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# Antioxidant activity of melanins derived from tea: comparison between different oxidative states

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**Abstract**

The antioxidant activity of tea melanin (TM) derived from tea leaves was studied. The influence of TM on the kinetics of the free-radical oxidation of β-carotene revealed essential differences between their oxidative states. The reduced preparation of TM delays the oxidation of β-carotene. The lag time increased in proportion to concentration of TM. The oxidized preparation of TM decreases the rate of β-carotene consumption, but does not affect the lag time. The kinetic characteristics of the antioxidant activity of TM can be explained by the participation of phenol and quinone groups in free-radical chain reactions. Antioxidant properties of TM can be utilized for prevention of free-radical oxidation of natural products. 

*Keywords:* Tea melanin; Antioxidant activity; β-Carotene oxidation

## 1. Introduction

Natural melanin from plants or animals possesses a broad spectrum of biological activity, which is believed to come from its antioxidant properties (Barr, 1983; Lukiewicz, 1972). These properties of melanin are of great interest because of implications for health maintenance (Gokce & Frei, 1996) and production of functional foods (Farr, 1997). The participation of melanin in free-radical reactions has been widely reported (Bilgihan, Bilgihan, Akata, Aricioglu, & Hasanreisoglu, 1995; Blarzino, Mosca, Foppoli, Coccia, De Marco, & Rosei, 1999; Mosca, Blarzino, Coccia, & Foppoli, 1998; Riley, 1997). It was found that the scavenging or chelating properties of melanin are mainly involved in their antioxidant activity. These properties can be utilized at the stage of initiating chain reactions by trapping of transitional metals or inactivating superoxide radicals. However, free-radical scavengers are not effective in preventing propagation of chain reactions due to their inability to react with peroxyl radicals (Denisov & Khudyakov, 1987). Nevertheless, the strong antioxidant activity of melanin indicates that it is probably able to terminate chain propagation. This possibility has not been investigated.

Earlier, we examined the antioxidant activity of melanin derived from tea leaves (Sava, Yang, Hong, Yang, & Huang, 2001). In the presence of tea melanin (TM), the oxidation of low-density lipoproteins exhibited a distinct lag phase, probably due to the termination of the propagation of chain reactions. However, a detailed study of the mechanism of antioxidant activity of TM, for this model reaction, was limited because of the interaction of TM with apolipoprotein B.

Additionally, it was found that the antioxidant activity of melanin depends on its oxidative state. The possibility of melanin existing in various degrees of oxidation is one of its fundamental properties (Crippa, Horak, Protta, Svoronos, & Wolfram, 1989; Horak & Gillette, 1971). This is caused by melanin's phenol-quinone structure. However, the role of such structural peculiarities in the antioxidant activity of melanin is not clear.

This paper is based on the comparison of antioxidant activity between the different oxidative states of TM and

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utilizes an alternative model reaction comprising pure lipids: β-carotene and linoleic acid (Pratt & Miller, 1984). These approaches reveal certain previously unrecognized aspects of the antioxidant activity of melanic pigments concerning the termination of free-radical chain propagation.

## 2. Material and methods

### 2.1. Chemicals

Fully fermented black tea produced in Miaoli, Taiwan was used. It was purchased from local retail shops. Synthetic melanin, Sephadex G-75, molecular size markers for gel-filtration chromatography, β-carotene, linoleic acid, Tween-20, gallic acid, and Folin-Ciocalteu's Phenol Reagent (FCPR) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were chemical reagent grade from Merck (Darmstadt, Germany). The organic solvents were of high-performance liquid chromatography (HPLC) grade.

### 2.2. Isolation and purification of TM

Isolation of TM was performed according to a scheme (Fig. 1) allowing preliminary extraction of tea polyphenols

