Research Article

Journal of PeptideScience

Received: 16 June 2008 Revised: 1 December 2008 Accepted: 3 December 2008 Published online in Wiley Interscience: 10 February 2009

(www.interscience.com) DOI 10.1002/psc.1116

The oxidation metabolites of endomorphin 1 and its fragments induced by free radicals

Pin Gong,[a] Fu Xin Chen,[a] Qianyu Zhao,[a] Guo Fen Ma[a] and Rui Wang[a,b]*

Endomorphin 1 (EM1), an endogenous μ-opioid receptor agonist, acts as a free radical scavenger *in vitro* and an antioxidant *in vivo*. The modification of EM1 by ROS and the properties of the OM attracted our attention. *In vitro* assays were performed via RP-HPLC, spectrophotometric measurements, EPR and amino acid analysis, Schmorl's reaction to define the formation of melanin-like compounds transformed from EM1, collectively named EM1–melanin and by solubility assay, radioligand-binding assay, NADH oxidation, superoxide anion scavenging assay to study some physical and chemical properties of EM1–melanin. Possible pathways of the formation of EM1–melanin were proposed. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: endomorphin 1; ROS; EM1–melanin; oxidative metabolite

Introduction

Melanins, widespread high-molecular-weight pigments in nature, are efficient photoprotective agents against solar radiation and have the capacity of scavenging ROS [1,2]. Melanins are classified into two major types, i.e. brown–black eumelanin composed of indole units derived from DOPA and yellow to reddish brown pheomelanin pigment composed of benzothiazine units derived from cysteinyldopas [3]. Melanins are found in the skin, in sensory organs and in some specific regions of the mammalian brain such as substantia nigra and locus coeruleus (as neuromelain) [4]. According to the general views, the starting materials for melanin synthesis are Tyr or cathecols which are converted by means of tyrosinase through the Mason–Raper pathway into the pigmented polymers [5]. In the last years, researches have demonstrated that opioid peptides such as esorphins, kyotorphins and enkephalins are substrates for mushroom tyrosinase and for plant and mammalian peroxidases and are converted into melanin-like compounds retaining the peptide moiety [6–8]. Moreover, a series of studies have documented that besides peptidases oxidative enzymes and ROS can also act upon enkephalins and also the Tyr amino-terminal peptides, Tyr-Gly and Tyr-Gly-Gly, produced *in vivo* by the action of peptidase [9], resulting in the formation of opio-melanin mainly due to the conversion of *N*-terminal Tyr to dopa [10–13]. These peptides exhibited a common chemical feature that the presence of a Tyr residue in the amino-terminus is essential for their polymerization [2,10–12].

EM1 (Tyr-Pro-Trp-Phe-NH_2) and EM2 (Tyr-Pro-Phe-Phe-NH_2) are endogenous μ-opioid receptor agonists [14]. In addition to its classical role in antinociception, we have found that EMs directly scavenge galvinoxyl radicals and AAPH-derived alkyl peroxyl radicals, inhibit LPO, DNA and protein oxidative damage [15] and that EMs inhibit the oxidation of human oxLDL induced by Cu^{2+} and AAPH in a concentration-dependent manner, due to their free-radical-scavenging activities [16]. We also demonstrated that EMs can effectively protect brain mitochondria oxidative stress induced by *in vitro* anoxia–reoxygenation [17]. More recently, we found that EM1 can function as an antioxidant *in vivo* against oxidative damage induced by cadmium intoxication [18]. Thus, the fact that EM1 acts as an antioxidant *in vitro* and *in vivo* and bears the *N*-terminal Tyr in common prompted us to investigate whether EM1 and also its fragments Tyr-Pro-Trp-Phe-OH, Tyr-Pro-Trp-OH, Pro-Trp-Phe-NH_2, produced by the action of peptidase [19,20], act like enkephalin to react with ROS to produce melanin-like oxidative products. So far, *in vitro* experiments, including EPR, HPLC, UV-VIS measurement, amino acid analysis and Schmorl's reaction, solubility assay, radioligand-binding assay, NADH oxidation and superoxide anion assay, were performed to evaluate the formation and the properties of the OMs. AAPH and H_2O_2–Fe^{2+} were

* *Correspondence to: Rui Wang, School of Life Science and State Key Laboratory of Applied Organic Chemistry, Key Laboratory of Preclinied Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Institute of Biochemistry and Molecular Biology, Lanzhou University, Lanzhou 730000, China. E-mail: wangrui@lzu.edu.cn*

a *School of Life Science and State Key Laboratory of Applied Organic Chemistry, Key Laboratory of Preclinied Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Institute of Biochemistry and Molecular Biology, Lanzhou University, Lanzhou 730000, China*

b *State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, Gansu 730000, China*

