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Raspberry seed extract effect on the ferroxidase activity of ceruloplasmin isolated from plasma

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ABSTRACT

In this study, the effect of the raspberry seed extract (RSE) on the ferroxidase activity of ceruloplasmin (Cp) isolated from healthy male subject serum was analyzed. The ferroxidase activity of Cp was determined by spectrophotometry using Fe(II) – histidine complex and ferrozine as a chromogenic reagent. The ferroxidase activity of ceruloplasmin was demonstrated in dose-dependent way within the range 22–66 μg/ml. The effect of RSE on Fe(II) concentration, measured as the decrease of Fe(II) concentration in samples and expressed as ΔFe(II), was found to be a dose-dependent within the range 1.20–51.56 μg dm/ml. The ferroxidase activity of Cp was influenced by the RSE within its studied range. However, the addition of the highest concentration of RSE (51.56 μg dm/ml) to the sample containing the highest level of Cp (66 μg/ml) did not affect its ferroxidase activity. It may be suggested that the competition for Fe(II) as the substrate limits the effect of RSE on Cp activity and causes no further changes in Fe(II) elimination.

1. Introduction

Ceruloplasmin (Cp), the main copper-binding plasma protein, plays the principal role in the oxidation and reduction processes in blood. The ability to oxidize Fe(II) to Fe(III) with reduction of molecular oxygen to water prevents releasing of partially reduced species of oxygen, i.e. superoxide anion, hydrogen peroxide, hydroxyl radical. This ferroxidase activity of Cp results in the uptake of Fe(III) by transferrin and the inhibition of the oxidation of various biomolecules, mainly lipids and proteins. The oxidase activity of Cp in plasma, measured with diamines as substrates, was found to be elevated during infection and inflammation (Louro, Cocho, Mera, & Tutor, 2000), in critical lower limb ischemia (Iskra & Majewski, 1999), colorectoral cancer (Zowczak et al., 2001), and suggested to be a biomarker of the acute phase reaction(see Table 1).

Cp and the other endogenic (albumin, bilirubin, uric acid, enzymes: superoxide dismutase and glutathione peroxidase) or exogenic (vitamins (A, E, C, β-carotene) biological substances demonstrate antioxidant activity. The same role is performed by some components of dietary beverages and products, such as red wine, green tea, fruit and vegetables, rich in polyphenolic compounds. There is still increasing interest in health benefits of polyphenol-rich food. Many of them may reduce the risk of coronary heart disease, cancer, allergy and inflammation (Brown, 1999; Visioli, Borsani, & Galli, 2000). Results of many studies have suggested an association between consumption of polyphenol-rich food and beverages and their profilactive role, and connected their beneficial effects with the antioxidant activity, namely their ability to scavenge hydroxyl radicals (Hanasaki, Ogawa, & Fukui, 1994) and superoxide anions (Magnani, Gaydou, & Hubaud, 2000).

Polyphenols represent the chemical structure with the hydroxy groups of the catechol-like moiety that donate hydrogen and is oxidized itself to semiquinone radical. Thus, the dihydroxy group is required for the participation of a polyphenol in the redox system and its antioxidant activity. On the other side, the semiquinone radical may accept hydrogen and participate in the mechanism of prooxidation, acting as an oxidant agent. The dual behavior of the polyphenolic compounds is still less explored than their antioxidant function. The prooxidant activity appears to be responsible for the harmful biological effects of polyphenols. In the presence of some metal ions polyphenols may play an important role in the mutagenesis or promotion of cancer (Sahu & Gray, 1996), and the oxidative damage of DNA and lipids *in vitro* (Yamashita, Tanemura, & Kawanishi, 1999).

The antioxidant or the prooxidant activity of phenolic compounds depends on many factors such as pH of the solution, their chelating properties and bioavailability. The presence of compounds of both reducing and oxidative properties in blood and other biological fluids may affect their antioxidant/prooxidant activity while competing for the substrate, i.e. free radicals, reactive oxygen species and transition metal ions. The nature of their contacts may depend on some chemical factors such as concentration, polarity and the oxidation state of antioxidants, or

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physiological factors such as their localization, the activity of, the rate of pathological changes.

Several reports have indicated interaction between antioxidants endogenously produced and derived from the diet (Chan, Chow, & Chiu, 1999; Machlin & Bendich, 1987). Gutteridge demonstrated that the ferroxidase activity of Cp was inhibited when the molar ratio of ascorbate to Cp was greater than 200 (Gutteridge, 1991). The inhibitory effect of ascorbate was observed at pH 6.0, but not at its physiological value (7.4) (Lovstad, 1997). The interaction between phenolic compounds of biological significance and ceruloplasmin in plasma or other antioxidants of blood plasma are poorly studied. It may be expected that the supplementation of humans with the exogenic antioxidants might affect the activity of the redox system and the total antioxidant status of a body. The competition for the limited amount of substrates might also take place and change the antioxidant barrier of a body.

Polyphenol-rich berries were shown to be effective antioxidants, and the extracts of raspberries, blackberries, blueberries, cranberries, and elderberries contain phenolic acids and flavonoids (Bagchi, Sen, Bagchi, & Atalay, 2004; Han, Shen, & Lou, 2007). Phenolic acids derived from hydroxybenzoic acid and hydroxycinnamic acid account for about one third of the total intake of dietary polyphenols (Han et al., 2007). The dietary sources of phenolic acids are fruit juices, and fruit or seed extracts where their concentration depends on a fruit cultivar and even the type of fruit tissue (localization). For example, ellagic acid was higher in the extract of raspberry seeds than of pulps (Juranic et al., 2005).