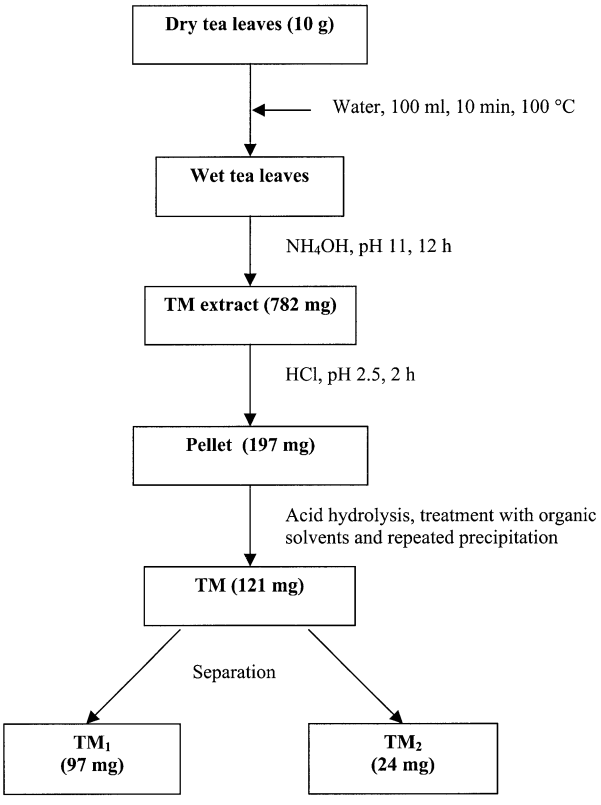


Fig. 1. The procedure for extraction and separation of TM.

(Sava et al., 2001). In short, tea leaves were treated with boiling water at a ratio of 1:10 (w/v) for 10 min, followed by filtration. The usual tea polyphenols were removed in the filtrate. The solid matter was immersed in water at 40 °C at a ratio 1:10 (w/v), followed by the addition of 10% $NH_4OH$ to adjust the pH to 10.5. Extraction time was 12 h to avoid excessive oxidation of TM. After extraction, the mixture was filtered and centrifuged at 15,000 ***g*** for 30 min to obtain the TM extract. This extract was acidified with 2 N HCl to pH 2.5 and centrifuged at 15,000 ***g*** for 15 min to give a pellet. Acid hydrolysis was employed to remove carbohydrates and proteins (Harki, Talou, & Dargent, 1997). According to this, TM was boiled in 7 M HCl during 2 h, followed by centrifugation at 10,000 g for 10 min and washing with water. Organic solvents (chloroform, ethyl acetate and ethanol) were used to remove lipids. In practice, 100 mg of TM were placed into 50 ml of appropriate organic solvent, and shaken during 30 min. TM was collected after filtration.

The product thus obtained was dissolved in 0.2% $NH_4OH$, and the solution was subjected to repeated precipitations, carried out according to a previously reported procedure (Sava et al., 2001). Four precipitations were employed to free TM from low molecular mass impurities and to improve its homogeneity. Each precipitation included acidification of TM solution with 2 N HCl to pH 2.5, incubation at room temperature for 2 h, and centrifugation at 15,000 ***g*** for 15 min. Products were collected and analysed.

Finally, TM was separated on a Sephadex G-75 column (1.6×40 cm) in 50 mM phosphate buffer (pH 7.5) at a flow rate of 0.5 ml $min^{-1}$. Fractions were monitored at 280 nm. To evaluate the molecular mass (MM) of TM, a Sephadex G-75 column was calibrated with bovine serum albumin (MM 66,000), carbonic anhydrase (MM 29,000), cytochrome C (MM 12,400), and aprotinin (MM 6500).

### 2.3. Oxidation-reduction of TM

Different oxidative states of TM were generated by oxidizing or by reducing the original samples. $Ti^{+3}$ was used as the reducing agent (Horak & Gillette, 1971). A 10 ml aliquot of TM suspension in distilled water, containing 100 mg of substance, was combined with 20 ml solution containing 0.1 M sodium oxalate and 1 M sulfuric acid. Then, 5 ml of 80 mM $TiCl_3$, dissolved in dilute sulfuric acid (1:10 by volume), were added. This mixture was incubated for 120 min and the reduced preparation of TM was separated by centrifugation. The reduced TM was washed with diluted sulfuric acid and plenty of water under $N_2$. Finally, the reduced TM was dialysed against milli-Q water for 24 h.

Molecular oxygen was used as the oxidizing agent. The reduced preparation of TM was suspended in distilled

water at a final concentration of 10 mg/ml and then dissolved at pH 12 using $NH_4OH$. The solution thus obtained was placed in a tightly closed vessel supplied with a stirrer and connected to an oxygen gauge. The incubation temperature was set to 40 °C. The solution was saturated with oxygen and the stirring speed was adjusted as necessary for steady oxidation. The reaction mixture was incubated for 72 h. The oxidized sample of TM was precipitated by acidification with 2 N HCl to pH 2, centrifuged, and the pellet washed with distilled water.