Abbreviations used: *EM1, Endomorphin 1; OMs, oxidative metabolites; ROS, reactive oxygen species; EMs, endomorphins; oxLDL, low density lipoprotein; AAPH, 2,2′-azobis (2-amidino-propane) dihydrochloride; HO•, hydroxyl radical; HOSu, N-hydroxysuccinimide; DCC, N,N′-dicyclohexylcarbodiimide; NBT, nitroblue tetrazilium chloride; NADH, β-nicotinamide adenine dinucleotide reduced form; PMS, phenazine methosulfate; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; [^{3}H] DAMGO, [^{3}H][D-Ala^2, N-methyl-Phe^4, Gly-ol^5]enkephalin; EPR, electron paramagnetic resonance; Dopa, dihydroxyphenyl alanine; LPO, lipid peroxidation.*

employed to initiate free radical reactions *in vitro*. To the best of our knowledge, this is the first-ever study regarding the OMs of EM1 *in vitro*.

Materials and Methods

Drugs and Animals

Peptides were synthesized following the reported methods [21]. Briefly, we employed a solution methodology using Boc-amino protection groups, HOSu and DCC as coupling reagents. Deprotection of Boc-amino protecting groups was accomplished with 50% TFA in CH_2Cl_2. All peptides were purified by preparative RP-HPLC on a Delta C18 column (Waters, 5 μm 300 Å 7.8 × 300 mm) with a linear gradient of 20–100% B in 30 min at a flow rate of 2.4 ml/min (A is 0.05% TFA in water and B contains 0.05% TFA in acetonitrile). Each peptide was more than 95% pure as determined by analytical RP-HPLC on a Delta C18 column (Waters, 5 μm 300 Å 3.9 × 150 mm) using a linear gradient of 20–100% B in 30 min at a flow rate of 0.6 ml/min, with UV detection at 280 nm. Molecular weights of all synthetic peptides were confirmed by ESI-MS (Mariner 5074).

AAPH, NBT, NADH, PMS and DPPH were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Radioligands [^{3}H] DAMGO were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other reagents were analytic grade products.

Male Wistar rats (250 g) were housed in the temperature- and humidity-controlled room with a 12-h light/dark cycle and a free access to standard pellet diet and drinking water. The study was approved by the Ethics Committee of Lanzhou Medical College. All the experiments were carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

OMs Production

Two models were employed to produce OMs: (1) 20-mg peptide was incubated at 37 °C in the presence of 233-mg AAPH in 10 ml of 50-mM phosphate buffer at pH 7.4 and (2) 1-mM peptide was incubated at 37 °C in the presence of 0.2-mM ferrous ammonium sulfate, 0.2-mM EDTA and 5-mM H_2O_2 in 50-mM phosphate buffer at pH 7.4. The concentration of H_2O_2 was measured using the molar extinction coefficient of 72.4 M^{-1} cm^{-1} at 230 nm [22].

After 24-h incubation, the reaction was stopped by adding 1-M HCl, and the mixtures were centrifuged at 12 000 g for 30 min. The supernatant was removed. The pellet was washed three times with 1-ml and 0.1-M HCl, centrifuged as above, and dialyzed overnight against de-ionized water. After lyophilization, a yellow-to-brown powder was obtained, which was stored in −20 °C until use [10].

HPLC Analysis and Spectrophotometric Measurements

HPLC analysis was performed with a Waters-millipore apparatus. Samples were applied on a reverse-phase condition (A = 0.05% TFA in water and B = 0.05% TFA in acetonitrile, 1 ml/min, a linear gradient of 20–100% B in 30 min). Aliquots of the incubation mixtures were collected every hour, filtered and injected in the column. The amount of effluents, monitored at 280 nm, was calculated by automatic peak area integration.

Spectrophotometric measurements were performed with a Hitachi model 557 UV spectrometer (Hitachi High Technologies, Japan) in 1-cm thermostatic cuvettes at 37 °C. Parallel samples without substrate were used as controls.

EPR Measurements

EPR studies were carried out according to the literature method [23]. Dried samples were examined at 25 °C using a Buker ER 200D-SRC spectrometer at X band. All EPR measurements were carried out at a modulation amplitude of 5 G and a microwave power of about 10 mW. DPPH was used as standard for g-value measurements.

