphenols is not an artifact due to peptide determination in the presence of these compounds or an artifact due to an aspecific effect depending on surface phenomena or solubility problems because it is present also when a soluble protein such as denatured hemoglobin is utilized.

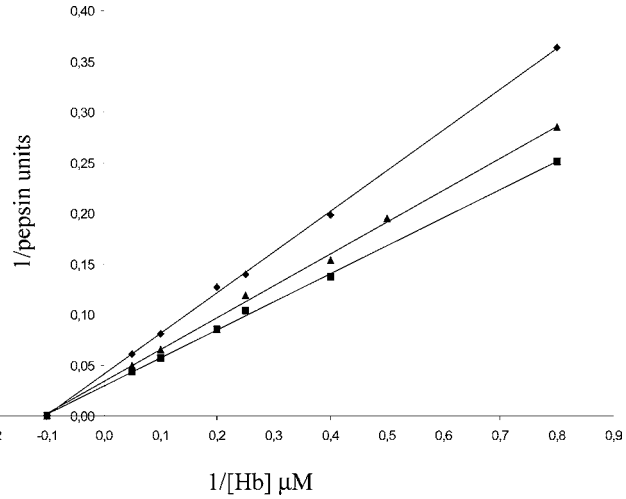

Figure 6. Effect of resveratrol and quercetin on pepsin enzymatic activity. The pepsin activity was measured at different hemoglobin concentrations as substrate and by recording the absorbance at 280 nm after precipitation of polyphenols with 1% PVP as described under Materials and Methods: (◆) 5% DMSO; (■) 0.1 mM resveratrol in 5% DMSO; (▲) 0.1 mM quercetin in 5% DMSO. Data are means ± SD ($n = 10$).

The tested polyphenols and the beverages increase by 50−300% the initial velocity, and the activating effect is concentration dependent.

The tested polyphenols (catechin, EGCG, quercetin, and resveratrol) are well-known for their antioxidant and biological activities (*10*, *11*). They are also able to inhibit several enzymes through different mechanisms including V_{max} decrease of glutathione reductase (*37*), K_m increase of xanthine oxidase (*38*), and the binding at specific sites of the enzyme involved in its mechanism of action as observed with fatty acid synthase, ribonucleotide reductase, and matrix metalloproteinases (*39*−*41*). The polyphenols tested, despite their different chemical structures, show a positive effect on pepsin activity that is concentration dependent. The activating effect is observed with the three substrates tested. The results show that their order of effectiveness in increasing the initial velocity with the three substrates tested is resveratrol ≥ quercetin > EGCG > catechin. In the same order they increase the V_{max} with denatured hemoglobin as substrate. If, however, we measure the total amount of the formed peptides after 180 min of digestion, the following rank order was observed: EGCG > resveratrol ≥ quercetin > catechin. This observation suggests that they not only increase the initial velocity but also decrease the inhibition byproducts that are observed during pepsin digestion, and in our experimental conditions this is observed after 90 min of reaction. As shown in **Figure 1**, catechin and quercetin have the same diphenylpropane ($C_6C_3C_6$) skeleton, with identical arrangements of the five hydroxyl groups. However, quercetin also contains the 2,3-double bond in the C ring and the 4-oxo function. This structural difference confers an enhancement of the positive effect on the initial velocity, on the V_{max}, and on the inhibition byproducts. EGCG differs from catechin in the presence of a third hydroxyl group in the B ring that goes to make a galloyl moiety and for the presence of another galloyl moiety esterified at the 3-OH group in the C ring. The presence of the galloyl moieties confers an enhancement of the positive effect on the initial velocity that is, however, lower than that observed with quercetin and a greater effect on the inhibition byproducts. Resveratrol is a stilbene with a different structure with respect to flavonoids that has two aromatic rings and three hydroxyl groups. Despite this structural difference, resveratrol

is the most effective in enhancing pepsin activity. The effect of these phenolic compounds on pepsin activity does not seem to be related to their antioxidant potential because resveratrol is less effective as a radical scavenger than EGCG, quercetin, or catechin (*10*, *42*, *43*). The rank order of effectiveness in increasing the initial velocity may be at least partially related to the polyphenol solubility because the more water-soluble compounds (catechin and EGCG) are less active than resveratrol and quercetin.