Finally, the reduced and oxidized samples of TM were suspended in distilled water and then 0.1 M NaOH was added dropwise to adjust to the required pH. The solutions thus obtained were filtered through a Nalgene 0.45 μm syringe filter. All operations were conducted under $N_2$.

The monitoring of the oxidative transformation of TM was conducted spectrophotometrically, using a JASCO V-530 UV-Visible Spectrophotometer (Jasco Ltd., Great Dunmow, UK). Differential absorbance measurements were carried out according to Korshin, Benjamin, and Li (1999). For this purpose the aqueous solution (100 mg/l) of each TM tested was divided into two, one being adjusted to pH 12 and the other to pH 7. The solutions at pH 7 were used as a blank. Spectra were plotted as differential absorbance versus wavelength.

### 2.4. Physico-chemical characterization of TM

Physical and chemical characteristics of TM were obtained according to procedures used previously (Paim, Linhares, Magrich, & Martin, 1990; Nicolaus, 1968; Prota, 1992). Ultraviolet (UV) absorption spectra were recorded on a JASCO V-530 UV-Visible Spectrophotometer. Infrared (IR) spectra were recorded in KBr discs on a Perkin-Elmer spectrometer (Model 1600 FT). Solubility in water, aqueous acid, and in common organic solvents and oxidative bleaching, by means of $KMnO_4$, $K_2Cr_2O_7$, NaOCl and $H_2O_2$, and a positive reaction for polyphenols, were used as the primary characteristics of TM.

Total phenol groups were assayed quantitatively by absorbance at 765 nm using Folin–Ciocalteu reagent. In short, 1 ml of Folin–Ciocalteu's phenol reagent, of 1:10 dilution, was added to 0.2 ml of TM. Then, 0.8 ml of 7.5% (w/v) sodium carbonate were added and mixed in. The mixture was left for 30 min for colour development and, after repeated mixing, the absorbance ($A_{765}$) was read against milli-Q water. The concentration of phenol groups was calculated from a standard curve obtained by subjecting various amounts of gallic acid to the same treatment as the test samples. The equation for the standard curve was as follows:

$$PG = 95.1.\ A_{765} - 2.6$$

where PG is concentration of phenol groups, mg/l.

### 2.5. Evaluation of antioxidant activity of TM

The antioxidant activity of MP was evaluated using the coupled oxidation of β-carotene and linoleic acid (Pratt & Miller, 1984). One millilitre of β-carotene solution in chloroform (5.2 mg/ml) was mixed with 40 mg linoleic acid and 400 mg of Tween-20. The chloroform was removed by rotary evaporation at 40 °C and 100 ml of distilled water were slowly added to the residue with vigorous agitation to form an emulsion.

A 5 ml aliquot of emulsion was placed in tubes containing 0.2 ml of the TM solution at the various concentrations and in the different oxidative states. The tubes were incubated in a water bath at 50 °C with constant shaking. The reaction was carried out in darkness, using intensive saturation with oxygen. Consumption of β-carotene was measured at 470 nm at 30-min intervals against the blank (the emulsion without β-carotene).

## 3. Results and discussion

TM was isolated from tea leaves according to the previously reported procedure (Sava et al., 2001), with minor modifications. In particular, extraction time was diminished to 12 h to avoid excessive oxidation. Gel-filtration on Sephadex G-75 disclosed the heterogeneity of natural TM. Fig. 1 depicts the scheme for the extraction and separation of TM, which yielded one major (88%) and one minor fraction with average molecular masses of $14 \pm 3$ kDa ($TM_1$) and $8 \pm 3$ kDa ($TM_2$), respectively. The average yield, based on the dry tea leaves extracted, was 0.97% for $TM_1$ and 0.24% for $TM_2$. The molecular mass of $TM_2$ corresponds to the apparent molecular mass of TM, derived previously from the oxidized tea polyphenols (Sava et al., 2001).