Amino Acid Analysis

Ten-milligram EM1 or OMs from EM1 was hydrolyzed in 6-M HCl under vacuum for 22 h at 120 °C. However, the Trp content was determined by means of alkaline hydrolysis [24] by dissolving the samples in 4-M NaOH and hydrolyzing for 22 h at 120 °C. Amino acids were assayed by using an automatic and high-speed amino acid analyzer (Model 835-50, Hitachi Ltd. Japan) as described by Di Girolamo *et al.* [25].

Schmorl's Reaction

Schmorl's reaction was carried out as described by Ragnelli *et al.* [26]. OM from EM1 was mixed 1 : 1 with ferricyanide solution (three parts 1% ferric chloride and one part freshly prepared 1% potassium ferricyanide) [27]. The reaction was also performed with a suspension of synthetic melanin that had been formed from the auto-oxidation of 5-mM l-DOPA [28]. As controls, the distilled water and EM1 solution also were diluted (1 : 1) with the ferricyanide solution. Twenty minutes after incubation, the controls, the reacted OMs or synthetic dopa-melanins were dropped onto slides and photographed.

Physical and Chemical Properties of OMs

Solubility Test

The solubility of OMs were tested by dissolving 0.1 mg samples in 2 ml of the following solutions respectively: 0.05-M glycine–HCl buffer (pH 2–4), 0.05-M phosphate buffer (pH 5–7.4), 0.05-M Tris–HCl (pH 8–12), 1-M HCl, 1-M KOH. Solutions were kept in the dark in 37 °C for 1 h. The optical density of the supernatant was read at 330 nm after being centrifuged at 2000 g for 10 min [29].

Radioligand-Binding Assay

Membranes were prepared from eight Wistar rat brains (without cerebellum) according to the reported method [30] and then combined together. All binding experiments were performed in 50-mM Tris–HCl buffer, pH 7.4, at a final volume of 0.5 ml containing 300–500 μg/ml protein (protein concentration was determined by the method of Bradford [31]. In competition experiments, incubations were performed in 0.5 nM [^{3}H] DAMGO for 1 h. Incubations were started by the addition of membrane suspension in a rotating incubator at 25 °C and terminated by rapid vacuum filtration through GF/C filters using cell harvester. The filters were washed three times with 6 ml of ice-cold buffer

and then dried for 1 h at 80 °C. The radioactivity was measured by a Wallac Microbeta 1450 Trilux scintillation counter (GE Healthcare) after 12 h of incubation in the scintillation cocktail. The extent of nonspecific binding was determined in the presence of 10-μM naloxone. Data were expressed as percentage of the maximum specific binding and were expressed as means ± SEM for at least three independent experiments. K_i values were calculated according to the equation of Cheng and Prusoff [32].

NADH Oxidation

The oxidation of NADH was followed by measuring the absorption decrease at 340 nm for 1 h at 25 °C. The reaction mixture contained 0.1-mM NADH whose concentration was determined using a molar extinction coefficient of $\varepsilon = 6200\ M^{-1}\ cm^{-1}$ at 340 nm, in 3 ml, 0.05-M phosphate buffer, pH 7.4. The reaction was started by addition of 0.5-mg OMs. Blank without NADH was instantaneously ran. The results were expressed as percentage of the initial absorbance value [33].

Superoxide Anion-Scavenging Ability

The effect of OMs as superoxide anion scavenger was assayed through the inhibition of NBT reduction by NADH in the presence of PMS.

Incubation mixtures contained: 73-μM NADH, 15-μM PMS, 50-μM NBT and the indicated amount of OMs in 2 ml, 0.05 M, pH 7.4 phosphate buffer at 25 °C. The control contained all the components except OMs. Absorbance variations (ΔAbsorbance/min) were determined at 560 nm, measuring the initial rate of superoxide-induced NBT reduction. The results were expressed as percentage of control value [34].

Stock solutions (1 mM) were freshly prepared everyday by dissolving PMS in ethanol, NBT in water and NADH in 0.05-M phosphate buffer, pH 7.4. OMs were dissolved in 0.05-M phosphate buffer, pH 7.4 and used as supernatant.

Statistical Treatment of Data

All the experiments were repeated at least three times with similar results. The data are presented as mean ± standard error (SEM). Mean values were statistically compared by one-way analysis of variance (ANOVA) and corresponding Student's *t*-test, $p < 0.05$ was taken to be statistically significant.

Results

OMs Production

The processes of OM production were monitored by HPLC and UV-VIS spectra. Because of their distinct reactivities, different incubation times were employed for both systems. However, a broad shoulder in the range of 280–310 nm can be observed in both incubation systems (Figure 1), and optical densities are increasing at 280 and 310 nm with time. When dissolving OM–EM1 from both systems in 1-M KOH, they displayed a monotonic increase in absorbance in the range of 250–600 nm (Figure 2). OMs of its fragments also showed similar tendency.

