Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 1057–1061
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A cytochrome *c*-enhanced peroxidation reaction with potential use in screening dietary antioxidants

Suiping Zhou[1], Yu Yao[1], Allan Davison, Amandio Vieira*

Laboratory for Metabolic and Endocrine Research, K9600 Simon Fraser University, Burnaby, BC, Canada V5A 1S6

Received 6 October 2002; received in revised form 25 August 2003; accepted 25 November 2003

Abstract

Reactive oxygen species (ROS) that result from events such as cellular respiration can cause damage to biological molecules and tissues. A variety of endogenous and dietary antioxidants function in moderating the extent of oxidative damage in the body. In this report, a pro-oxidant system is presented as an assay for screening possible antioxidant activities of dietary factors. The assay reaction involves peroxidatic oxidation of the redox indicator *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine (TMPD). It is shown that the reaction rate is enhanced by up to 10-fold in the presence of cytochrome *c* (cyt *c*), a mitochondrial electron transport protein. The extent to which selected dietary antioxidant factors inhibit the cytochrome *c*-enhanced peroxidatic oxidation of TMPD is also reported. Considering the known pathological consequences of mitochondrial membrane disruption and cytochrome *c* release in the cell, this reaction and assay may be of pathological and therapeutic relevance.

Keywords: Dietary antioxidants; Cytochrome *c*; TMPD; Ascorbate; Anthocyanins; Peroxidatic oxidation

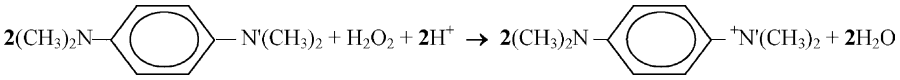
1. Introduction

Cytochrome *c* (cyt *c*) is a heme protein that functions normally in the transport of electrons from cytochrome *c* reductase (complex III) to cytochrome *c* oxidase (complex IV). Cyt *c* is located on the inner mitochondrial membrane at the inter-membrane space. In addition to its normal functions in electron transport, it has been reported that cytochrome *c* has the capacity to catalyze peroxidatic oxidations [1–4]. These include in vitro and in vivo reactions: e.g., the oxidation of electron donors such as 2-2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and luminol, and oxidation of lipids in phosphatidylcholine liposomes and rat heart mitochrondria [5–7]. While pathological significance for the peroxidatic activity of cytochrome *c* remains a topic of current research, there are several potential roles related to apoptotic death of cells [8–11] and to disruptions of the electron transport chain or mitochondrial membrane integrity [12–17].

In this report, a cytochrome *c*-enhanced peroxidation reaction is described and presented as a screening assay for putative antioxidants. The reaction involves the oxidation of *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine (TMPD):

* Corresponding author. Tel.: +1-604-291-4251; fax: +1-604-291-3040.
E-mail address: avvieira@sfu.ca (A. Vieira).
[1] Both authors contributed equally to this work.

0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.08.002



The reaction yields Wurster's blue, a chromogenic product that can be monitored spectrophotometrically. A basic characterization of the reaction rates is provided, as well as an indication of how dietary components such as anthocyanin-rich extracts and ascorbate may influence the extent and rates of this reaction. In relation to other assays of antioxidant activity (reviewed in [18]), the assay presented herein provides an additional or alternative method of testing putative antioxidants. It may also have particular relevance to antioxidants that target peroxidatic oxidations, and events related to mitochondrial dysfunction and release of cytochrome *c* (see [11,16,17,19] for examples of such events).

2. Materials and methods

N,*N*,*N*′,*N*′-Tetramethyl-1,4-phenylenediamine was obtained from Aldrich. Cytochrome *c* (equine heart), ethylenediaminetetraacetate (disodium salt), ascorbic acid and potassium phosphate were obtained from Sigma. TMPD solutions were prepared in potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA; all reactions were carried out in this buffer. Both the buffer and TMPD solutions were bubbled with argon gas during the experiments.

