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Isolation and characterization of melanic pigments derived from tea and tea polyphenols

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Abstract

The dark brown pigments derived from tea and tea polyphenols were studied. Physical and chemical properties revealed that pigments directly extracted from tea leaves and derived from tea polyphenols were similar to typical melanins. Further investigation showed that both melanic pigments possessed similar antioxidant capability, due to their chelating and scavenging properties. The inhibitory effect of melanic pigments, either from tea or tea polyphenols, was significantly stronger than that of non-treated tea polyphenols. According to these properties, we have extracted melanin from tea. In addition, oxidation of tea polyphenols also provided an alternative method to maximize the yields. The extracted melanin is an antioxidant, which interrupted free radical reactions at a step in the development chain by its scavenging properties and, at the step of initiation, by its ability to chelate metals.

Keywords: Tea polyphenols; Melanin; Antioxidant

1. Introduction

Natural antioxidants can be useful in the prevention of human diseases such as atherosclerosis and cancer (Ames, Shigenaga & Hagen, 1993; Witztum, 1994). Study of tea polyphenols (TPs) suggests that tea is one of the most abundant sources of natural antioxidants (Wiseman, Balentine & Frei, 1997). TPs possess significant antioxidant activity due to their ability to scavenge reactive oxygen species and chelate metal ions (Lorel et al., 1993). However, clinical tests revealed no significant antioxidant activity from consumption of tea (Princen et al., 1998). The contradiction could due to the heterogeneous nature of TPs, polymeric derivatives with a broad range of molecular mass and subsequent polymerization in vivo after consumption of tea. Some human plasma, bile, intestinal and pancreatic juices provide alkaline conditions, which can promote formation of polymeric polyphenols (Yoshino, Suzuki, Sasaki, Miyase & Sano, 1999).

Polymerization of TPs occurs in the presence of polyphenol oxidase (Halder, Tamuli & Bhaduri, 1998) contained in tea leaves and constitutes a spontaneous reaction during the industrial processing of tea, which is known as "fermentation". During "fermentation", some of the catechins combine to create complex theaflavins and other flavonoids that give distinctive flavour and colour to black tea. In addition to flavonoids, black tea beverage contains approximately 15% undefined condensation products (Wiseman et al., 1997). Some of these products are likely to be related to melanins. These dark pigments were found in different plants, such as chestnut, sunflower, beans (Nicolaus, 1968) and grapes (Zherebin, Makan, Sava & Bogatsky, 1982), which contain abundant polyphenols. However, the highly polymerized melanic pigments (MPs), formed in tea leaves, have not been investigated yet.

MPs may be extracted directly from tea by alkali or purified from oxidized TP. Our work focused on the isolation of MPs from tea and TPs, characterization of the physical-chemical properties compared to standard synthetic melanin and characterization of the antioxidant activities.

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2. Materials and methods

2.1. Chemicals

Chinese black tea was purchased from local retail shops. Synthetic melanin was purchased from Sigma Chemical Co. (St. Louis, MO). It was used without any further treatment as a reference. Nitro blue tetrazolium (NBT), βnicotinamide adenine dinucleotide reduced form (NADH), phenazine methosulfate (PMS), epigallocatechin gallate (EGCG), Folin-Ciocalteu's Phenol Reagent (FCPR), thiobarbituric acid (TBA), trichloroacetic acid (TCA), phosphate buffer saline (PBS), Tris-HCl buffer, ethylenediaminetetraacetic acid (EDTA), Superdex and Sephadex G-50 were purchased from Sigma Chemical Co. All other reagents were chemical reagent grade from Merck. The solvents were of high-performance liquid chromatography (HPLC) grade or the highest grade available.

2.2. Isolation of TP

Isolation of TP was carried out by a conventional technique (Vinson & Dabbagh, 1998). Aqueous infusions were prepared by extracting fully fermented black tea (Miaoli, Taiwan) with boiling water at volume ratio (solid/liquid) of 1:10 for 10 min followed by filtration to remove solid matter. TP were further purified by partitioning extract first between ethyl acetate and aqueous phase, and then between methylene chloride and water. The phases were taken in equivalent volumes. The final purified extract was obtained using a lyophilizing procedure. Total content of TP was measured with the FCPR using EGCG as the reference standard.

