PHENOLIC ACIDS IMPROVE
THE ANTIOXIDANT ACTIVITY
OF CERULOPLASMIN ISOLATED
FROM PLASMA OF HEALTHY
VOLUNTEERS AND ATHEROSCLEROTIC
PATIENTS

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Abstract

The aim of the present study was to estimate the ability of individual phenolic acids to eliminate Fe(II) ions from the solution. Moreover, the influence of phenolic acids on the ferroxidase activity of ceruloplasmin (Cp) isolated from blood plasma of healthy volunteers (Cp C) and patients with atherosclerosis obliterans (Cp AO) was established in vitro. Phenolic acids demonstrated a ferroxidase-like activity, i.e. the ability to eliminate Fe(II) ions, within the studied concentration range of 2.0-17.0 mol 10⁻⁵ dm⁻³, in the following order of decreasing effectiveness: caffeic acid (CA) > ellagic acid (EA) > chlorogenic acid (ChA) ≈ ferulic acid (FA) = p-coumaric acid (PcA) = sinapic acid (SA). The study showed that the ability of phenolic acids to eliminate Fe(II)
ions by oxidation or chelation was related to the structure of the former, to the presence of orto OH groups, especially. Furthermore, the effect of the molar ratio of phenolic acid to Fe(II) ion was observed. EA and ChA, both containing two orto-OH groups and the highest number of –OH groups (4 and 5, respectively), demonstrated the greatest ability to eliminate Fe(II) ions, especially at the Fe(II) to phenolic acid molar ratio of 6:1. Phenolic acids added to samples with a constant amount of Cp caused decrease in the concentration of Fe(II) ions. Therefore, it may be assumed that the addition of phenolic acids to Cp, and Cp^apo, even in low concentrations, caused a significant decrease in the concentration of Fe(II) ions.

**Key words:** caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, sinapic acid, p-coumaric acid, atherosclerosis obliterans, ceruloplasmin, ferroxidase activity.

**INTRODUCTION**

A growing interest in the protective properties of fruits and their components can be ascribed to their potentially beneficial influence on human health. Fruits are known to demonstrate anti-proliferative activities and their consumption is associated with a reduced risk of cardiovascular disease and some types of cancer (Losso et al. 2004, Estaquio et al. 2008). Bioactive compounds of fruits, such as phenolic acids, flavonoids, stilbenes and vitamins (Han et al. 2007, Lugasi et al. 2011, Bobinaité et al. 2012) exhibit various health promoting mechanisms and activities, i.e. antioxidant activity, anti-proliferative activity, free radical scavenging capacity, neuroprotective capacity (Amarowicz et al. 2007, Bowen et al. 2010, Fortalezas et al. 2010).

Polyphenols, a class of compounds present in fruits, are known to be good chelators and oxidants of Fe(II) ions, and may inhibit metal-dependent processes generating reactive oxygen species (ROS). Iron ions, Fe(II) particularly, exhibit the ability to generate free radicals (O_2^- or ‘OH), which are capable of oxidizing of biological molecules because of their high redox potential. In the Fenton reaction, Fe(II) is oxidized by hydrogen peroxide to Fe(III) and OH is produced. It has been demonstrated that Cu(I) and other transition metals can also react with hydrogen peroxide and generate ROS. The efficiency of this reaction increases in pathological conditions, often accompanied by an increased concentration of unbound Fe(II) ions. Oxidation of Fe(II) to Fe(III) ions followed by subsequent binding by transferrin and ferritin is catalyzed by ceruloplasmin (Cp), the most important plasma antioxidant.

The ferroxidase activity of Cp seems to be enhanced by polyphenols, yet the interaction between polyphenolic compounds and Cp or any other endogenous antioxidants remains understudied (Budzyn et al. 2009). In our previous study, a ferroxidase-like activity was demonstrated by raspberry seed extract (RSE) (Gryszczyńska et al. 2009). An addition of RSE to Cp, isolated from serum of healthy subjects, caused a dose-dependent decrease of Fe(II) concentration. This was due to polyphenolic compounds present in RSE, which eliminated Fe(II) ions by oxidation or chelation. The analysis of
the profile of phenolic compounds in extracts from different raspberry cultivars demonstrated that gallic acid (GalA), ChA, EA, 4-hydroxybenzoic acid (4-HbA), CA, PcA, FA, and gentisic acid (GenA) played the most significant antioxidant role among the phenolic acids (Moyer et al. 2002, Zhang et al. 2010).