Both fractions obtained were employed for the preparation of reduced and oxidized samples. Two reduced samples ($TM_{1r}$ and $TM_{2r}$) were prepared by treatment with $TiCl_3$ (Horak & Gillette, 1971) and two oxidized samples ($TM_{1ox}$ and $TM_{2ox}$) were prepared by oxidation with molecular oxygen.

All preparations of TM exhibited the physical and chemical properties of natural melanin previously reported (Bilinska, 1996; Ellis & Griffiths, 1974; Nicolaus, 1968; Paim et al., 1990; Prota. 1992). They were insoluble in both water and organic solvents: ethanol, hexane, acetone, benzene and chloroform. They dissolved only in alkali, precipitated in alkaline $FeCl_3$ and below pH 3, were bleached in $H_2O_2$, $KMnO_4$, $K_2Cr_2O_7$ and NaOCl, and produced a blue colour with $FeSO_4$/ferricyanide.

Treatment of TM fractions with $TiCl_3$ and oxygen gave samples with different oxidative states. $Ti^{+3}$ was fully removed from TM by subsequent treatment with sulfuric acid and dialysis, the ash content remaining unchanged from that determined initially, $0.09 \pm 0.01\%$.

Reduction and oxidation of TM caused changes in structure of functional groups, which were revealed by physico-chemical investigations. Some differences were found in the IR spectra of TM at 3450 $cm^{-1}$, attributed to stretching vibration of –OH groups. This band was much diminished in oxidized preparations of TM. More significant differences were apparent in the UV spectra of reduced and oxidized TM. As with other aromatic compounds, containing auxochromic groups, the UV spectrum of melanin varies with pH (Crippa et al., 1989). The influence of pH was distinct on the spectra of the reduced samples of TM, but the UV spectra of the oxidized samples remained practically constant over a wide range of pH. This dependence on pH may be due to dissociation of phenolic groups in reduced TM. The dissociation constants ($pK_d$), determined for the reduced samples $TM_{1r}$ and $TM_{2r}$ according to Albert and Serjant (1962) had values of $10.5 \pm 0.3$ and $10.7 \pm 0.3$, respectively. Such values are typical to those found for various phenolic groups (Labudzinska & Gorczynska, 1995).

Previous research (Horak & Gillette, 1971) has proved that phenolic groups of melanin are turned into quinone groups during the oxidation. We have observed that such transformation can be derived from UV spectra of TM. However, UV spectra were characterized by strong absorbance, which limited determination of the small contribution of functional groups. Therefore, we employed differential absorbance, a method that has been utilized for the study related compounds, such as humic acid (Korshin et al., 1999), and also for the analytical determination of phenolic groups (Nowicka-Jankowska, Gorczynska, Michalik, & Wieteska, 1986).

Differential spectra (pH 12 vs. pH 7) displayed two broad bands at 295 and 245 nm for both reduced and oxidized samples of TM (Figs. 2 and 3). The differential absorbance at 295 gradually decreased during oxidation of $TM_1$ and $TM_2$ preparations at the same time as that at 245 nm increased. The bands at 295 and 245 nm were changing in a compensatory manner during the reducing-oxidation cycle for both fractions of TM.

The UV band at 295 nm was consistent with data for related phenolic compounds (Escarpa & Gonzalez, 2001). Thus, differential absorbance at 295 nm was attributed to the effect of phenolic groups. Likewise, quinone groups were considered to be the cause of the differential absorbance at 245 nm. The band at 295 nm did not disappear completely, even in fully oxidized samples, which may reflect residual phenolic groups present in TM and unable to react with molecular oxygen.

Additional evidence about phenol groups was obtained using Folin–Ciocalteu's reagent. Recently, this method was quantitatively assessed as comparable to HPLC (Escarpa & Gonzalez, 2001). The total amount of phenolic groups in reduced preparations of $TM_{1r}$ was found to be 28.2 mg/g. The preparation of $TM_{2r}$ contained 84.4 mg/g of phenol groups. Oxidation decreased the amount of phenolic groups to 5.2 and 14.2 mg/g in $TM_{1ox}$ and $TM_{2ox}$, respectively. These data were correlated to the changing of relative differential absorbance at 295 nm (Figs. 2 and 3).