2.3. Isolation of natural MP

Extraction of MP was performed according to the basic procedure designed for grape melanin with minor adjustment (Zherebin et al., 1982). Wet tea leaves obtained after extraction of TP were immersed in water at the volume ratio 1:10, followed by addition of 10% NH_4OH to adjust the pH to 10.5 (final NH_4OH concentration was about 2%). After 36 h incubation at 40°C, the mixture was filtered, followed by centrifugation at 15,000 ***g*** for 30 min to obtain MP extract (Fig. 1). The latter was acidified with 2 N HCl to pH 2.5, incubated at room temperature for 2 h, and centrifuged at 15,000 ***g*** for 15 min to give a pellet of MP. To follow the accumulation of polymeric substances, aliquots of 9 ml were sampled from the extract periodically. Each aliquot was acidified with 1 ml 2 N HCl and centrifuged at 10,000 ***g*** for 10 min. Pellets were collected and analyzed.

2.4. Oxidation of TP and isolation of artificial MP

Purified TP (Fig. 1) were dissolved in de-ionized water at a final concentration of 7 mg/ml. The solution was adjusted to pH 11 with 10% NH_4OH and then placed in a tightly closed vessel supplied with stirrer and connected to an oxygen gauge. Incubation temperature was set to 40°C. The solution was saturated with oxygen, and the stirring speed was adjusted as necessary for steady oxidation.

After 72 h, the oxidized TP were acidified with 2 N HCl to pH 2, followed by centrifugation at 15,000 ***g*** for 15 min. The resulting pellet was dissolved in 1 N NH4OH and spun at 10,000 ***g*** for 15 min. The pH of the collected supernatant was adjusted to 2 by the addition

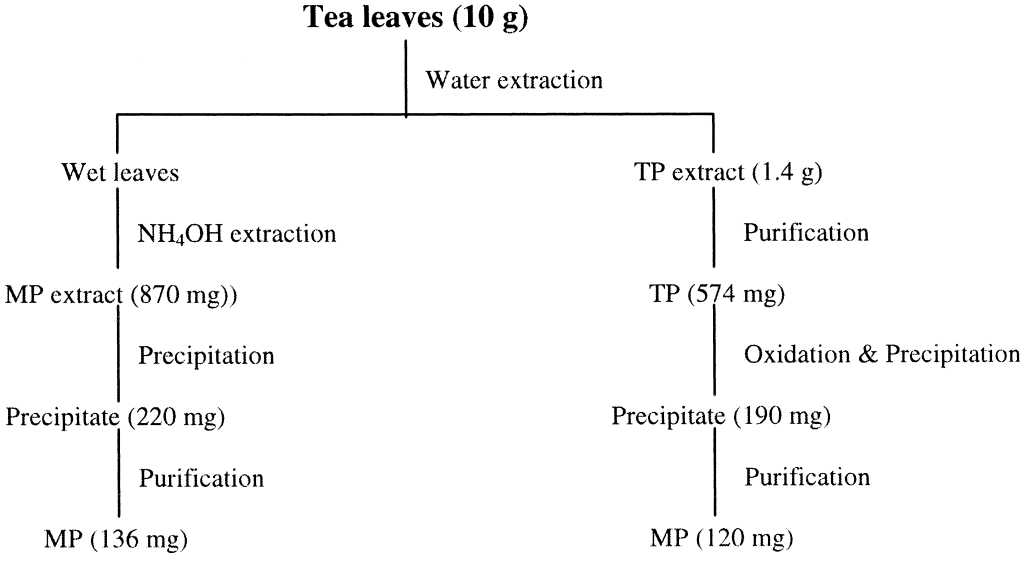

Fig. 1. The procedure for extraction of melanic pigments from tea and tea polyphenols.

of 2 N HCl, followed by centrifugation at 10,000 ***g*** for 15 min. The above precipitation procedure was repeated two more times.

