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Analytical Methods

Laccase-generated tetramethoxy azobismethylene quinone (TMAMQ) as a tool for antioxidant activity measurement

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ABSTRACT

The potential of laccase-generated tetramethoxy azobismethylene quinone (TMAMQ) for measuring antioxidant activity of a wide range of structurally diverse molecules present in food and humans was investigated for the first time. All the tested antioxidants including simple phenolics, polyphenols and vitamins quenched TMAMQ. The antioxidant activity of phenolics and polyphenolics depended on the position and number of hydroxyl groups on the benzene ring. Equally interesting was the ability of amino acids like cysteine, tryptophan and methionine as well as peptides (glutathione) and proteins (albumin) to quench TMAMQ, demonstrating the great potential of TMAMQ for analysis of antioxidant activity of serum samples. Further, TMAMQ is promising is a more reliable tool for measuring antioxidant activity of amino acids when considering conflicting reports on antioxidant activity of some of the amino acids. The extracts from various food samples showed varying antioxidant activity with highest for spinach (4.36 mg methanol extract/mmol TMAMQ) followed by kiwi (13.95 mg methanol extract/mmol TMAMQ) and lettuce (40 mg methanol extract/mmol TMAMQ). The use of the laccase generated TMAMQ can be exploited for the development of laccase based biosensors for complex and coloured samples thereby facilitating online monitoring of antioxidants in food, cosmetic and health industries.

1. Introduction

The human body is exposed to a large variety of reactive species (free radicals) from both endogenous and exogenous sources. Endogenous free radical species (superoxide, nitric oxide and hydrogen peroxide) are products of normal cellular function. These cellular functions include mitochondrial respiration (Serrano, Goni, & Saura-Calixto, 2007), activated phagocytes, arachidonic acid metabolism, ovulation and fertilization (Magalhaes, Segundo, Reis, & Lima, 2008; Singh, Sharad, & Kapur, 2004). Exogenous sources of free radicals include pollutants such as car exhaust, industrial contaminants encompassing many types of nitrogen reactive species, drugs and xenobiotics (toxins, pesticides, herbicides etc.) (Kohen & Nyska, 2002; Valko et al., 2007). Cell damage caused by free radicals has been implicated in the pathogenesis of at least 50 diseases conditions (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Halliwell, 1994). Similarly, in food, for example the oxidation of lipids by free radicals has historically been a major problem for food processing industries responsible for the formation of off-flavours and undesirable chemical compounds which may be detrimental to health (Jadhav, Nimbalkar, Kulkarni, & Madhavi, 1996).

To protect the cells and organs against free radicals, biological systems have evolved a highly sophisticated and complex antioxidant protection system. These antioxidants therefore constitute the body's first line of defence against free radical damage. The antioxidants include biologically built-in mechanism of neutralizing free radicals for example glutathione peroxidase, catalase, and superoxide dismutases, glutathione and albumin (Singh et al., 2004; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). The exogenous sources of antioxidants are mainly of dietary origin including vitamin C, tocopherols, carotenoids, flavonoids (Singh et al., 2004; Valko et al., 2006). Endogenous and exogenous antioxidants function interactively and synergistically to neutralize free radicals. When the availability of antioxidants is limited, cell damage and food oxidation occurs. Strangely, despite the well recognised importance of antioxidants for human health and food preservation, currently there is no nutritional standard index available related to antioxidants for food labelling because of the lack of standardised methods (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). However, recently determining antioxidant capacity has become a very active research topic as recently demonstrated by international efforts to standardise assay methods (Prior, Wu, & Schaich, 2005).

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Recently, we have discovered that laccase oxidised syringaldazine was a reliable tool for measuring antioxidant activity of vitamin C and vitamin E (Nugroho Prasetyo et al., 2009). The performance of laccase oxidised syringaldazine was comparable to the commercially available 2,2-diphenyl-l-picrylhydrazyl (DPPH) radical.

Syringaldazine is a yellowish compound which is converted to tetramethoxy azobismethylene quinone (TMAMQ) with maximum absorbance at 530 nm (deep purple colour) upon oxidation by laccases (Harkin, Larsen, & Obstharkin, 1974; Holm, Nielsen, & Eriksen, 1998). It is a well known laccase substrate which is also used to detect peroxidase activities. The reaction of laccase with syringaldazine first generates a free radical and loss of the second electron can either proceed enzymatically or by disproportionation forming a deep purple coloured quinone (TMAMQ – Fig. 1) which is not prone to polymerisation under appropriate conditions (Kuznetsova & Romakh, 1996; Thurston, 1994). Further, electrochemical and pulse radiolysis studies of the oxidation of syringaldazine confirmed a reversible two-electron-two-proton transfer leading to the formation of a deep purple compound (Hapiot, Pinson, Neta, & Rolando, 1993).

Syringaldazine can also be oxidised by chlorine and is currently used for the colorimetric determination of chlorine in water (Bauer & Rupe, 1971; Cooper, Roscher, & Slifker, 1982). This work, is the first to explore the ability of laccase generated TMAMQ to measure the antioxidant activity of a wide variety of structurally different antioxidants relevant to both the food industry and human health. The study is also extended to investigate the ability of laccase generated TMAMQ to measure antioxidant activity of crude extracts of known important dietary sources. This knowledge will allow the development of laccase based biosensors for complex thereby facilitating online monitoring of antioxidants in food, cosmetic and health industries.