The antioxidant effect of TM was evaluated using the coupled oxidation of β-carotene and linoleic acid (Pratt & Miller, 1984). The oxidation of β-carotene was retarded in the presence of TM. The kinetic curves (Figs. 4 and 5) obtained for equal concentrations of reduced and oxidized forms of TM differed in that of the reduced form of TM exhibited a lag phase (Fig. 4), whereas that the oxidized TM did not (Fig. 5).

The effect of the reduced form of TM can be depicted by two approximately linear parts of the kinetic curves, representing non-inhibited (the last part) and inhibited (the initial part) phases of oxidation (Fig. 4). The rates of non-inhibited ($W_0$) and inhibited ($W_{inh}$) oxidation were determined from the tangents to the linear parts of the kinetic curves (Le Tutour & Guedon, 1992). The position of the intersections of the two tangents corresponds to the end of the lag time ($\tau$) of oxidation.

The kinetic parameters of inhibited oxidation of β-carotene are given in Table 1. It was found that rates of non-inhibited phase ($W_0$) of the reaction were about the same as the rate observed for the control ($1.41 \pm 0.11$ μmol $l^{-1}$ $min^{-1}$). The rates of the inhibited phase of the reaction ($W_{inh}$) were substantially lower. The lag time ($\tau$) for $TM_2$ fraction was longer than for $TM_1$ (Fig. 4, Table 1). The lag time gave the linear correlations with the quantities of phenolic groups for both TM fractions:

$$\tau = 0.966 \cdot PG + 63.18 \qquad (r^2 = 0.8917)$$

The linear correlation was also revealed for lag time and concentration of TM (Table 1).

The results obtained can be interpreted on the basis of the mechanism of free-radical oxidation of lipids (Denisov & Khudyakov, 1987; Yanishlieva, Marinova, Gordon, & Raneva, 1999). The common scheme of free-radical oxidation of lipids (LH) includes a large set of elementary reactions. Briefly, it can be described by three stages: initiation (0), chain propagation (1–2), and chain termination (3):

$$2\ LH + O_2 \rightarrow 2\ L^{\bullet} + H_2O_2 \qquad \text{Initiation} \qquad (0)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \qquad \text{Propagation} \qquad (1)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \qquad \text{Propagation} \qquad (2)$$

$$LOO^{\bullet} + InH \rightarrow LOOH + In^{\bullet} \qquad \text{Termination} \qquad (3)$$

In principle, oxidation could be stopped if alkyl radicals ($L^{\bullet}$) could be scavenged efficiently, thus preventing reaction (1). However, reaction (1) is extremely fast