During the course of oxidation, aliquots of 9 ml were periodically sampled from the oxidized mixture to follow the accumulation of polymeric substances. Each sample was acidified with 1 ml 2 N HCl and centrifuged at 10,000 ***g*** for 10 min. The pellets were collected and analyzed.

2.5. Purification of MP

The extracted MP was purified (Fig. 1) by acid hydrolysis, organic solvent treatment and repeated precipitation. The acid hydrolysis was employed to remove carbohydrates and proteins. Organic solvents were used to wash away lipids. Multiple precipitation allowed separation of MP from low molecular-mass polyphenols.

The samples of MP were hydrolyzed with 7 M HC1 for 2 h at 100°C, followed by centrifugation at 10,000 ***g*** for 10 min, and washing with water. The non-hydrolysable MP were washed sequentially with chloroform, ethyl acetate and ethanol and then dried. The solid matter of each sample was re-dissolved in 1 N NH_4OH and mixture was centrifuged at 6000 ***g*** for 10 min. The supernatant was acidified with 1 N HCl and the precipitate was washed with water. The re-precipitation was repeated four times. The residue was washed with water until the reaction for chloride ions was negative.

MP solution was prepared by the following procedure. The precipitate was dissolved in water made slightly alkaline with 0.5 N NH_4OH to pH 9, incubated at 50°C for 1 h and centrifuged at 10,000 ***g*** for 15 min. The ammonia was removed by rotary evaporation under reduced pressure to a final pH of 7.5. The content of MP was measured with the FCPR using EGCG as the standard.

2.6. Physico-chemical characterization of MP

Physical and chemical characteristics of MP were obtained according to different procedures (Nicolaus, 1968; Paim, Linhares, Magrich & Martin, 1990; Prota, 1992). Solubility in water, aqueous acid, and in common organic solvents; 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 primary characteristics of MP. The elemental composition was determined with a Vario EL analyzer (EAS GmbH, Germany). Ultraviolet-visible (UV) absorption spectra were recorded on a JASCO V-530 UV-Visible Spectrophotometer. Infrared (IR) spectra were recorded on a Perkin Elmer spectrometer (Model 1600 FT). KBr samples were obtained from uniformly prepared mixture contained 2 mg of sample and 150 mg spectrometric grade KBr. MP were chromatographed through Sephadex G-50 in 50 mM Tris-HC1 (pH 7.8) at a flow rate of 1 ml min^{-1}. The column's dimensions were 1.640 cm. Fractions were monitored at 280 nm.

2.7. Assessment of antioxidant properties of MP

Antioxidant properties of MP were investigated, based on the hypotheses of initiation of free radical chain reaction (chelating Fe^{2+}), chain development (scavenging superoxide radicals) and lipid peroxidation [oxidation of low-density lipoproteins (LDL)].

Chelating activities of MP and TP was evaluated by the ability to complex with Fe^{2+} under alkaline condition (Iwasa & Torii, 1962). 0.25 ml of 6.96 mM ferrous sulfate, 0.25 ml of 14.2 mM potassium sodium tartrate, 0.5 ml of sample solution, and 0.5 ml of water were added to 1.5 ml of 25 mM phosphate buffer (pH 8.0). The formation of blue complexes was measured by absorbance at 540 nm. The chelating activities were expressed as the absorbance ratios of MP-Fe^{2+} complexes to the absorbance of TP-Fe^{2+} complex. The sample concentrations were equivalent to 25 μM EGCG according to the analysis with FCPR.

Scavenging activity against superoxide anions was measured by the ability to inhibit the reduction of NADH and PMS mediated by NBT (Nishikimi, Rao & Yagi, 1972). Superoxide anions were generated in assays containing 0.5 ml of 15 νM PMS, 0.5 ml of 200 μM NBT, 0.5 ml of 750 μM NADH and 0.5 ml of sample solution and incubated at 25°C. The reaction was monitored at 560 nm, and the inhibition ratio was calculated by using the initial rate of reaction. The results were expressed as percentage inhibition. The sample concentrations were equivalent to 25 μM of EGCG according to the analysis with FCPR.