In the HPLC chromatogram, under the above-described conditions, the retention time of EM1 was 12.7 min. The peak represented EM1 significantly decreased with the increasing incubation time, while some new peaks with shorter retention time

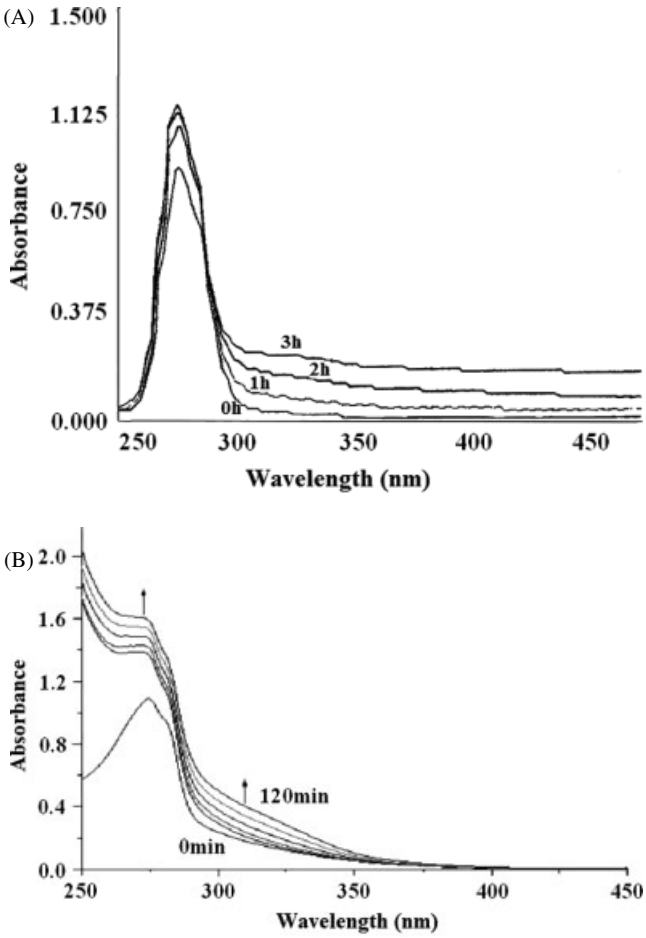

Figure 1. Spectral modifications of EM1 oxidized by different free radical systems. (A) Incubation mixture contained 1-mM EM1 and 20-mM AAPH in 50-mM phosphate buffer, pH 7.4. Spectra were recorded every hour, from 0 to 3 h. (B) Incubation mixture contained 1-mM EM1 and 0.2-mM ferrous ammonium sulfate, 0.2-mM EDTA and 5-mM H_2O_2 in 50-mM pH 7.4 phosphate buffer. Spectra were recorded at 0, 15, 30, 60, 90 and 120 min, respectively. The arrow is indicative of the absorbance increasing following the time elapsed. Reference cuvette contained all the regents except EM1. The experiment was repeated at least three times with similar results.

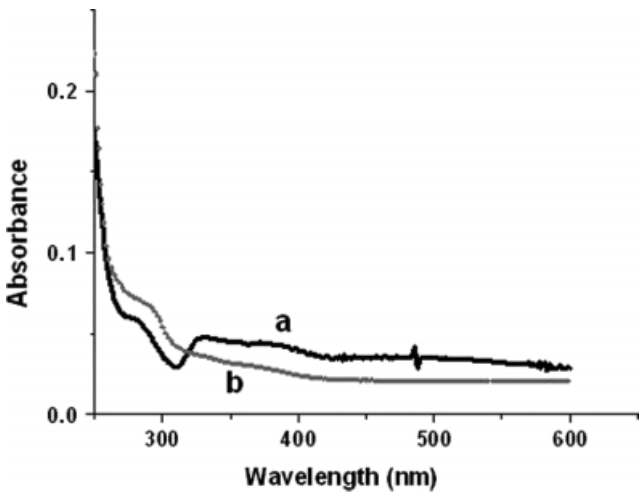

Figure 2. Absorption spectra of OMs from AAPH system (a) and from Fenton system (b). OMs dissolved in 1-M KOH.