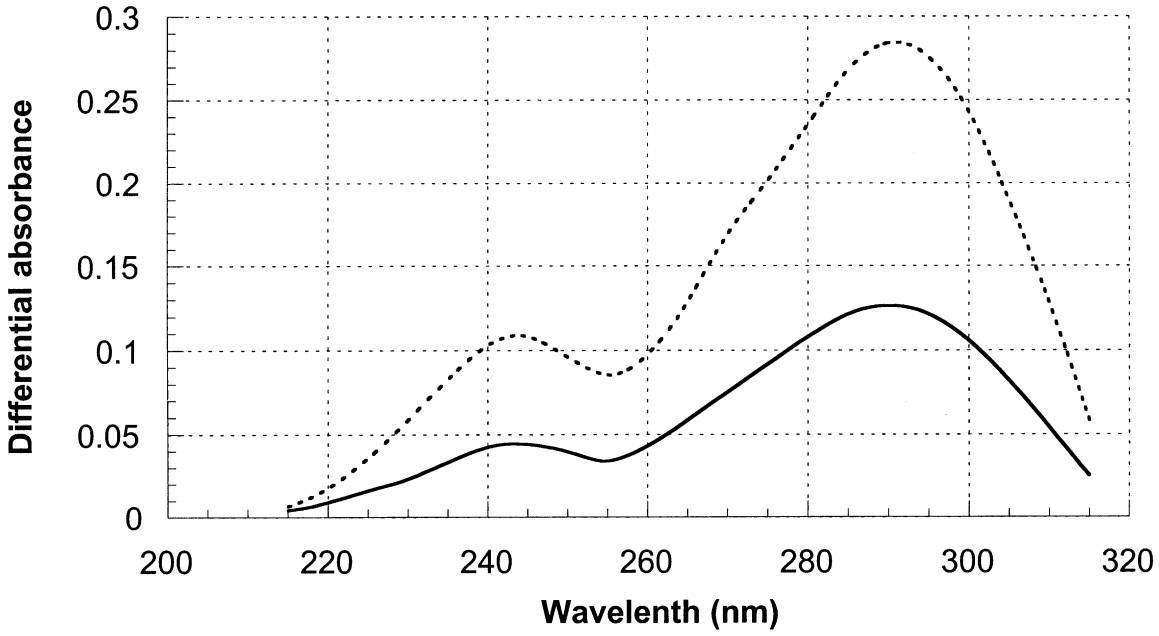


Fig. 2. Differential spectra (pH 12 vs. pH 7) of reduced fractions of TM (0.1 mM). Solid and dashed lines represent $TM_{1r}$ and $TM_{2r}$, respectively.

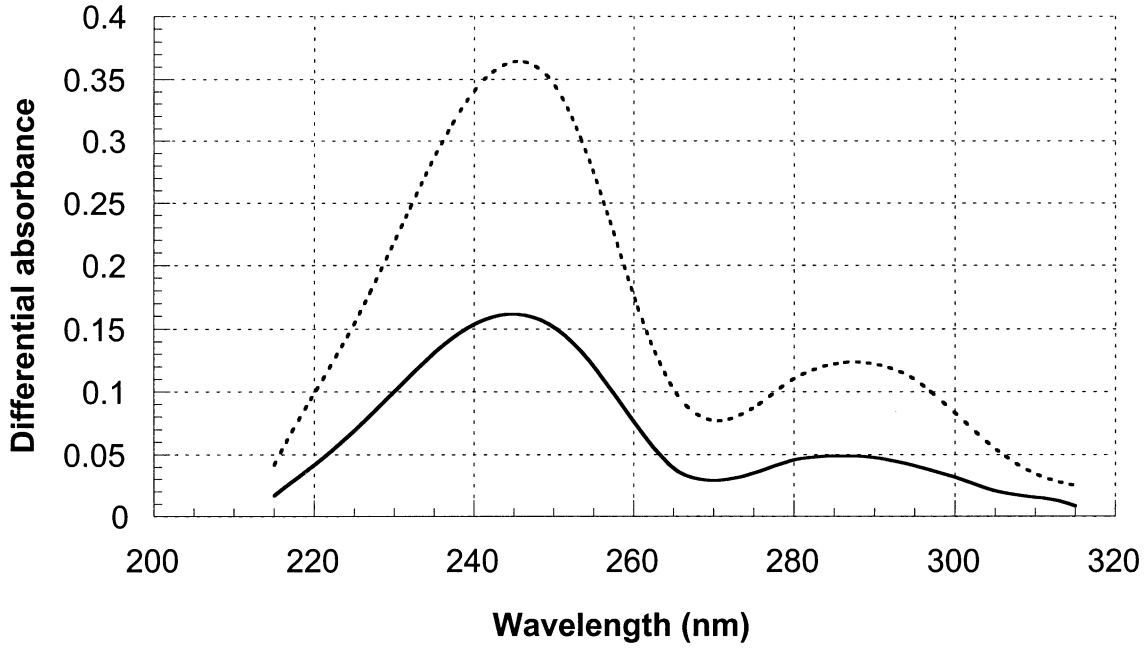


Fig. 3. Differential spectra (pH 12 vs. pH 7) of oxidized fractions of TM (0.1 mM). Solid and dashed lines represent $TM_{1ox}$ and $TM_{2ox}$, respectively.

(Denisov & Khudyakov, 1987). Therefore, free-radical scavengers cannot be effective if there is oxygen present. A peroxyl radical (LOO•) can abstract an atom of hydrogen from a lipid (LH) due to reaction (2), which causes propagation of the free-radical chain. An inhibitor (InH) has to provide a more easily abstractable hydrogen atom than those of the lipids. Thus, competitive reaction (3) provides replacement of active peroxyl radical with non-active radical (In•) that cannot continue the chain.