LDL was isolated from porcine plasma in the density range 1.019–1.063 g cm^{-3} by ultracentrifugation, as previously described (Huang, Wang, Wang, Sun, Chu & Mao, 1999). Porcine plasma was isolated from animal blood by centrifugation for 15 min at 3000 *g* and 4°C in the presence of 0.3 mM EDTA, followed by ultracentrifugation at 40,000 ***g*** for 20 h at 10°C. Lipids of very low density were removed as the top layer. Potassium bromide was added to a final density of 1.063 g cm^{-3} followed by ultracentrifugation at 40,000 ***g*** and 10°C for 20 h. Typically, 3 ml of 95% pure LDL was derived as the top layer from each preparation of 50 ml porcine serum. The purity of LDL was accentained by HPLC using Superdex gel filtration. The isolated LDL was dialysed against PBS to remove KBr. Protein concentration was measured by absorbance at 280 nm. Typical protein concentrations were about 5 mg ml^{-1} using this procedure.

The resistance of LDL to copper-mediated oxidation was determined as previously described (Huang et al.,

1999) with minor modifications. In short, 250 μg of LDL was incubated with 500 μM of cupric sulfate in PBS at a final volume of 50 μl. The concentrations of TP and MP added to the LDL were equivalent to 25 μM of EGCG. The mixture was incubated at 37°C. Reaction was terminated by the addition of an excess of EDTA.

TBA was employed to monitor oxidation. The colorimetric TBA assay (Huang et al., 1999) with minor modifications was utilized to define lipid peroxidation. Fifty microlitres of sample were added to 300 μl of 20% of TCA, followed by 300 μl of 0.67% TBA in 0.05 N NaOH and mixed vigorously. The mixture was incubated 30 min at 90°C to complete the TBA reaction with malondialdehyde (MDA), followed by centrifugation at 10,000 ***g*** for 3 min. Absorbance was measured at 540 nm using an ELISA plate reader (MR 7000 4186). Accumulation of MDA was monitored at 5-min intervals for 2 h. The parameters determined from the LDL-oxidation profile were lag time and oxidation rate. The lag time was determined as the intercept of the baseline and the propagation phase of the oxidation curve, and was expressed in minutes. The oxidation rate was equal to the slope during the propagation phase. All experiments were performed in triplicate.

3. Results

During the extraction of TP, the mean yield of crude tea extract (dry matter) was 14%, which itself contained 41% TP measured by FCPR (Fig. 1). The oxidative polymerization of TP in alkaline media depended on the concentration of dissolved oxygen. At lower stirring speed the oxygen consumption rate increase, based on the stirring speed (Fig. 2), indicated that the oxidation is limited by oxygen diffusion from gas phase to liquid. The oxygen consumption reached a plateau at higher stirring speed and was determined by the chemical reactivity of TP. In any case, increasing of the stirring speed changed the mode of oxidation from diffusion to kinetic mode. The kinetic mode was essential to maximize the yield of TP polymeric products.

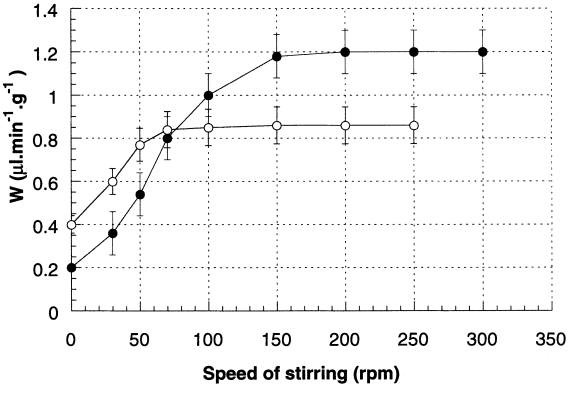

Fig. 2. Dependence of the oxygen consumption rates (W) on the speed of stirring (rpm) and concentration of tea polyphenols in oxidizing mixture at 40°C. Concentration of TP is 7 mg/ml (filled circles) and 5 mg/ml (open circles). Values are means of three measurements.