Therefore, ChA, CA, EA, FA, PcA, and SA, as the most prevalent and effective components of plants and plant products, were selected for this study.

A particularly intensified production of free radicals and ROS occurs as a result of upsetting the balance between antioxidative and prooxidative processes. This imbalance, accompanied by lower activity of antioxidant enzymes and reduced concentration of low-molecular antioxidants, plays a significant role in the pathogenesis of many diseases. Oxidative stress is one of the risk factors of many pathological conditions, including atherosclerosis obliterans (AO) (Majewski et al. 2007). The occlusion of the lower limbs due to atherosclerosis may lead to moderate or critical ischaemia of the lower limbs. Reperfusion injuries and ischaemic changes in tissues entail the formation of various reactive oxygen species (ROS). The latter are resistant to elimination, due to insufficient adaptive mechanisms of the organism, and are produced when the body’s antioxidant status is significantly deteriorated (Harris 1992).

The analysis of plasma from patients with and without atherosclerosis, carried out in the present study, is expected to clarify to what extent phenolic compounds support enzymes or low-molecular antioxidants in their fight against free radicals and ROS, in a situation when the disease causes an excessive use or an insufficient production of endogenous antioxidants.

The purpose of the study was to determine individual abilities of these compounds to eliminate free Fe(II) ions, and to assess their effect on the ferroxidase activity of Cp isolated from blood plasma of healthy volunteers (CpC) and patients with atherosclerosis obliterans (CpAO) in vitro.

**MATERIAL AND METHODS**

**Chemicals**

(NH₄)₂Fe(SO₄)₂ · 6H₂O (Mohr’s salt), (NH₄)₂SO₄, NaCl, KH₂PO₄, K₂HPO₄, CH₃COOH, CH₃COONa, chloroform and ethanol were purchased from POCh Spółka Akcyjna (Gliwice, Poland). 3-(2-Pyridyl)-5,6-diphenyl–1,2,4-triazine-4’4’”-disulfonic acid sodium salt (ferrozine), histidine, DEAE-Sephadex A-25 chloride form, CA, EA, ChA, PcA, SA, FA, apotransferrin, acetic acid, sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents and solvents were of analytical grade of purity.
Preparation of phenolic acid solutions

The basic solutions of CA, ChA, FA, PcA were prepared in high purity water. The basic solution of SA was prepared in methanol: H$_2$O (1:4,v:v) and EA was dissolved in methanol.

Isolation and purification of ceruloplasmin from human plasma samples

For the purpose of the study, Cp was isolated from blood plasma samples of healthy volunteers and patients with AO (the volume of 0.1 dm$^{-3}$ combined from 20 subjects in each studied group). Prior approval for the study had been given by the Ethics Committee of Poznan University of Medical Sciences (decision No 614/12). Cp$_C$ and Cp$_{AO}$ were isolated and purified according to the procedure detailed previously (Gryszczyńska et al. 2009). The purification procedure yielded an essentially pure preparation of Cp$_C$ and Cp$_{AO}$, and the absorbance $A_{610}/A_{280}$ ratio reached the value of 0.036 and 0.031, respectively. Cp$_C$ and Cp$_{AO}$ solutions were prepared in PBS buffer (0.05 mol dm$^{-3}$, pH 7.38). PBS was chosen because the phosphate buffer represents one of the main components of the physiological buffering system.

The ferroxidase activity Cp$_C$ and Cp$_{AO}$

The ferroxidase activity of Cp was measured spectrophotometrically, using the Fe(II)-histidine complex and ferrozine [disodium salt of 3-(2-pyridyl)-5,6-bis (4-phenylosulfonic acid)-1,2,4-triazine] as a chromogenic reagent. Histidine, a possible chelator of Fe(II) in vivo, was chosen to bind Fe(II) in a stable and low-molecular complex in order to avoid iron autooxidation in vitro. In the presence of Cp, Fe(II) ions are oxidized to Fe(III) and the remaining amount of Fe(II) forms a more stable complex with ferrozine, yielding a product measured spectrophotometrically (Juan, Aust 1998). The ferroxidase activity of Cp$_C$ and Cp$_{AO}$ in the concentration range of 22-66 mg dm$^{-3}$ was measured according to the procedure described by Gryszczyńska et al. (2009) and expressed as a change in Fe(II) concentration in a sample.