It follows from the kinetic curve (Fig. 4), that the reduced form of TM behaves as a free-radical chain-breaking antioxidant, which can compete with the β-carotene substrate (LH) for the chain-propagating peroxyl radicals (LOO•), generated by the oxidizing system according to reaction (1). The interaction of active peroxide radicals (LOO•) and inhibitory fragments (InH) of the macromolecule of TM prevents the propagation of the free-radical oxidation of β-carotene. Thus, replacement of the active peroxide radical LOO•

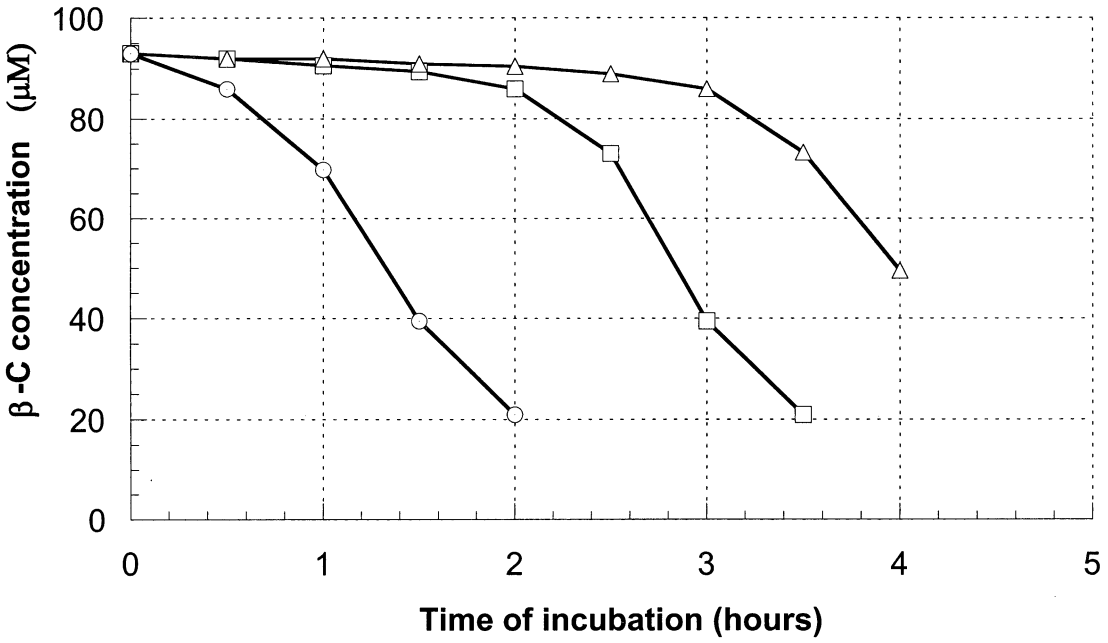


Fig. 4. Kinetic curves of β-carotene consumption at presence of 0.2 μM reduced fractions of $TM_{1r}$ (squares) and $TM_{2r}$ (triangles). Circles represent control (oxidation without TM).