The kinetic data that we have obtained with soluble denatured hemoglobin show that the K_m for the substrate is not changed, whereas the V_{max} of the reaction is increased. Pepsin activity follows a simple Michaelis−Menten kinetic, suggesting that k_3 is increased by polyphenols.

At this moment we are not able to establish which is the mechanism of activation of pepsin by polyphenols. Pepsin is a member of the family of aspartyl proteases that contain in the catalytic site two aspartyl residues. Pepsin shows preference toward the substrates containing amino acids with large nonpolar side chains, such as phenylalanine or tyrosine. It was suggested that pepsin binds to a nonpolar side chain of its substrate by means of a hydrophobic bond in a so-called hydrophobic binding site (*44*). Because this binding appears to determine the site of peptic hydrolysis in a protein substrate, it must be considered the first step in the mechanism of pepsin catalysis (*44*). The proposed mechanism of pepsin catalysis is based on the consideration that one aspartyl residue in the catalytic site is protonated and the other is unprotonated. There is a general agreement that, in the enzyme−substrate complex, there is a concerted abstraction of a proton from water by the aspartate ion corresponding to one of the active site aspartyl residues, transfer of the hydroxyl group of water to the carbonyl carbon of the substrate to form a tetrahedral intermediate, and cleavage of the C−N bond by transfer of a proton from the other protonated aspartyl residue (*45*).

The kinetic of denatured hemoglobin shows that the K_m for substrate is not changed by polyphenols, suggesting that their possible binding to the denatured hemoglobin does not change its affinity for enzyme. The binding to the substrate may change, however, the k_3 of the reaction, increasing, for example, the release of the reaction products.

Another possible target of the polyphenols is the enzyme. Because the mechanism of catalysis of pepsin involves two aspartyl residues and not cysteine residues, we consider it to be unlikely that the action of polyphenols on pepsin is related to the antioxidant activity of these compounds. Furthermore, resveratrol exhibits a greater activating effect on pepsin than catechin but is less effective as radical scavenger. Because polyphenols are able to interact with proteins (*46*), these compounds could bind pepsin and change its three-dimensional conformation to make it more active. A decrease of pepsin autolysis by polyphenols, although possible, cannot explain our results in which initial velocity is measured, whereas it may contribute when longer times of incubation are considered. We have measured the pepsinogen content of the pepsin that we have used. Because the pepsinogen content is <1% of the pepsin activity, its possible conversion to pepsin by polyphenols may give only a negligible effect on the total pepsin activity.

This work, to our knowledge, is the first study that describes an effect of activation of polyphenols and related beverages on pepsin. We need more information about the interactions between polyphenols and pepsin and substrates, and further investigations and experiments will be indispensable to achieve better understanding of the mechanism of activation of pepsin by polyphenols and related beverages.

Whatever is the mechanism of activation of pepsin, our observation is relevant for protein digestion because it suggests a beneficial effect of beverages containing polyphenols on gastric digestion.

Tea is one of the most popular beverages in the world because of its attractive flavor and aroma, and it is rich in phenolic compounds. We have tested the effect of green tea on pepsin activity both in the digestion with insoluble azocasein and in the digestion conducted with meat. The results show that green tea is able to enhance the activity of pepsin in both digestion systems according to the results obtained with simple catechins.

Red wine is a popular beverage commonly consumed with meals, especially in combination with meals rich in meat. We have already demonstrated a significant positive relationship between the iron and protein contents of foods and the polyphenolic content in accompanying wines (*6*). Red wines, which are rich in polyphenols, are preferentially combined with foods that are rich in iron and proteins. This relationship between the iron content of foods and the polyphenolic content of wines is very favorable because polyphenols are very effective in binding iron and copper, therefore preventing peroxidative stress during digestion due to liberation of iron and copper from proteins during their hydrolysis. Also, the positive correlation between the protein content of foods and the polyphenolic content of wines is very favorable because the high content of proteins partially compensates for the antinutritional effect of the tannin component of red wine polyphenols due to the ability of these compounds to bind and precipitate proteins. In this work we demonstrated that red wine is able to enhance the activity of pepsin both with meat and with insoluble azocasein despite the presence of tannins. The activating effect on pepsin due to the phenolic components of red wine contributes to compensate not only the antinutritional properties of tannins toward proteins but also the inhibitory effect of ethanol on pepsin activity.