The effect of phenolic acids on the ferroxidase activity of Cp$_C$ and Cp$_{AO}$

The effect of phenolic acids on the ferroxidase activity of Cp$_C$ and Cp$_{AO}$ was measured in a mixture of Cp$_{AO}$ or Cp$_C$ of a given concentration (22; 33; 44; 66 mg dm$^{-3}$) and variable concentrations of phenolic acids (2.0-17.0 mol 10$^{-5}$ dm$^{-3}$). Appropriate volumes of Cp and phenolic acid solution were added to test tubes and handled according to the procedure described by Gryszczyńska et al. (2009).

Determination of the ability of phenolic acids to oxidize Fe(II) ions

The ability of phenolic acids to oxidize Fe(II) ions was measured according to the procedure described by Johanson et al. (1967). This micromethod
was used for the determination of the serum concentration of ceruloplasmin and based on the oxidation reaction of Fe (II) to Fe (III) by Cp under defined conditions. The absorbance of orange complex of Fe(III):transferrin was measured spectrophotometrically at $\lambda=460$ nm. This method was applied in order to test the capacity of phenolic compounds to oxidize Fe(II) ions. The concentration of Fe(III):transferrin was calculated from the formula:

$$\text{Fe(III):transferrin} = \frac{[(A - A_0) - (C - C_0)]}{\epsilon \cdot t \cdot m} \quad (\mu\text{M})$$

where:
- $A_0$ – absorbance value at 0 minute,
- $A$ – absorbance value after 1 minute of reaction,
- $C_0$ – absorbance value of blank sample at 0 minute,
- $C$ – absorbance value of blank sample after 1 minute of reaction,
- $\epsilon$ – micromolar absorption coefficient, $\epsilon=0.0025 \ (\text{dm}^3 \ \mu\text{mol}^{-1} \text{cm}^{-1}),$
- $t$ – reaction time in minutes,
- $m$ – concentration of phenolic acid (µmol dm$^{-3}$).

The study of the ability of phenolic compounds to oxidize Fe(II) ions was carried out for two molar proportions of Fe(II) ions to phenolic acids: 6:1 (A) and 1:1 (B). The solutions were prepared by combining 0.00017 dm$^{-3}$ of acetate buffer (1.2 M; pH 6.0), 0.000250 dm$^{-3}$ apotransferrin solution (2.0%), and an appropriate volume of phenolic acid solution, subsequently replenished with water to obtain 0.0007 dm$^{-3}$ of mixture in a cuvette. The cuvette was placed in a spectrophotometer for 3 minutes at 30ºC. Then, 0.0003 dm$^{-3}$ of Fe(II) ion solution (Mohr’s salt, 0.22 mmol dm$^{-3}$) was added to the cuvette and the initial absorbance was measured immediately ($A_0$). The measurement was repeated after 1 minute ($A$). At the same time, a blank was prepared by combining 0.00017 dm$^{-3}$ of buffer solution, 0.00025 dm$^{-3}$ of apotransferrin solution and 0.00028 dm$^{-3}$ of water. The initial absorbance ($C_0$) was measured following the addition of Fe(II) ion solution to the blank and then the measurement was repeated after 1 minute ($C$).

**Statistical analysis**

The results were expressed as means ± standard deviation. Statistical differences were estimated by using Student’s $t$-test. The significance level was accepted at $p < 0.01$.

**RESULTS AND DISCUSSION**

The study showed the cooperation of Cp with phenolic acids in Fe(II) ions elimination from solution and the effect of chronic arterial occlusion of the lower limbs due to atherosclerosis on the ferroxidase activity of Cp. The
ferroxidase activity of CpC (Figure 1a), expressed as ΔFe(II), was lower than that of CpAO in the dose of 66 mg dm$^{-3}$ (Figure 1b) – $p < 0.001$.