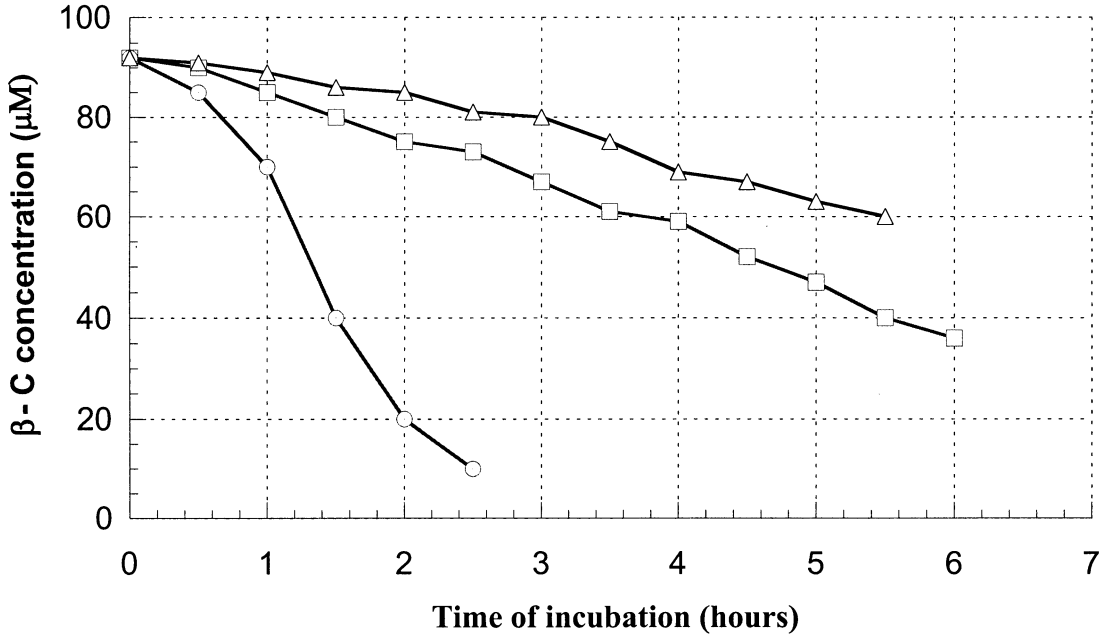


Fig. 5. Kinetic curves of β-carotene consumption in the presence of 0.2 μM oxidized fraction of $TM_{1ox}$ (triangles) and $TM_{2ox}$ (squares). Circles represent control (oxidation without TM).

by the less active (macromolecular) radical In• causes the lag in β-carotene oxidation. During this period, the oxidation ran slowly with rate $W_{inh}$. After the inhibitory capacity of TM was exhausted, the initial rate $W_0$ of β-carotene oxidation was resumed.

The lack of phenolic groups in oxidized TM changed the kinetic behavior of β-carotene oxidation. The lag phase was not observed (Fig. 5). The oxidized TM only diminished consumption of β-carotene. The rates of inhibited oxidation ($W_{inh}$) were left approximately constant during the inhibitory process and were inversely dependent on the concentration ($C_{ox}$) of fully oxidized TM:

$$W_{inh} = K/C_{ox}$$

where $K = (4 \pm 0.8) \times 10^{-14}$ mol $l^{-1}$ $min^{-1}$

Table 1
Kinetic parameters of the antioxidant effect of reduced fractions of TM

| TM fractions | Concentration | | $\tau$[a] (min) | $W_{inh}$ (μ mol $l^{-1}$ $min^{-1}$) | $W_0$ (μmol $l^{-1}$ $min^{-1}$) | $W_0/W_{inh}$ |
|---|---|---|---|---|---|---|
| | TM (μM) | PG (μg/l) | | | | |
| $TM_{1r}$ | 0.05 | 10.8±1.2 | 63±4 | 0.18±0.02 | 1.65±0.13 | 9.2 |
| | 0.1 | 24.5±2.6 | 87±6 | 0.15±0.02 | 1.51±0.1 | 10.1 |
| | 0.2 | 45.1±4.1 | 135±8 | 0.13±0.01 | 1.53±0.12 | 11.8 |
| $TM_{2r}$ | 0.05 | 36.7±4.0 | 84±6 | 0.084±0.005 | 1.32±0.08 | 15.7 |
| | 0.1 | 62.5±5.3 | 126±9 | 0.062±0.007 | 1.24±0.09 | 20 |
| | 0.2 | 138±11.6 | 191±15 | 0.051±0.005 | 1.13±0.08 | 22.1 |

[a] Means±S.D. ($n=4$).

Previous research (Denisov & Khudyakov, 1987) showed that this kind of dependence reflects the participation of quinones (Q) in free-radical reactions. In this case, quinones slow down oxidation, owing to their reaction with alkyl radicals (L•).

$$L^{\bullet} + Q \rightarrow LQ^{\bullet} \quad (4)$$

$$LQ^{\bullet} + L^{\bullet} \rightarrow LQL \quad (5)$$

Kinetic curves obtained with oxidized TM (Fig. 5) can be explained by assuming that reaction (5) follows reaction (4) and can produce the free-radicals (LQ•) of macromolecular nature, which are less active than the alkyl radicals (L•) from β-carotene, thus reducing the generation of peroxyl radicals (LOO•) due to reaction (1). In this case, quinone groups of TM can act as chain-transfer agents.

Thus, the kinetic peculiarities of TM's inhibitory reaction can be explained by the participation of both phenol and quinone groups in free-radical chain reactions. Phenolic groups of TM can terminate propagation of a free-radical chain, causing delay of oxidation, and quinone groups can transfer the free-radical chain, decreasing rate of β-carotene oxidation.

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