It is interesting to note that both wine and tea are two beverages very utilized during meals. Wine is also used in soaking and cooking several meat dishes. The activating effect on peptic digestion may be one reason for their popularity as beverages and in cooking.

LITERATURE CITED

(1) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr. Rev.* **1998**, *56* (*11*), 317−333.

(2) Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113−4117.

(3) Lopez, M.; Martinez, F.; Del Valle, C.; Orte, C.; Miro, M. Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography. *J. Chromatogr.* **2001**, *922*, 359−363.

(4) Shi, J.; Yu, J.; Pohorly, J. E.; Kakuda, Y. Polyphenolics in grape seeds—biochemistry and functionality. *J. Med. Food* **2003**, *6* (*4*), 291−299.

(5) Benassi, M. T.; Cecchi, H. M. Method development for the simultaneous determination of carboxylic acids, phenolic compounds and sorbic acid in white wine. *J. Liq. Chromatogr. Relat. Technol.* **1998**, *21*, 491−501.

(6) Ronca, G.; Palmieri, L.; Maltinti, S.; Tagliazucchi, D.; Conte, A. Relationship between iron and protein content of dishes and polyphenol content in accompanying wines. *Drugs Exp. Clin. Res.* **2003**, *29* (5/6), 271−286.

(7) Sanchez-Moreno, C.; Satuè-Gracia, M. T.; Frankel, E. N. Antioxidant activity of selected Spanish wines in corn oil emulsions. *J. Agric. Food Chem.* **2000**, *48*, 5581−5587.