We monitored formation of polymeric substances during the oxidation process (Fig. 3). At 40°C, absorbance (*A*) and yield of precipitated substances increased as oxidation proceeded and both curves reached saturation level in 48 h. However, *A* did not increase proportionally to the accumulation of polymeric substances. Therefore, *A* is caused, not only by the accumulation of polymeric material, but also by changes in the molar absorbance.

Employing ammonia, as an addition to the water for extraction, allowed extra 8.7% yield (Fig. 1). The kinetic behaviour of MP accumulation under direct extraction is depicted in Fig. 3. The yield increased rapidly and reached a plateau after 12 h. The absorbance increased proportionally (not displayed). The further slow accumulation of MP above the plateau level may be due to the formation of polymeric compounds by TP oxidation.

To evaluate the molecular mass (MM) of MP, a Sephadex G-50 column was calibrated with dextran blue (MM 2,000,000 kDa), aldolase (MM 158,000 kDa), bovine serum albumin (MM 66,000 kDa), cytochrome C (MM 12,400 kDa), and vitamin B12 (MM 1360 kDa) as size markers. The apparent molecular mass of MP was estimated by formula:

$$MM = 10^{(4.2\text{-}Ve/20)}$$

where Ve is elution volume (ml).

The apparent molecular masses for MP derived from tea and TP were 143 and 83 kDa, respectively.

The amorphous dark-brown pigments extracted from oxidized TP and directly from tea presented all the physical and chemical properties common to natural melanins 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, bleached in H_2O_2, $KMnO_4$, $K_2Cr_2O_7$ and NaOCl, and produced a blue colour in $FeSO_4$/ferricyanide.

The two polymeric substances derived from tea and TP were compared with standard synthetic melanin. Some of their characteristics are summarized in Table 1, which shows the similarity between MP derived from tea or from TP and synthetic melanin. The elemental compositions obtained for various batches of MP extracted directly from tea (C = 54.23±6.34%; N = 3.17± 0.52%; H = 4.23±0.54%) and of MP derived from TP (C = 49.81±5.11%; N = 1.96±0.45%; H = 3.81±0.68%) were comparable to each other and to synthetic melanin

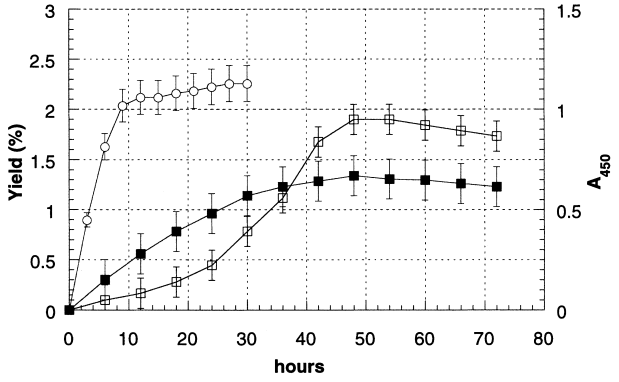

Fig. 3. Accumulation of melanic pigmants (MP) during the direct extraction of MP (open circles represent yields) and during oxidation of tea polyphenols (open squares represent yields; filled squares represent A_{450}). The yields are calculated from the dry weights of MP and weight of initial tea. Values are means of four measurements.

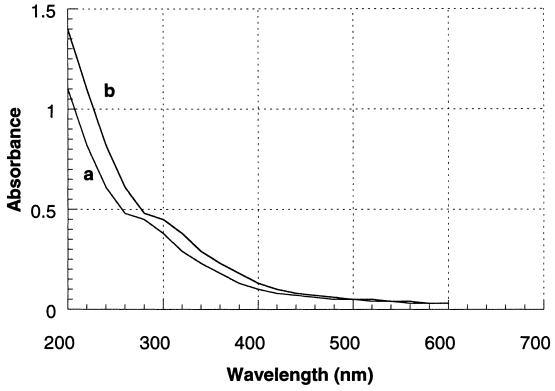

Fig. 4. UV spectra of melanic pigments (MP) derived from (a) tea and (b) tea polyphenols. Concentration of MP was 100 mg/ml.