It is well known that polyphenols can act as antioxidants by radical scavenging, transient metal ion chelation, and inhibition of xanthine oxidase activity. Furthermore, curcumin, quercetin, and resveratrol increase the concentration of glutathione and the activities of antioxidant enzymes: glutathione peroxidase, superoxide dismutase, catalase *in vivo* and *in vitro* (Han et al., 2007). Some authors recognize that chelation is the main mechanism of polyphenols action (Rice-Evans, Miller, & Paganga, 1996) rather than free radical scavenging (Ferrali et al., 1997; Lopes, Schulman, & Hermes-Lima, 1999).

In plasma, Fe(II) ions are oxidized to Fe(III) by Cp, and chelated or oxidized by polyphenols, thus the possible relation and competition of Cp and polyphenols in the elimination of Fe(II) needs to be studied. The aim of the present study was to investigate the effect of complex mixture of polyphenolic compounds on the antioxidant activity of Cp. Therefore, the effect of raspberry seed extract on the ferroxidase activity of Cp isolated from a serum sample of a healthy subject was investigated.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

$(NH_4)_2$ $Fe(SO_4)_2$ $6H_2O$ (Mohr's salt), $(NH_4)_2$ SO_4, NaCl, KH_2PO_4, K_2HPO_4, CH_3COOH, CH_3COONa, chloroform, 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, hexane, Folin & Ciocalteu's phenol reagent, DEAE-Sephadex A-25 chloride form, caffeic acid, ellagic acid, quercetine were purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents and solvents were of analytical grade of purity.

2.1.2. Preparation of raspberry seed extract

Raspberry seeds, obtained as the waste from the food industry (Zakłady Przemysłu Owocowo-Warzywnego Kotlin Sp. z o.o.), were first dried, comminuted, defatted with hexane and finally extracted by using 80% aqueous ethanol. The solvent was evaporated at 45 °C and dry residue was dissolved in 96% ethanol. The ethanol solution of raspberry seed extract contained 17.8 mg of dry mass/ml. The total content of phenolic compounds determined by using the Folin–Ciocalteu method (Singleton & Rossi, 1965), and expressed in mg of caffeic acid was 2370.5 mg per 100 g of dry matter of seed.

For further studies, the raspberry seed extract was prepared as a solution diluted in PBS (0.05 M, pH 7.38) in a ratio 1:100 (v/v).

2.1.3. Isolation and purification of ceruloplasmin from human serum samples

Ceruloplasmin was isolated from healthy male subject serum according to the procedure based on the protein precipitation with ammonium sulfate followed by adsorption and elution from DEAE-Sephadex A-50 (Hilewicz-Grabska, Zgirski, Krajewski, & Płonka, 1988). The proteolytic degradation of Cp was inhibited by the addition of aprotinin and 6-aminocaproic acid (EACA) to serum. Aprotinin, the inhibitor of several serine proteases, and 6-aminocaproic acid (plasminogen inhibitor, binds and inactivates carboxypeptidase B) prevent Cp degradation, and as a result the ferroxidase activity of isolated Cp was stable within the period of the study. The structural integrity of Cp and the presence of copper ions are required for the enzyme to effectively catalyze iron loading into transferrin and ferritin (Van Eden & Aust, 2000). Thus, metal-chelating agent as EDTA was not used since it. An efficient metal-chelating agent may cause the loss of copper ions, and the irreversible decrease in Cp activity (Sokolov, Zacharova, Shavlovskii, & Vasil'ev, 2005). Methylparaben (a bacteriostatic agent and preservative) and gentamicin (a bactericidal antibiotic) were added to preserve and conserve the sample.

In brief, DEAE-Sephadex A-25 chromatography, eluted with 0.2 M acetate buffer (pH 5.5 contained 0.125 M NaCl) were applied sequentially. To the pooled fractions from the previous step ammonium sulfate was added to remove globulins and precipitated with ammonium sulphate to reach 58% saturation. The sediment was dissolved in H_2O, precipitated with ethanol–chloroform (9:1, v/v). The final product was dialysed overnight against 0.05 M phosphate buffer, pH 6.82. Purified Cp sample was conserved by chloroform. The purification procedure yielded essentially pure preparation of Cp, and the absorbance ratio A_{610}/A_{280} ratio reached the value of 0.044. Although Sokolov, Zacharova, Shavlovskii, and Vasil'ev (2005) obtained higher value of the ratio (0.050 and 0.052) for Cp preparation isolated on PR-Sepharose column (PR – protamine, i.e. salmon protamine used to form a complex Cp–PR) but for Cp isolated from large volumes of blood plasma (higher than 200 ml). In the present study smaller volume of plasma was used (100 ml). The purity of Cp was expressed as the ratio of protein concentration measured at 610 nm and 280 nm, and calculated as 95.5%. Cp concentration in a phosphate buffer (0.05 M, pH 7.38) with 0.15 M NaCl was 1.17 mg/ml. The choice of the environment for studying the interaction between Cp and RSE was one of the basic questions in this study. The PBS was chosen because the phosphate buffer represents one of the main components of physiological buffering system. The acetate buffer (Sokolov et al., 2005) or Hepes (Welch, Davis, & Aust, 2002) may be more convenient for the measurement of the ferroxidase activity of Cp isolated from serum of different species and used for studies performed in at lower pH value (5.5) than physiological range (slightly above 7.0). Moreover, the mixture of Cp and RSE solutions contain 50 mM of NaCl added to reach equal volume of each sample.

2.2. Methods

2.2.1. The ferroxidase activity

The measurement of the ferroxidase activity is based on the reaction between Fe(II)-histidine complex and ferrozine [disodium