The abilities of individual phenolic acids to eliminate Fe(II) and their effect on the ferroxidase activity of both CpC and CpAO was analyzed. CA, ChA and EA were shown to be more capable of eliminating Fe(II) ions (at 17.0 mol 10$^{-5}$ dm$^{-3}$) when compared to SA, PcA and FA. The addition of individual phenolic acids to the solution with a constant amount of CpC or CpAO caused a significant decrease in Fe(II), shown as ΔFe(II) in Figure 1a and Figure 1b, respectively. The effect of phenolic acids on the ferroxidase activity of CpC or CpAO was dose-dependent for each of Cp concentration. However, the addition of higher concentrations (6.0 mol 10$^{-5}$ dm$^{-3}$ and more) of phenolic acids, especially CA, EA and ChA, to the samples with 66 mg dm$^{-3}$ of CpC or CpAO caused less efficient Fe(II) elimination in both groups.

Fig. 1. The effect of phenolic acids (17 mol 10$^{-5}$ dm$^{-3}$) on the ferroxidase activity of CpC (a) or CpAO (b) (66 mg dm$^{-3}$) (* $p < 0.001$ – significant differences vs samples with constant amount of CpC or CpAO without phenolic acids); CA – caffeic acid, EA – ellagic acid, ChA – chlorogenic acid, SA – sinapic acid, PcA – $p$-coumaric acid, FA – ferulic acid
The highest concentrations of Fe(II) eliminated by phenolic acids with and without the presence of $C_{PC}$ or $C_{PAO}$ in a model solution were evaluated. For this purpose, the parameters of the regression curves of the reciprocal of concentration of the selected phenolic acids and the reciprocal of $\Delta Fe(II)$ were calculated (Table 1). The maximum decreases in Fe(II) concentration obtained for CA, EA, ChA, PcA, SA and FA were 64.10; 48.80; 7.44; 0.40; 0.40; 0.40 $\mu$mol dm$^{-3}$, respectively. On the other hand, in the presence of $C_{PC}$ or $C_{PAO}$, the calculated $\Delta Fe(II)_{\text{max}}$ values were lower for CA, EA, ChA and higher for SA, PcA, and FA.

In the next step, the ability of individual phenolic acids to oxidize Fe(II) and the formation of Fe(III)-transferrin complex were estimated. It was shown (Figure 2.) that EA and ChA demonstrated the greatest ability to oxidize Fe(II) ions and thus binding Fe(III) with transferrin. The lower capacity was calculated for CA and the lowest ability to oxidize Fe(II) ions was shown by SA, PcA and FA. Moreover, the ability to eliminate Fe(II) ions was related to the Fe(II)/
phenolic acid molar ratio. The analysis of data presented in Figure 2 shows that the 6:1 molar ratio is much more favourable than the 1:1 one.

The chosen phenolic acids differ in terms of their chemical structure, i.e. the number of OH groups in a molecule, mutual arrangement thereof, and the presence of aromatic rings. Therefore, some differences in the reactivity of phenolic acids with Fe(II) ions were expected to occur.

Fig. 2. Formation of a Fe(III):transferrin complex due to the oxidation of Fe(II) ions in the presence of phenolic acids and at the Fe(II)/phenolic acid molar ratio of 6:1(a) and 1:1(b). (*p < 0.001 significant difference vs. figure a for ellagic acid; ** P<0.01 significant difference vs. figure a for chlorogenic acid)
The results demonstrated that all phenolic acids were able to eliminate unbounded Fe(II) ions by oxidation or chelation, but in different manner. The addition of CA, EA and ChA, even in low concentrations (2.0; 4.0; 6.0 mol 10⁻⁵ dm⁻³), to solution containing Cp caused a significant increase in ΔFe(II). When the concentration of phenolic acids increased, along with the concentration of Cp or Cp₄o, the competition for the substrate, i.e. free Fe(II) ions, was shown to have intensified. The oxidative activity of Cp₄o was higher when compared to Cp. The study also revealed that values of ΔFe(II)max obtained for samples containing only CA, EA, or ChA were higher than for samples in which either Cp₄o or Cp were present. Although these considerations confirm the competition of CA, EA, ChA and Cp for Fe(II) ions, it is very likely that the addition of the phenolic acids at higher concentrations might even replace Cp as an antioxidant. This effect may be particularly desirable in chronic disease, which probably entails a greater exploitation of antioxidants and necessitates a higher activity of antioxidant enzymes. The next chosen phenolic acids, SA, PcA, and FA, showed significantly weaker ability to eliminate free Fe(II) ions, which confirms that Cp plays a predominant role in decreasing the concentration of Fe(II) ions in the experimental model. The presence of one OH group only, and thus the lack of an ortho-OH group arrangement, seems to exert an influence on the ability of these acids to chelate or oxidize Fe(II) ions.