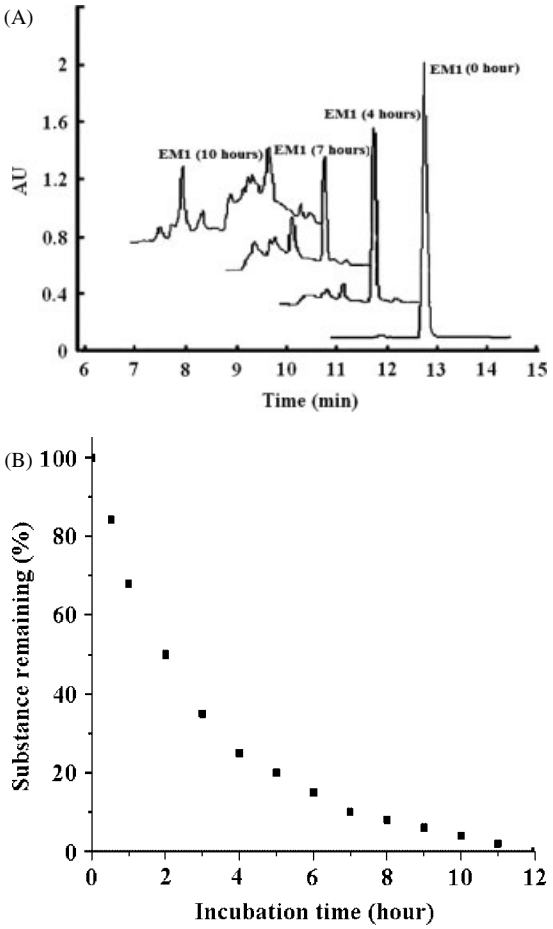

Figure 3. (A) HPLC profile of EM1 oxidized by AAPH at different time. Incubation mixture contained 1-mM EM1, 20-mM AAPH in 50-mM phosphate buffer, pH 7.4. (B) The decay of EM1 oxidized by AAPH measured by HPLC. The reported percent values were calculated taking as 100% the initial amount of substrate. Absorbance was read at 280 nm. The experiment was repeated at least three times with similar results.

than that of EM1 emerged (Figure 3(A)). The decay of EM1 measured by RP-HPLC is shown in Figure 3(B). In the presence of AAPH, the peak of EM1 disappeared completely after about 10 h. While in the absence of AAPH, EM1 retained after 12 h incubation (data not shown).

After an incubation time of 24 h, it was surprising to find that the oxidation products were polymerized, obtaining a yellow-to-brown solid, which could be easily separated by centrifugation. Similar OMs were obtained under both incubation conditions. It has to be pointed out that when Pro-Trp-Phe-NH_2 is incubated under both systems, the precipitation of the pigment in acid medium does not taken place and cannot be obtained by centrifugation.

EPR and NADH Oxidation

OMs from EM1 exhibited an unresolved single electronic paramagnetic resonance signal (Figure 4).

The NADH-oxidizing ability of OMs was tested, however, the absorbance of NADH in the presence of OMs is not susceptible to reduction during the first hour (data not shown).

Schmorl's Reaction

After schmorl's reaction, there was no difference in the color between ferricyanide solution alone and ferricyanide solution with EM1, only stained light yellow, showing EM1 does not influence the Schmorls reaction. Synthetic melanin from auto-oxidation of L-DOPA stained dark green–blue. OMs arising from EM1 with free radicals stained light blue–green (Figure 5).

Solubility of OMs

The solubility of OMs was tested in 1-M HCl, 1-M KOH and different pH buffers. All OMs were insoluble in acidic medium, but soluble in KOH. It was interesting to find that OMs from peptides whose *C*-terminus is $-NH_2$ could not dissolve in phosphate buffer (pH = 7.4), whereas those whose *C*-terminus is –OH were soluble.

The solubility behaviors of OMs in different pH solvents are shown in Figure 6. It was seen that OMs arising from Tyr-Pro-Trp-Phe-NH_2, Trp-Pro-Tyr-Phe-NH_2 showed similar solubility properties and OMs derived from Tyr-Pro-Trp-Phe-OH, Tyr-Pro-Trp-OH exhibited identical solubility behavior.

Amino Acid Analysis and Radioligand-Binding Assay

In order to determine the modification of amino acid residues during the oxidation, EM1 and its OM were subjected to hydrolysis. The results are shown in Table 1. The *N*-terminal Tyr content of OMs from EM1 was reduced by $57 \pm 3\%$ compared with that of the EM1 ($p < 0.01$) and the remaining Tyr residues in the backbone of the polymer could be released after 22-h hydrolysis. Approximately 90% of the Trp content of OMs has been transformed ($p < 0.001$), suggesting that Tyr and Trp residues might be the main targets attacked by the free radicals. Since *N*-terminal Tyr is essential for the binding of μ-opiate receptor, the capacity of OMs radioligand binding was tested.