(8) Frankel, E. N.; Waterhouse, A. L.; Teissedre, P. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of low-density lipoproteins. *J. Agric. Food Chem.* **1995**, *43*, 890−894.
(9) Prior, R. L.; Cao, G. Antioxidant capacity and polyphenolic components of teas: implications for altering in vivo antioxidant status. *Proc. Soc. Exp. Biol. Med.* **1999**, *220*, 255−261.
(10) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20* (*7*), 933−956.
(11) Ross, J. A.; Kasum, C. M. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **2002**, *22*, 19−34.
(12) Orgogozo, J. M.; Dartigues, J. F.; Lafont, S.; Letenneur, L.; Commenges, D.; Salamon, R.; Renaud, S.; Breteler, M. B. Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Rev. Neurol.* **1997**, *153* (*3*), 185−192.
(13) Conte, A.; Pellegrini, S.; Tagliazucchi, D. Synergistic protection of PC12 cells from β-amyloid toxicity by resveratrol and catechin. *Brain Res. Bull.* **2003**, *62*, 29−38.
(14) Subbaramaiah, K.; Chung, W. J.; Michaluart, P.; Telang, N.; Tanabe, T.; Inoue, H.; Jang, M.; Pezzuto, J. M.; Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J. Biol. Chem.* **1998**, *273*, 21875−21882.
(15) Kuroda, Y.; Hara, Y. Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutat. Res.* **1999**, *97*, 436−469.
(16) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghom, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 217−220.
(17) Dell'Agli, M.; Buscialà, A.; Bosisio, E. Vascular effects of wine polyphenols. *Cardiovasc. Res.* **2004**, *63* (*4*), 593−602.
(18) Visioli, F.; Borsani, L.; Galli, C. Diet and prevention of coronary disease: the potential role of phytochemicals. *Cardiovasc. Res.* **2000**, *47*, 419−425.
(19) Lapidot, T.; Harel, S.; Granit, R.; Kanner, J. Bioavailability of red wine anthocyanins as detected in human urine. *J. Agric. Food Chem.* **1998**, *46*, 4297−4302.
(20) Simon, C.; Barathieu, K.; Laguerre, M.; Schmitter, J. M.; Fouquet, E.; Pianet, I.; Dufourc, E. J. Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. *Biochemistry* **2003**, *42*, 10385−10395.
(21) Sarni-Manchado, P.; Cheynier, V.; Moutounet, M. Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* **1999**, *47*, 42−47.
(22) Kanner, J.; Lapidot, T. The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biol. Med.* **2001**, *31*, 1388−1395.
(23) Natella, F.; Belelli, F.; Gentili, V.; Ursini, F.; Scaccini, C. Grape seed proanthocyanidins prevent plasma postprandial oxidative stress in humans. *J. Agric. Food Chem.* **2002**, *50*, 7720−7725.
(24) Hodgson, J. M.; Puddey, I. B.; Croft, K. D.; Burke, V.; Mori, T. A.; Caccetta, R. A.; Beilin, L. J. Acute effects of ingestion of black and green tea on lipoprotein oxidation. *Am. J. Clin. Nutr.* **2000**, *71*, 1103−1107.
(25) Griffiths, D. W. The inhibition of digestive enzymes by polyphenolic compounds. *Adv. Exp. Med. Biol.* **1986**, *199*, 509−516.
(26) Rohn, S.; Rawel, H. M.; Kroll, J. Inhibitory effects of plant phenols on the activity of selected enzymes. *J. Agric. Food Chem.* **2002**, *50*, 3566−3571.
(27) Morel, I.; Lescoat, G.; Cogrel, P.; Sergent, O.; Pasdeloup, N.; Brissot, P.; Cillard, P.; Cillard, J. Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochem. Pharmacol.* **1993**, *45*, 13−19.
(28) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *45*, 1173−1178.
(29) Anson, M. L. The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. *J. Gen. Physiol.* **1938**, *22*, 79−89.
(30) Bradford, M. A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(31) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(32) Herriott, R. M. Swine pepsin and pepsinogen. *Methods Enzymol.* **1955**, *2*, 3−7.
(33) The United States Pharmacopoeia Inc., Rockville, MD, 2000.
(34) Siebert, K. J.; Lynn, P. Y. Comparison of polyphenol interactions with polyvinylpyrrolidone and haze-active protein. *J. Am. Soc. Brew. Chem.* **1998**, *56* (*1*), 24−31.
(35) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin−Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.
(36) Sapan, C. V.; Lundblad, R. L.; Price, N. C. Colorimetric protein assay techniques. *Biotechnol. Appl. Biochem.* **1999**, *29*, 99−108.
(37) Zang, K.; Yang, E. B.; Tang, W. Y.; Wong, K. P.; Mack, P. Inhibition of glutathione reductase by plant polyphenols. *Biochem. Pharmacol.* **1997**, *54*, 1047−1053.
(38) Lin, J. K.; Chen, P. C.; Ho, C. T.; Lin-Shiau, S. Y. Inhibition of xanthine oxidase and suppression of intracellular reactive oxygen species in HL-60 cells by theaflavin-3,3′-digallate, (−)-epigallocatechin-3-gallate, and propyl gallate. *J. Agric. Food Chem.* **2000**, *48*, 2736−2743.
(39) Wang, X.; Song, K. S.; Guo, Q. X.; Tian, W. X. The galloyl moiety of green tea catechins is the critical structural feature to inhibit fatty acid synthase. *Biochem. Pharmacol.* **2003**, *66*, 2039−2047.
(40) Fontecave, M.; Le Piovre, M.; Elleingand, E.; Gerez, C.; Guittet, O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett.* **1998**, *421*, 277−279.
(41) Grimm, T.; Schäfer, A.; Högger, P. Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (picnogenol). *Free Radical Biol. Med.* **2004**, *36* (*6*), 811−822.
(42) Belguendouz, L.; Fremont, L.; Linard, A. Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochem. Pharmacol.* **1997**, *53*, 1347−1355.
(43) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. A. Polyphenolic flavanols as scavengers of aqueous phase radical and as chain-breaking antioxidant. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.
(44) Tang, J. Competitive inhibition of pepsin by aliphatic alcohols. *J. Biol. Chem.* **1965**, *240*, 3810−3815.
(45) Fruton, J. S. A history of pepsin and related enzymes. *Q. Rev. Biol.* **2002**, *77* (*2*), 127−147.
(46) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenols/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.

Received for review March 23, 2005. Accepted August 2, 2005.

JF058074N