The elimination of Fe(II) ions by chelation with phenolic acids was confirmed by using ferrozine. The presence of the phenolic acid perturbs the Fe(II)-ferrozine complex formation and indicates their higher chelating activity (Güleİ 2006). Various studies have demonstrated that compounds containing two or more –OH, -SH, -COOH, -C=O, -S- groups show a metal chelating ability (YuAN et al. 2005, Andjelković et al. 2006). However, oxidation of Fe(II) is an important mechanism in the elimination of this ion. Phenolic acids increase the rate of Fe(II) oxidation to Fe(III) and form complexes with Fe(II), thus preventing the Fenton reaction, which was suggested by Lopes and co-workers (Lopes et al. 1999). In this study, oxidation of Fe(II) ions by phenolic acids was proven by measuring the concentration of Fe(III):transferrin complex. Higher concentrations of this complex were observed in the presence of EA, ChA and CA than of SA, PcA and FA. The results of other studies indicate that phenolic acids containing gallolyl moieties i.e. 3 adjacent OH groups, favour the autooxidation of Fe(II) ions. This moiety is present both in CA and ChA, thus the highest concentration of Fe(III):transferrin complex in their presence was found. EA, which is a dimer of GalA, demonstrates the highest efficiency to oxidize of Fe(II) ions, as revealed by Chvátalová (Chvátalová et al. 2008). The presence of two ortho-OH groups in the structure of EA may favour the oxidation of Fe(II) ions. It seems probable that acids containing one OH group only (3-hydroxybenzoic acid, 4-HbA and 4-hydroxy-3-methoxybenzoic acid) decrease the oxidation of Fe(II) ions with an efficiency similar to that of ascorbic acid. This observation is important for SA, PcA and FA, derivatives of cinnamic acid, containing only
one OH and or one methoxyl group. Furthermore, our results indicate that the ability of phenolic acids to oxidize Fe(II) ions depends on their molar ratio, too. The oxidation of Fe (II) is favoured when the Fe(II) to phenolic acid ratio is higher (i.e. equals 6:1), which leads to the more efficient binding of Fe(III) by apotransferrin. Phenolic acids favour the oxidation of Fe(II) when their concentration is much lower than the initial concentration of Fe(II) ions. It was shown that CA and protocatechuic acids are the best oxidants if their concentration constitutes only 0.4% of the initial concentration of Fe(II) (Chvátalová et al. 2008).

CONCLUSIONS

The ability of phenolic acids to chelate or oxidize Fe(II) ions, followed by Fe(III) ions binding to apotransferrin and ferritin, may represent a beneficial mechanism that could assist in the elimination of unbound Fe(II) ions by ceruloplasmin in the human body. Phenolic acids added to solution of Cp_m and Cp_c at variable concentrations increase the elimination of Fe(II) ions and demonstrate a ferroxidase-like activity in the order of decreasing effectiveness: CA>EA>ChA>FA =PcA=SA.

It was shown that the ability of phenolic acids to eliminate Fe(II) ions by oxidation or chelation was associated with their structures, the presence of orto-OH groups in particular. Furthermore, the influence of phenolic acid/Fe(II) ion ratio on the oxidative ability of phenolic acids was observed. EA and ChA, containing two orto-OH groups and the highest number of –OH groups (4 and 5, respectively), show the highest ability to eliminate Fe(II) ions, especially at the Fe(II)/phenolic acid molar ratio of 6:1.

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