The binding of EM1, Tyr-Pro-Trp-Phe-OH, Trp-Pro-Tyr-Phe-NH_2 and the OMs from EM1, Tyr-Pro-Trp-Phe-OH, Trp-Pro-Tyr-Phe-NH_2 to μ opioid receptors in membrane preparations from rat brain have been studied using [^{3}H]DAMGO as tracers. The concentration of OMs from Trp-Pro-Tyr-Phe-NH_2 required to inhibit 50% of the specific binding of 0.5 nM [^{3}H]DAMGO (IC_{50}) is about 3.56×10^{-8} g/ml. In contrast, much higher concentrations are required when OMs from EM1 and Tyr-Pro-Trp-Phe-OH inhibit the specific binding of [^{3}H] DAMGO, IC_{50} are 5.9×10^{-7} ($p < 0.01$) and 11.17×10^{-6} g/ml ($p < 0.05$), respectively. The comparison of IC_{50} and K_i values for OMs from EM1, Tyr-Pro-Trp-Phe-OH and Trp-Pro-Tyr-Phe-NH_2 are summarized (Table 2). The binding abilities of EM1, Tyr-Pro-Trp-Phe-OH and Trp-Pro-Tyr-Phe-NH_2 are also listed for comparison.

Superoxide Anion-Scavenging Assay

OMs of EM1 function as superoxide anion scavenger in a concentration-dependent manner (IC_{50} = 57 µg), which is depicted in Figure 7(B). OMs of the other peptides exhibit similar activities (data not shown). The parallel comparison among OMs arising from the series of peptides at 50 µg is shown (Figure 7(A)). The inhibition percentages of the NBT reduction rate by 50 µg OM-EM1 and OM-Tyr-Pro-Trp-Phe-OH are 50.5 and 42.9% ($p < 0.001$), respectively, whereas other OMs showed lower inhibitory activity.

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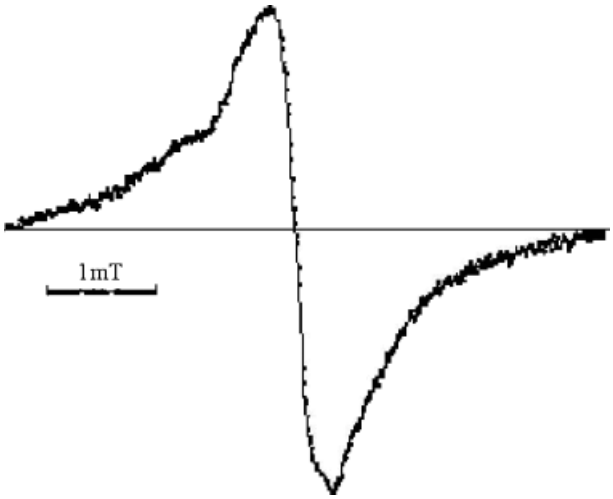

Figure 4. X band EPR spectrum of solid EM1–OM. EPR spectrum was recorded at 25 °C on a Buker ER 200D-SRC spectrometer at X band with the following instrument settings: microwave power, 10 mW; modulation amplitude, 5 G; time constant, 200 ms; scan time, 200 s. G value is 2.0039.

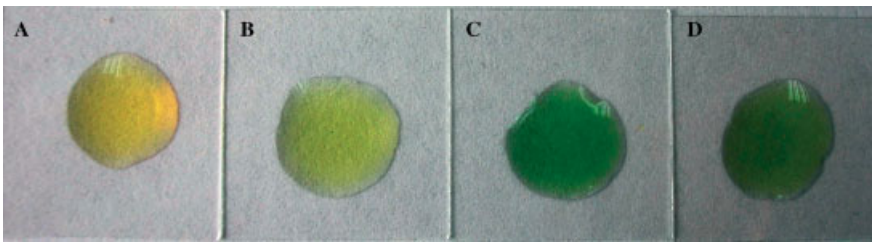

Figure 5. Schmorl's reaction of OM from EM1 and synthetic melanin. After Schmorl's reaction, ferricyanide solution with distilled water (A) and ferricyanide solution with EM1 (B) all stained light yellow; ferricyanide solution with synthetic melanin from auto-oxidation of L-DOPA (1 mg/ml) stained dark blue–green (C); ferricyanide solution with OM from EM1 reacting with ROS (1 mg/ml) stained light blue–green (D). The experiment was repeated at least three times with similar results.