Table 1
Comparative characteristics for melanic pigments (MP) and synthetic melanin[a]

Tests	MP derived from tea	MP derived from oxidized tea polyphenols	Sigma's melanin
Solubility in H_2O at 25°C	N	N	N
Solubility in organic solvents	N	N	N
Solubility in 1 N NH_4OH at 25°C.	43 mg/ml	54 mg/ml	20 mg/ml
Precipitation by HCl	P	P	P
Reaction with $KMnO_4$	P	P	P
Reaction with NaOCl	P	P	P
Reaction with H_2O_2	P	P	P
Reaction with $K_2Cr_2O_7$	P	P	P
Reaction with $FeCI_3$	P	P	P

[a] N, negative response; P, positive response

(Nicolaus, 1968). The major difference was found in values of N%. Before the acid hydrolysis, contents of nitrogen in both MP was about 6%. After the multiple hydrolysis, N% was reduced to a constant level. It was found that acid hydrolysis led to a loss of contaminated proteins. Also, acid hydrolysis decreased ash content from an initial 0.72±0.11% to 0.09±0.01%.

The low ratios of H/C indicate the aromatic nature of MP. The residual amount of nitrogen can be connected to its incorporation into the backbone of MP. Such an amount of nitrogen is not enough to suggest an indole melanin nature. However, MP may contain some indole since peroxidase oxidation of tyrosine has been demonstrated (Digendra & Mukherjee, 1973). Also, MP may contain caffeine fragments, which were verified owing to the presence of trimethylxanthine derivatives discovered in melanin hydrolysis products.

Solutions of MP in 0.1 M phosphate buffer, pH 8.0, exhibited strong absorbance (Fig. 4) over a wide spectral range. The spectra of MP extracted from tea and derived from TP were similar. The spectra showed a distinct shoulder at 275–280 nm that is typical (Prota, 1992) for melanins. The plots of logarithm absorbance versus wavelength, obtained for samples with a concentration of 100 mg/ml, gave lines with negative slopes. The slopes were −0.0013, −0.0019, and −0.0018 nm^{-1} for MP derived from tea, MP derived from TP, and for synthetic melanin, respectively, similar to melanins extracted from *Epicoccum nigrum* and *Vertieillium dahliae* (Ellis & Griffiths, 1974).

IR-spectroscopy was employed to study structural characteristics of MP (Bilinska, 1996; Paim et al., 1990; Flip, Haider, Beutelspacher & Martin, 1974). IR spectra for MP derived from tea and TP were very similar to each other (Fig. 5a and b). Both spectra showed similar bonding characteristics to the spectrum of synthetic melanin (Fig. 5c).

The major differences were at 2920 and 2850 cm^{-1}, assigned to stretching vibrations of aliphatic CH groups. These bands were much reduced in synthetic melanin (Fig. 5c). The multiple treatment of both MP with organic solvents (chloroform and ethyl acetate) reduced, but did not abolish the absorbance. Another difference was found at 1720 cm^{-1}, assigned to the free carboxylic group COOH. Chelating Fe^{2+} by MP completely eliminated the band at 1720 cm^{-1} (results not shown) but generated two new bands at 1560 and 1380 cm^{-1}. Acid treatment of MP restored the band at 1720 cm^{-1}. An important feature in the IR spectrum of MP was the presence of a wide band at 3450 cm^{-1}, attributed to stretching vibrations of OH and NH_2 groups. A strong band at 1650 cm^{-1} was due to vibrations of aromatic C=C, or of C=O groups. After acid hydrolysis of MP, the broad band at 3450 cm^{-1} and the band at 1650 cm^{-1} were reduced, a phenomenon possibly caused by reaction between phenolic and carboxylic groups to form lactones.

We explored the efficiency of MP in protecting LDL against oxidation induced by copper ions. The kinetics of LDL oxidation monitored by the generation of MDA

is depicted in Fig 6. The concentration of MDA reached a constant level in 30 min. Incorporation of TP had no significant effect on LDL oxidation, employing MP strongly reduced this oxidation. At a concentration equivalent to 25 μM of EGCG, MP displayed a lag time of 18–26 min (Table 2).