Dietary antioxidant samples were obtained (from fruit, unless otherwise noted) by three methods: (i) *Pisum sativum* (seed, commercial pea) and *Fragaria ananassa* (commercial strawberry) samples were prepared by homogenization of 50 g of commercial frozen material in 50 ml of distilled, deionized water, followed by centrifugation (8000 rpm for 25 min); the supernatant was stored at −80 °C. (ii) *Vaccinium myrtillus* (wild bilberry) samples were prepared by resuspending 400 mg of dried material in 1 ml of distilled, deionized water. After shaking and vigorous vortexing, insoluble material was removed by centrifugation (5000 × *g*, 1 min); the supernatant was stored in aliquots at −80 °C. Total anthocyanin content of the *Vaccinium* sample was calculated from a standard curve using cyaninidin-3-*o*-β-glucoside as the standard. (iii) For the *Citrus reticulata* (Mandarin orange) sample, fresh juice without pulp was used in the assay. Vitamin C (ascorbate) concentrations in the dietary samples were calculated based on the analysis provide by the USDA Food Composition Database.

For TMPD peroxidation reactions, 25 μl of a 1.2 mM cytochrome *c* stock solution was incubated with 20 μl of a 750 mM hydrogen peroxide stock solution for 10 min in a cuvet, and the stated amount of antioxidant(s) was added. Buffer (50 mM potassium phosphate containing 0.1 mM EDTA, pH 7.4) was then added to the cuvet to make a total volume of 2.96 ml, followed by the addition of 40 μl of a 30 mM TMPD solution. The final concentrations for TMPD, H_2O_2, and cytochrome *c* in the cuvet were 400 μM, 5 mM, and 10 μM, respectively. The oxidation of TMPD was followed at 630 nm, with a Beckman spectrophotometer equipped with a chart recorder and computer online data acquisition. Initial rates were obtained from the slopes of a first order plot of the initial parts of each reaction.

Unless otherwise noted, error bars in the graphs represent standard errors of the respective means (*n* is given in the figures). Results were considered statistically significant if Student's *t*-test results gave a *P*-value less than or equal to 0.05.

3. Results

3.1. Cytochrome c greatly enhances the peroxidatic oxidation of TMPD

Fig. 1A shows the rates of TMPD peroxidation, and cytochrome *c*-enhanced TMPD peroxidation. The rate of the latter reaction (i.e., with cytochrome *c* and hydrogen peroxide) is approximately 10-fold higher than that of the former reaction (i.e., with only hydrogen peroxide). As additional controls, the rate of TMPD oxidation in the presence and absence of cytochrome *c* (i.e., no hydrogen peroxide) is shown (Fig. 1A); these control rates are about three-fold lower than the rate achieved in the presence of hydrogen peroxide and about 20-fold lower than the maximal rate achieved in the presence of both cytochrome *c* and hydrogen peroxide.

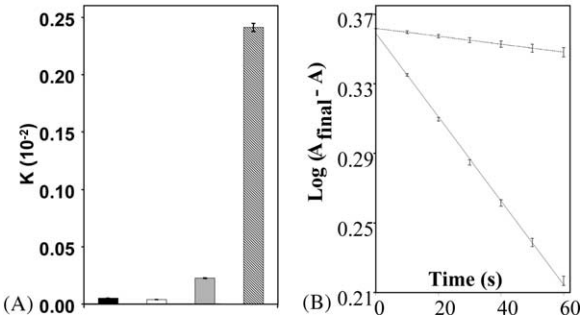


Fig. 1. Effect of cytochrome *c* on the oxidation of tetramethyl-1,4-phenylenediamine. (A) Reaction rates are shown for TMPD alone (black), TMPD + cytrochrome *c* (white), TMPD + hydrogen peroxide (shaded), and TMPD + hydrogen peroxide + cytochrome *c* (striped). The rate with cytochrome *c* and hydrogen peroxide is approximately 10-fold higher than that with hydrogen peroxide alone, and approximately 20-fold higher than that with cytochrome *c* alone. (B) The oxidation of TMPD was measured (see Section 2) either in the presence of hydrogen peroxide (upper line) or hydrogen peroxide and cytochrome *c* (lower line). The rate with cytochrome *c* is approximately 10 times higher than that without cytochrome *c*. The log of final (maximal) absorbance minus absorbance at the given times is shown as a function of reaction time.

Fig. 1B also shows a comparison of the reaction rates with hydrogen peroxide alone and with both hydrogen peroxide and cytochrome *c*; again, the former reaction is about 10-fold slower than the latter. With respect to time, Fig. 1B shows that both the peroxidation and cytochrome *c*-enhanced peroxidation of TMPD are first order reactions. Measurements up to 400 s were performed in some cases and always displayed first order kinetics (data not shown).

