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Original Contribution

Cellular and plasma antioxidant activity assay using tetramethoxy azobismethylene quinone

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ABSTRACT

The kinetics of the reduction of enzymatically generated tetramethoxy azobismethylene quinone (TMAMQ), a newly developed antioxidant activity assay method, by pure cellular and plasma antioxidants was studied. Further, the potential application of TMAMQ to the estimation of the antioxidant activity of clinical serum samples was investigated. The highest reduction rate (k) was obtained with ascorbic acid (1.11×10^{-2} μM^{-1} s^{-1}) and glutathione showed the lowest (2.94×10^{-5} μM^{-1} s^{-1}). Comparing TMAMQ and the commercially available antioxidant method Total Antioxidant Capacity clearly shows a similar trend, although the values differ. This study also shows that TMAMQ is highly sensitive (only a minute plasma sample was required) and reproducible, and the reaction proceeds until steady state (until all antioxidants have reacted). TMAMQ is very stable in acetonitrile (>3 months), making it a highly flexible method because it can be easily adapted for analysis of just a single sample or for high-throughput analysis. This has direct implications on reducing costs and experimental steps. TMAMQ is therefore a highly promising antioxidant activity assay method for cellular and plasma antioxidant activity assay.

The human body is constantly exposed to a large variety of reactive species (free radicals), which under certain conditions exceed the antioxidant capacity of the body, resulting in oxidative stress. Oxidative stress has been implicated in various pathophysiological conditions, including atherosclerosis, cancer, neurological disorders, diabetes, ischemia/reperfusion, and aging [1–4]. However, to protect cells and organs against free radicals, biological systems have evolved a highly sophisticated and complex antioxidant system [5]. These antioxidants constitute the body's first line of defense against free radical damage. The antioxidants include a biologically built-in mechanism of neutralizing free radicals comprising enzyme systems (superoxide dismutase, glutathione peroxidase, catalase) and nonenzymatic antioxidants [glutathione, ascorbic acid (vitamin C), uric acid, albumin, γ- and α-tocopherol (vitamin E), carotenoids, flavonoids, etc.].

Among the nonenzymatic antioxidants, glutathione and vitamins C and E play a central and complementary role in quenching free radicals in and around the cell. For example, glutathione and vitamin C are complementary water-soluble antioxidants that scavenge reactive oxygen species (ROS) in the fluid outside and within the cell. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids, capable of neutralizing ROS in aqueous phase to prevent their entry into cells [6]. Glutathione is the major soluble antioxidant in the cell compartments comprising cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) [7]. Unlike glutathione and vitamin C, vitamin E is the most effective lipid-soluble antioxidant present in human cells [8]. Vitamin E as an antioxidant is involved in protecting membrane fatty acids from oxidants. Ascorbic acid and α-tocopherol are believed to be regenerated by reduction with glutathione [9].

In blood, albumin is code-named the “sponge or tramp streamer” of the circulation because of its ability to mop up a wide range of antioxidants and bind many foreign molecules, metals and organic molecules alike. Its antioxidant activity is estimated to account for more than 70% of that in serum [10]. Another important serum antioxidant is uric acid, which in humans is also claimed to contribute to nearly half of the antioxidant capacity of blood plasma [11].

Given the importance of the antioxidants described above, any method that can accurately monitor the levels of these cellular and plasma antioxidants will be a welcome development in the fight against oxidative stress-related diseases. Measurement of the antioxidant status of biological fluids could be used as an early warning sign

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("biomarker") of possible disease. However, although several methods have been developed and are commercially available, they are hampered by questionable sensitivity, unreliability, nonreproducible results, and procedural difficulties, all making it difficult to have a standardized internationally accepted method [12–14]. For example, the total peroxyl radical trapping assay developed by Wayner et al. [15] (the most widely used method 2 decades ago) has a serious problem in that the oxygen electrode used does not maintain its stability during the whole incubation time. On the other hand, comparison of the oxygen radical absorbance capacity assay (ORAC) with the Randox Trolox equivalent (Randox TEAC) and the ferric-reducing ability (FRAP) assays showed no correlation among them, rendering the results questionable for practical applications [16].

In continued efforts to develop an effective total antioxidant biomarker, we recently developed a highly promising antioxidant activity method based on laccase-generated tetramethoxy azobismethylene quinone (TMAMQ) [5]. In the subsequent screening studies, we showed that TMAMQ was reduced by all known and tested antioxidants, including glutathione, uric acid, albumin, and vitamins C and E [14]. In this study the kinetics of the reduction of TMAMQ by cellular and plasma antioxidants (glutathione, uric acid, vitamins C and E) was investigated by plotting the initial velocities of the reaction as a function of the concentration of reactants. The initial velocity was defined as the initial slope of a graph of the concentrations of reactants as a function of time measured over a range of times such that only a small fraction of TMAMQ has been reduced [17]. Preceding the kinetic study, the influence of pH and solvent on TMAMQ stability was investigated to define its working pH range. Finally, the ability of TMAMQ to measure the antioxidant activity of clinical samples was compared with a commercially available method (based on the chromogenic substrate tetramethylbenzidine monitored at 450 nm) used for measuring the total antioxidant activity of serum and EDTA plasma.