The interaction of MP with superoxide free radicals was also investigated. The formation of superoxide radicals was monitored by the accumulation of NBT diformazane at 560 nm. The kinetic curve of its accumulation is representative of a reaction of the first order. Addition of MP decreased the initial rate of diformazane formation. Incorporation of MP, derived from TP at a concentration equivalent to 25 μM of EGCG, inhibited this reaction by 84%. The initial rate of NBT reduction depended linearly on the concentration of MP (Fig. 7). Comparative data, representing scavenging activities of both MPs and TP, are summarized in Table 2.

Chelating activity against Fe^{2+} was also determined. All data obtained were normalized (Table 2) with the chelating activity of TP set at 1. Relative Fe^{2+}-chelating activity represents the absorbance ratio of Fe^{2+}-MP to Fe^{2+}-TP. MP directly extracted from tea, displayed higher chelating ability than MP derived from TP (3.2 versus 2.2).

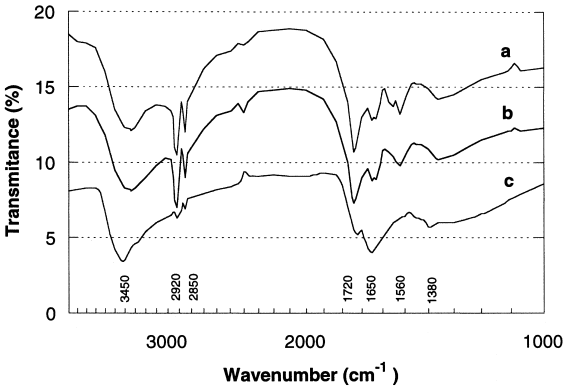

Fig. 5. IR spectra of melanic pigments derived from (a) tea and (b) tea polyphenols in comparison with (c) synthetic melanin.

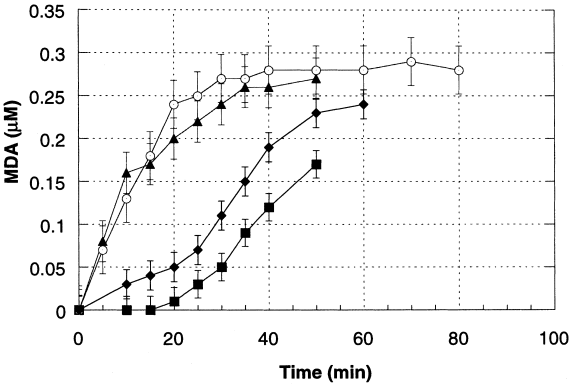

Fig. 6. Kinetic of low-density lipoproteins oxidation in presence of tea polyphenols (TP) (filled triangles), of melanic pigments (MP) derived from TP (filled squares) and of MP derived from tea (filled diamonds). All compounds were taken in equal concentrations of 25 μM. Control represented by open circles. Values are means of three measurements.

Table 2
Comparison of antioxidant activities of tea polyphenols (TPs) and melanic pigments (MP)

Tested compounds	Lag time of LDL oxidation, min	Scavenging activity (% of inhibition)	Relative Fe^{2+} chelating activity
TP	0	45±5	1
MP derived from TP	26±3[a]	84±7	2.2±0.3
MP extracted from tea	18±3	68±8	3.2±0.4

[a] Values are mean ± S.D. ($n=3$).

4. Discussion

In this study, we have isolated melanins from tea and also from tea polyphenols for the first time. Characterization of these tea melanins indicated that they possessed physical and chemical properties very similar to melanins extracted from other sources.