3.2. The cytrochrome c-enhanced reaction can be used for testing putative dietary antioxidants

Fig. 2 shows an application of the cytochrome *c*-enhanced peroxidation reaction in terms of assessing the antioxidant potential of a known dietary antioxidant source, *V. myrtillus*. The kinetic spectrophotometric data are indicated for two reactions: (i) TMPD + hydrogen peroxide + cytochrome *c*, and (ii) TMPD + hydrogen peroxide + cytochrome *c* + anthocyanin-rich dietary extract. The anthocyanin-rich extract (prepared from the *Vaccinium* as describe in Section 2) reduced the extent of cytochrome *c*-enhanced peroxidation of TMPD by about three-fold.

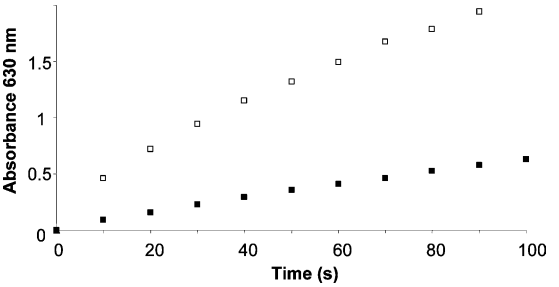


Fig. 2. Kinetics of cytochrome *c*-enhanced TMPD oxidation and of the inhibition of oxidation by an anthocyanin-rich extract. The assay was carried out as described in Section 2 either with (■) or without (□) the presence of a *V. myrtillus* aqueous extract (equivalent to a 10 mg sample of fresh material; compare with amounts of other dietary samples in Fig. 3) containing a final assay concentration of 12.5 μg/ml total anthocyanins. The figure shows a typical kinetic spectrophotometric recording for the first 100 s of the assay.

Aqueous extracts prepared from other dietary sources were also examined using the cytochrome *c*-enhanced peroxidation reaction as an assay. The extent to which oxidation can be inhibited by various concentrations of *F. ananassa* and *P. sativum* extracts is shown in Fig. 3. Per unit weight, the *Fragaria* extract was more potent than that from *Pisum*: 50% inhibition of oxidation was achieved with about 4 times lower concentration of *Fragaria* sample compared to *Pisum* sample (Fig. 3).

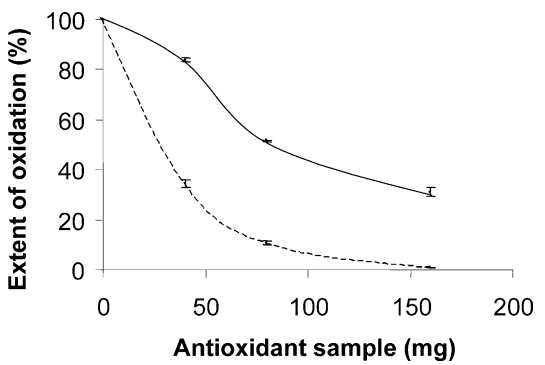


Fig. 3. A comparison of the antioxidant activities of dietary samples, aqueous extracts of *F. ananassa* and *P. sativum*. The samples were prepared as described in Section 2, and were added to the assay in three different concentrations. The *Fragaria* samples (- - -) exhibited greater antioxidant activity than those from *Pisum* (—) against the cytochrome *c*-enhanced peroxidation reaction.