2. Materials and methods

2.1. Chemicals and enzyme

All the used antioxidants molecules were of analytical grade. The phenolics were purchased from Sigma–Aldrich, Steinheim, Germany while the flavonoids were purchased from Carl Roth GmbH, Karlsruhe Germany. All the other chemicals were purchased from Merck, Darmstadt, Germany. The *Trametes hirsuta* laccase was produced and purified as previously described by Almansa, Kandelbauer, Pereira, Cavaco, and Guebitz (2004). Food samples of onion (*Allium cepa*), green and fermented tea (*Camellia sinensis*), *kiwi* (*Actinidia arguta*), apple (*Malus domestica*), carrot (*Daucus carota subsp. sativus*), roasted coffee (*Coffea robusta*), lettuce (*Lactuca sativa*), spinach (*Spinacea oleracea*), pumpkin seed (*Curcubita maxima*), tomato (*Solanum lycopersicum*) and garlic (*Allium sativum*) were purchased from local markets in Graz, Austria.

2.2. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (ε_{436} = 29,300 M^{-1} cm^{-1}) as a substrate at 436 nm in 50 mM sodium succinate buffer at pH 4.5 and 30 °C (Nugroho Prasetyo et al., 2009). The spectrophotometric measurements were done by recording the absorbance in the time scan mode for 2 min using a Hitachi U-2001 UV–vis spectrophotometer.

2.3. Generation of TMAMQ

TMAMQ stock solutions were prepared by incubating syringaldazine (0.17 mM) with 50 μl of laccase (20 nkat ml^{-1}) in 50 mM sodium succinate buffer at pH 4.5. The reaction mixture (1 ml) was incubated at 30 °C for 10 min while shaking at 140 rpm in a thermomixer (Eppendorf AG, Germany). The oxidation process was monitored at 530 nm using a Hitachi U-2001 UV–vis spectrophotometer in disposable cuvettes of 1 cm pathway. To this stock solution of laccase generated TMAMQ, methanol was added to a final concentration of 80% (v/v) to stop laccase activity and to stabilize the TMAMQ (Nugroho Prasetyo et al., 2009).

2.4. Determination of antioxidant activity of pure molecules

Different concentrations of pure antioxidant molecules dissolved in methanol (0–20 μM) were added to 800 μl of TMAMQ dissolved in 80% methanol (final absorbance of 0.8) in order to obtain a dose response curve. The mixture was thoroughly mixed and incubated at 30 °C while shaking in a thermomixer (Eppendorf AG, Germany) at 140 rpm until full completion of the reaction as evidenced by no further decrease in absorbance. The degree of decoloration of the solution indicates the scavenging efficiency of the added antioxidant sample. The stoichiometrical reduction of TMAMQ (μM of TMAMQ reduced by 1 μM antioxidant) was then calculated from a dose response curve of added antioxidant.

2.5. Preparation of food samples and extraction of antioxidants

2.5.1. Recovery of simple phenolic compounds

Food samples were purchased fresh from local markets, and the edible parts blended using a blender GT800 (Uetendorf, Switzerland) and then freeze-dried. The procedure followed for extraction of antioxidants was as previously described by Saura-Calixto, Serrano, and Goni (2007) with slight modifications. The first extraction procedure involved suspending 0.5 g of freeze dried food sample in 20 ml of 98% methanol/water (50:50 v/v) in a 250 ml Erlenmeyer flask and incubating at 25 °C while mixing at 150 rpm for 1 h. The mixture was then centrifuged at 2500 g for 20 min and the supernatant recovered. The residue was then washed with 20 ml of 98% acetone/water (70:30 v/v) and centrifugation repeated as described above to maximally recover remaining antioxidants as recommended by previous researchers (Perez-Jimenez et al., 2008). The antioxidant activity of the different extracts was determined separately after adjusting pH to 7 using NaOH to stabilize the extracts (Perez-Jimenez et al., 2008).

2.5.2. Recovery of hydrolysed tannins

The residues arising from methanol extracts were mixed with 20 ml of methanol and 2 ml of concentrated sulphuric acid. Sam-

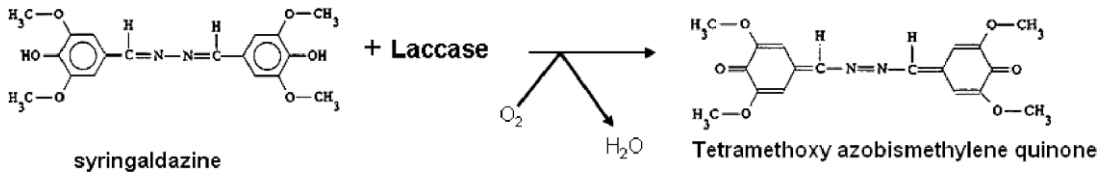

Fig. 1. Laccase oxidation of syringaldazine results in the formation of a water molecule and tetramethoxy azobismethylene quinone (TMAMQ).