UV spectra (Fig. 4) of extracted MP resembled the spectrum of synthetic melanin, taken as the reference material. IR spectra (Fig. 5) also presented all the basic characteristics of synthetic melanin. Some minor differences were found in the area of 2920–2850 cm^{-1}, probably due to aliphatic impurities, treatment with several organic solvents reducing the absorption. The polymeric compounds obtained, displayed similarity in elemental composition and possessed all basic physical and chemical properties specific to melanin pigments (Table 1). They were insoluble in water, acids and organic solvents. They were soluble in alkaline media and could be

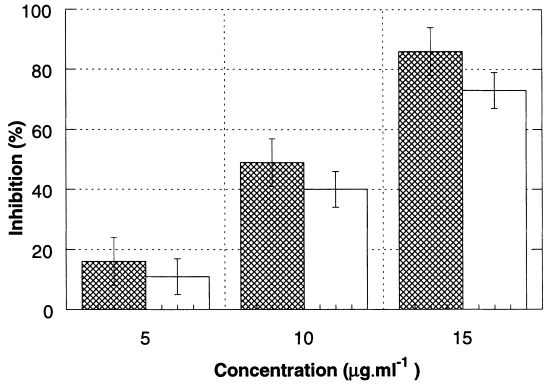

Fig. 7. Inhibitory effect of different concentrations of melanic pigments derived from tea (open bars) and from tea polyphenols (filled bars) on the reaction of nitro blue tetrazolium reduction mediated by superoxide radicals. Values are means of three measurements.

precipitated by acidification to below pH 3. The compounds also gave all the qualitative reactions, usually employed for identification of melanins: formation of coloured complexes with FeSO4 and bleaching by different oxidizing agents (H_2O_2, $KMnO_4$, $K_2Cr_2O_7$ and NaOCl).

Melanin pigments could be formed during the growth of tea plant or the subsequent "fermentation". Melanin formation in tea is probably caused by the abundance of polyphenols and the presence of specific enzymes, such as polyphenol oxidase (Halder et al., 1998). During the "fermentation" of tea, these enzymes come into contact with the polyphenols and accelerate their oxidation. The experimental results showed it possible to produce melanin by non-enzymic oxidation of tea polyphenols. The polymeric compounds isolated and purified from the oxidation mixture of TP were almost identical to the compounds directly extracted from tea. The yield of directly extracted MP was 1.35%, slightly surpassing the yield of MP (1.2 %) obtained by oxidation of TP.

Polyphenols of high molecular mass showed advantages against low molecular mass compounds. For instance, polymeric polyphenols from oolong tea have been found to inhibit glucosyltransferase (Hamada et al., 1996). This might be due to their lower susceptibility to enzymic hydrolysis and glucosylation in vivo. Thus, such compounds could work more effectively and longer compared to polyphenols of low molecular mass. Also, high molecular mass polyphenols possess stronger chelating properties than low molecular mass ones (Yoshino et al., 1999).

We have studied the antioxidant activity of MP obtained on the basis of the LDL oxidation model. The tea melanin retarded the oxidation of LDL. We showed that MP derived from oxidized TP possessed a slightly greater protective activity compared to MP obtained by direct extraction from tea (Fig. 6). However, TP showed a lack of antioxidant activity in this model, probably due to specific properties of porcine LDL. The hydrophilic properties of TP may also play a negative role here by preventing their incorporating into the lipophilic LDL. Melanins are hydrophilic compounds as well, but they might bind to apolipoprotein B due to their strong protein-binding property (Prota, 1992).

To understanding these results better we have characterized the chelating ability of MP in relation to Fe^{2+}. We showed that the chelating power of MP was higher than that of TP (Table 2). In addition to chelating activity, studies of the interaction of MP with superoxide free radicals showed that the scavenging activity might play a role in the protective property. MP gave a significant inhibition of the free radical reaction of NBT (Table 2). The scavenging activity of MP extracted from tea was 1.87 times higher than that of TP, the corresponding figure for MP derived from TP being 1.51 times.

The antioxidant activity of MP may well be due to a combination of chelating and scavenging characteristics. Thus, MP can be considered as an antioxidant, which can interrupt free radical reactions at the step of chain development (due to the scavenging properties) or at the step of the initiation of free radical reactions (due to chelating of metals). Obviously, the overall antioxidant activity of tea comprises both that of TP and that of MP. The activity of MP may be based on several structural features and these require further study. Since MP possess an inhibitory effect, which is stronger than that of TP, they could well lead to the development of new health products.

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