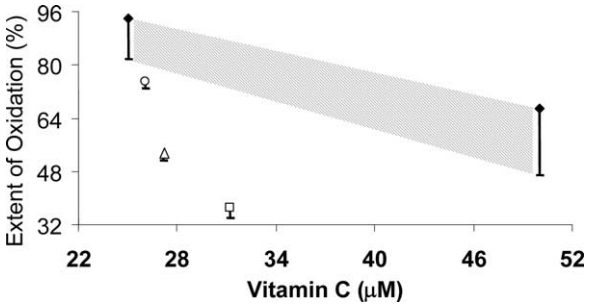


Fig. 4. Antioxidant activity of ascorbate and of various dietary samples relative to their ascorbate concentration. Sample amounts that gave ascorbate concentrations between 25 and 50 μM in the assay were selected, and the extent of oxidation in the presence of each of the samples (○, △, □) was compared with that of ascorbate itself in the same concentration range. The ascorbate inhibitory zone is represented by the shaded area that lies between the 25 and 50 μM mean ascorbate inhibition values (◆) with SEM error bars shown). Only the dietary sample prepared from *Citrus* (○) exhibited antioxidant activity that could be accounted for based almost entirely on its ascorbate content. Both *Pisum* (△) and *Fragaria* (□) samples had antioxidant activities approximately 40 and 50% greater, respectively, than what would be expected based only on their ascorbate content.

The cytochrome *c*-enhanced peroxidation reaction was also inhibited by ascorbate (vitamin C): 30% inhibition was observed with 50 mM ascorbate[2] (Fig. 4). The inhibition of oxidation observed with the *Pisum* and *Fragaria* samples, however, was much greater than that which one would expect if all antioxidant activity in these samples was due only to their content of ascorbate. In other words, the *Pisum* and *Fragaria* samples plotted in Fig. 4 lowered the extent of oxidation to 52 and 36%, respectively; but one would have expected a lowering of the extent of oxidation to only about 90 and 80%, respectively, based on the ascorbate content of the samples. In contrast to the *Pisum* and *Fragaria* samples, a dietary antioxidant sample prepared from a *C. reticulata* exhibited antioxidant activity close to that which one would expect based only on the ascorbate content of the sample (Fig. 4).

Although not plotted in Fig. 4, a preliminary estimate for the *Vaccinium* sample based on its known antioxidant activity (e.g. Fig. 2) and approximate vitamin C content [20] indicates that a sample containing 25 μM ascorbate would lower the extent of oxidation to less than 1%. Thus, of the four different samples tested, *V. myrtillus* is likely to be the most potent inhibitor of the cytochrome *c*-enhanced oxidation of TMPD and the sample for which ascorbate makes the smallest contribution to total antioxidant activity.

4. Discussion

Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals, and the superoxide anion can cause damage to cells and tissues. The oxidative and peroxidative reactions promoted by these ROS are normally kept under control by antioxidants. In addition to the body's endogenous antioxidant chemicals and enzymes, a variety of dietary antioxidants including some vitamins and flavonoids function in moderating the extent of oxidative damage to the body's tissues. In this report, a peroxidatic oxidation reaction that is catalyzed by a mitochondrial electron transport protein, cytochrome *c*, is characterized and presented as a possible assay for screening antioxidant activities of dietary factors.

Several examples of the application of the assay for testing dietary antioxidant activities are presented. Flavonoids such as anthocyanins are recognized as potent dietary antioxidants [20–23]; and an anthocyanin-rich *Vaccinium* extract greatly reduced the extent of cytochrome *c*-enhanced TMPD oxidation: over 60% inhibition with a sample equivalent to 10 mg of fresh material. In this context, it is of interest to note that *F. ananassa* fruit also contains anthocyanins at approximately 10-fold lower concentration relative to *V. myrtillus* [20,22]; with *Fragaria*, the same 60% inhibition was reached using approximately four-fold more material (40 mg) relative to *Vaccinium*. Consistent with this latter result, previous tests using a different antioxidant activity assay (ORAC) suggest that *V. myrtillus* has about three-fold more activity than *Fragaria* ([20] and references therein). Relative to the *Vaccinium* and *Fragaria* extracts, a lesser antioxidant activity was observed in our assay for an extract from *P. sativum*, a species that is deficient in anthocyanin antioxidants but contains ascorbate, tannins and other antioxidant phenolics.

All of the dietary samples tested herein contain also at least one additional antioxidant, ascorbate (as

[2] This is the inhibition at $t = 120$ s of the assay, a time used arbitrarily for comparison with other antioxidant activities. As the assay progressed, the inhibitory activity of ascorbate diminished.

well as trace quantities of vitamin E). Only with the *Citrus* sample, however, could it be suggested that the antioxidant activity was due almost entirely to the ascorbate content. With the *Fragaria* extract, in contrast, the ascorbate content is likely to account for less than half of the observed antioxidant activity. Also, in *V. myrtillus* and other *Vaccinium* species, ascorbate is likely to account for less than 5% of total antioxidant activity ([20]; see also Section 3). The remainder of the antioxidant activities in *Fragaria*, *Vaccinium*, and *Pisum* samples are due to other phytochemicals.