Discussion

Our previous works have demonstrated that EM1 can function as an antioxidant *in vitro* and *in vivo* by scavenging free radicals, and great attentions were paid on the identification of OMs from EM1 and its fragments with ROS. The results presented in this paper provide evidence that EM1 can interact with ROS, furthermore, the data support the hypothesis of ROS-mediated synthesis of opiomelanins from EM1 and its fragments.

It was demonstrated that ROS attack on proteins converts intramolecular Tyr residues into dopa which can be involved in redox reactions [35,36]. Lunt and Evans (1976) have proposed that Tyr can function as a substrate of tyrosinase, ultimately forming melanin through the following sequence: Tyr → dopa → dopachrome → melanin [37]. Rosei *et al*. also demonstrated the occurrence of further oxidative modifications of dopa residues in peptides such as enkephalin which are converted into melanin pigments when the oxidized Tyr is amino-terminal [12,13]. Since EM1 bears the common *N*-terminal Tyr-like enkephalins, the possibility that EM1 can be transformed to melanin-like compound by the action of ROS cannot be easily ruled out. Pigments produced from enkephalins and their derivatives bearing *N*-terminal Tyr are different kinds of melanin-like compounds collectively named opio-melanin [10–12]. The physical and chemical properties of OMs from EM1 and its fragments obtained in the present work are listed in Table S1 (see supporting information) [5,23,29] and compared with those of dopa-melanin, formed from the autoxidation of dopa [38], and opio-melanin.

It can be seen from Table S1 that the characteristics of OMs from EM1 and Trp-Pro-Tyr-Phe-NH_2 containing $-NH_2$ in the

Table 1. Chemical hydrolysis of EM1 and OMs from EM1

Area (%) normalized to Pro	Tyr	Pro	Trp	Phe-NH_2
EM1	101 ± 2	100 ± 1	96 ± 5	97 ± 3
EM1–OMs	43 ± 3***	100 ± 1[a]	10 ± 4**	97 ± 2[a]

Hydrolysis was performed with 10-mg EM1 or EM1–OMs in 6-M HCl or 4-M NaOH under vacuum at 120 °C for 22 h. Values are means ± SEM from three different independently determinations.
[a] There is NO significant difference *versus* that of EM1.
*** $p < 0.001$ *versus* that of EM1.
** $p < 0.01$ *versus* that of EM1.

Table 2. Summary of IC_{50} and K_i values in rat brain membrane receptor binding studies, using [^{3}H] DAMGO as tracers

Substrate	IC_{50} (g/ml ± SE)	K_i (g/ml ± SE)
EM1	$2.78 \pm 0.21 \times 10^{-9}$	$1.44 \pm 0.11 \times 10^{-9}$
Tyr-Pro-Trp-Phe-OH	$5.21 \pm 0.35 \times 10^{-6a}$	$2.69 \pm 0.18 \times 10^{-6a}$
Trp-Pro-Tyr-Phe-NH_2	$3.4 \pm 0.15 \times 10^{-7b}$	$1.76 \pm 0.08 \times 10^{-7b}$
OMs from EM1	$5.9 \pm 0.3 \times 10^{-7d}$	$3.05 \pm 0.15 \times 10^{-7d}$
OMs from Tyr-Pro-Trp-Phe-OH	$11.17 \pm 3.6 \times 10^{-6c}$	$5.77 \pm 1.86 \times 10^{-6c}$
OMs from Trp-Pro-Tyr-Phe-NH_2	$3.56 \pm 1.25 \times 10^{-8}$	$1.84 \pm 0.65 \times 10^{-8}$

Values are means ± SEM for three different independent determinations.
[a,**] $p < 0.01$ *versus* that of Trp-Pro-Tyr-Phe-NH_2.
[b,*] $p < 0.05$ *versus* that of Trp-Pro-Tyr-Phe-NH_2.
[c,**] $p < 0.01$ *versus* that of OMs of Trp-Pro-Tyr-Phe-NH_2.
[d,*] $p < 0.05$ *versus* that of OMs of Trp-Pro-Tyr-Phe-NH_2.