Materials and methods

Enzyme and chemicals

All antioxidant molecules were of analytical grade. The cellular and plasma antioxidants were purchased from Sigma–Aldrich (Steinheim, Germany). All the other chemicals were purchased from Merck (Darmstadt, Germany). *Trametes modesta* laccase was produced and purified as previously described by Nyanhongo et al. [18].

Production of TMAMQ

TMAMQ was produced as previously described [5,14]. Briefly, syringaldazine (0.17 mM) was incubated with 50 µl of laccase (20 nkat ml^{-1} determined as previously described by Nugroho Prasetyo et al. [5]) in 50 mM sodium citrate buffer at pH 4.5 for 10 min at 30 °C while shaking at 140 rpm using a Themomixer (Eppendorf AG, Hamburg, Germany). The reaction was then immediately stopped by freezing in liquid nitrogen followed by lyophilization using a Labconco freeze dry system/FreeZone 4.5-L Benchtop Model 77500 (Vienna, Austria). The freeze drier was operated at a temperature of −48 °C and at a vacuum pressure of 3×10^{-4} mbar. The freeze-dried TMAMQ was then dissolved in ice-cold methanol and centrifuged at 20,000×*g* for 30 min to precipitate the enzyme. The resulting supernatant containing TMAMQ was used for antioxidant activity assay.

Effect of pH and solvent on TMAMQ

Buffer solutions of 50% v/v buffer/50 mM methanol were prepared using sodium citrate buffer for pH 4–6, tris(hydroxylmethylaminomethane–hydrochloric acid for pH 7–9, sodium orthophosphate for pH 11–12, and potassium chloride for pH 12–13. The effect of pH on TMAMQ stability was studied by dissolving freeze-dried TMAMQ in the various pH's above and recording the wavelength scans immediately. The buffers containing TMAMQ at various pH's were then further incubated for 3 h at 25 °C while changes in the chromatograms were monitored by measuring the wavelength scan in the range 200–900 nm every hour. The effects of solvents were investigated by dissolving and incubating lyophilized TMAMQ in the respective solvent (methanol, ethanol, ethyl acetate, acetonitrile) at 4 °C, −20 °C, and room temperature. The residual TMAMQ was measured at defined times during the incubation period.

Monitoring the kinetics of the reduction of TMAMQ

Spectrophotometric data were acquired by incubating various TMAMQ concentrations (in excess with respect to antioxidants) with various concentrations of the individual antioxidants. The rate of reduction of TMAMQ was monitored at 530 nm using either a stopped-flow spectrometer (PBP Spectra Kinetic 05-109 monochromator from Applied Photophysics, UK) or the above-mentioned UV–Vis spectrometer (Hitachi U-2001). The temperature of all the reagents was first adjusted to 25 °C and the temperature in the cell kept constant by means of a thermoset bath. A typical procedure consisted of adding a freshly prepared solution of the antioxidant to a freshly prepared solution of TMAMQ in methanol and placing it in the spectrometer cell. Spectra were recorded every 0.5 s until the reaction reached a plateau.

Data analysis

The exact concentration of TMAMQ was calculated from a calibration curve recorded for each pH value. For each antioxidant concentration tested, data were fitted by using the software program Origin Pro 7.5 to obtain the kinetic parameters and confirmed by mathematical derivation. The kinetic parameters were estimated using curve fittings achieved through least-square regression analysis and yielded optimized values for the parameters.

Antioxidant activity measurement of plasma samples

A commercial antioxidant activity assay, Total Antioxidant Capacity (TAC) (LDN, Nordhorn, Germany) was used. The handling and measurements were conducted as described in the accompanying manual. For TMAMQ, standards, controls, and serum samples were first diluted by transferring 5 µl into 200 µl of double-distilled water and then 60 µl of the diluted samples was transferred into 340 µl of TMAMQ, total volume 400 µl. Samples were incubated until the reaction reached a plateau (approximately 20 min) before reading the absorbance at 530 nm using a TECAN Infinite M200 plate reader (Tecan Austria GmbH, Grödig, Austria).

Results and discussion

The effect of pH on TMAMQ

The importance of pH on electron transfer reactions in measuring antioxidant activity has been established [19–21]. Therefore the study of the effect of pH on TMAMQ is extremely important to define its effective working pH range. TMAMQ was stable in a broad pH range spanning from pH 4.0 to 7.5 (Fig. 1).

The change in color coincided with the change in maximum absorbance as shown in Fig. 1. TMAMQ is purple within the pH range 4.0–7.5 and shifts to yellow in the pH range 8.0–11.0 and intense yellow at pH 12.0–14.0. Interestingly, TMAMQ is stable over the human physiological pH of 7.4 (Fig. 1). This observation also coincides well with several studies that reported that most antioxidants are