The tests that were carried out on purified and crude dietary samples and reported herein serve as examples of some of the potential uses of the assay for screening putative antioxidant factors. The assay also has the advantage that it is simple and does not require expensive equipment. The fact that cytochrome *c*-enhanced oxidative damage may be of significance in neurodegenerative illnesses such as Parkinson's and in situations where mitochondrial membrane integrity is disrupted [16,17,19,24], gives this assay potential additional value in terms of screening for antioxidant factors that may have an influence over the course of some pathological events.

References

[1] Y. Nakamura, T. Samejima, K. Kurihara, M. Tokjo, K. Shibata, J. Biochem. 48 (1960) 862–869.
[2] T. Flatmark, Acta Chem. Scand. 20 (1966) 1470–1475.
[3] V.E. Shashoua, Arch. Biochem. Biophys. 111 (1965) 550.
[4] I.L. Nantes, A. Faljioni-Alario, A.E. Vercesi, K.E. Santos, E.J. Bechara, Free Radic. Biol. Med. 25 (1998) 546–553.
[5] R. Radi, L. Thomson, H. Rubbo, E. Prodanov, Arch. Biochem. Biophys. 288 (1991) 112–117.
[6] R. Radi, K.M. Bush, B.A. Freeman, Arch. Biochem. Biophys. 300 (1993) 409–415.
[7] P.J. O'Brien, Biochem. J. 100 (1966) 68P.
[8] X. Lui, N.C. Kim, J. Yang, R. Jemmerson, X. Wang, Cell 96 (1996) 147–157.
[9] S. Schendel, M. Montal, J.C. Reed, Cell Death Differ. 5 (1998) 372–380.
[10] H. Zou, Y. Li, X. Liu, X. Wang, J. Biol. Chem. 274 (1999) 11549–11556.
[11] D.R. Green, J.C. Reed, Science 281 (1998) 1309–1312.
[12] S.A. Cook, P.H. Sugden, A. Clerk, Circ. Res. 85 (1999) 940–949.
[13] V.P. Skulachev, FEBS Lett. 423 (1998) 275–280.
[14] G. Petrosillo, F.M. Ruggiero, M. Pistolese, G. Paradies, FEBS Lett. 509 (2001) 435–438.
[15] N. Das, S. Gupta, S. Mazumdar, Biochem. Biophys. Res. Commun. 286 (2001) 311–314.
[16] M. Hashimoto, A. Takeda, L.J. Hsu, T. Takenouchi, E. Masliah, J. Biol. Chem. 274 (1999) 28849–28852.
[17] M. Deshmukh, J. Johnson, Neuron 21 (1998) 695–705.
[18] I.I. Koleva, T.A. van Beek, J.P. Linssen, A. de Groot, L.N. Evstatieva, Phytochem. Anal. 13 (2002) 8–17.
[19] J. Cai, J. Yang, D.P. Jones, Biochim. Biophys. Acta 1366 (1998) 139–149.
[20] R. Prior, G. Cao, A. Martin, E. Sofic, J. McEwan, C. O'Brien, N. Leschner, M. Ehlenfeldt, W. Kalt, G. Krewer, C. Mainfield, J. Agric. Food Chem. 46 (1998) 2686–2693.
[21] C. Ramirez-Tortosa, O.M. Andersen, P.T. Gardner, P.C. Morrice, S.G. Wood, S.J. Duthie, A.R. Collins, G.G. Duthie, Free Radic. Biol. Med. 31 (2001) 1033–1037.
[22] H. Wang, G.H. Cao, R.L. Prior, J. Agric. Food. Chem. 45 (1997) 304–309.
[23] T. Tsuda, K. Shiga, K. Ohshima, S. Kawakishi, T. Osawa, Biochem. Pharmacol. 52 (1996) 1033–1039.
[24] E. Masliah, E. Rockenstein, I. Veinbergs, M. Mallory, M. Hashimoto, A. Takeda, Y. Sagara, A. Sisk, L. Mucke, Science 287 (2000) 1265–1269.