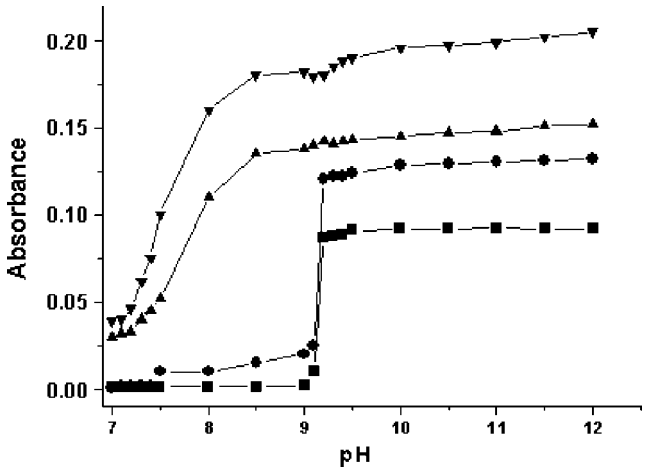

Figure 6. Solubility behaviors of various OMs. OMs (0.1 mg) were dissolved in 0.05-M glycine–HCl buffer (pH 2–4), 0.05-M phosphate buffer (pH 5–7.4), 0.05-M Tris–HCl (pH 8–12). OMs studied were Trp-Pro-Tyr-Phe-NH_2-OM [filled square (■)]; Tyr-Pro-Trp-Phe-NH_2-OM [filled circle (●)]; Tyr-Pro-Trp-OH-OM [filled triangle (▲)]; Tyr-Pro-Trp-Phe-OH-OM [filled inverted triangle (▼)].

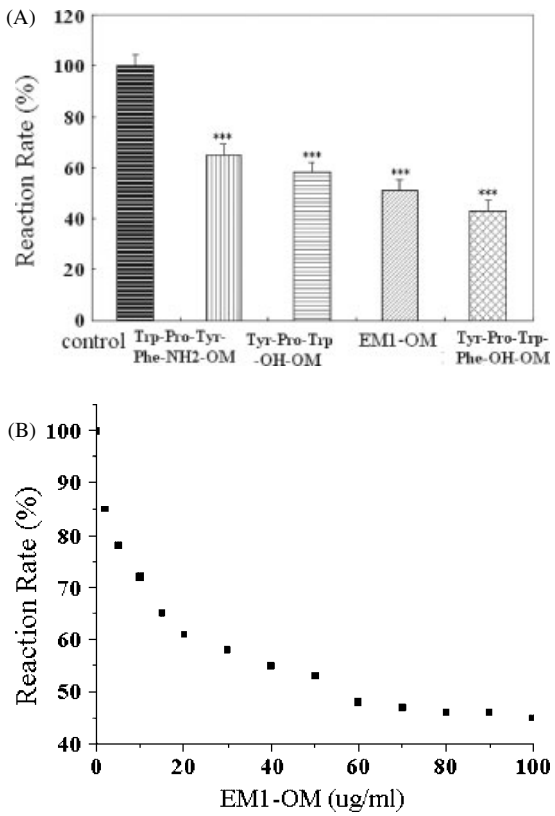

Figure 7. (A) Superoxide scavenging activity of various OMs from EM1 and their fragments. Incubation mixtures contained: 73-μM NADH, 15-μM PMS, 50-μM NBT, and 50 μg of OMs in 1 ml of 0.02-M Tris–HCl buffer, pH 8.0. (B) Superoxide scavenging activity of EM1–OM as a function of concentration. Incubation mixtures contained: 73-μM NADH, 15-μM PMS, 50-μM NBT, and the indicated amount of OMs in 1 ml of 0.02-M Tris–HCl buffer, pH 8.0. Reaction rate, measured as ΔAbsorbance/min at 560 nm, is expressed as percentage of control value. The experiment was repeated at least three times with similar results, data are presented as mean ± SEM, [***] $p < 0.001$ *versus* control.

C-terminal are more similar to that of dopa-melanin, while OMs from Tyr-Pro-Trp-Phe-OH and Tyr-Pro-Trp-OH whose *C*-terminal is –OH corresponds more closely to opio-melanin. Therefore, pigments derived from EM1 and its fragments were collectively named EM1–melanin.

In the study of the time course of the incubation process, we found that the absorbance value was modified at 280 and 310 nm and was increased along with time (during the initial incubation time), suggesting the formation of dopa and dopachrome derived from Tyr, which coincide with the observation that when EM1 was incubated with free radical generators, darkening of the solutions can be seen within 2–3 h after the start of incubation. On the other hand, there are no major peaks in the range of 250–600 nm when dissolving OMs in KOH solvent, probably indicating all dopa and dopachrome are converted completely after incubation.

The results of the HPLC study (which monitored changes in EM1 content) also provide direct evidence for the interaction of EM1 and free radicals. Since free radicals gradually deplete EM1 with time (Figure 3), the final products have been isolated by HPLC and identified by ESI-MS, because they are polymers, no molecular weight of these metabolites can be determined [10–12].

The fact that ferricyanide reacts with the dark brown solution containing OMs resulting in the blue–green color as is typical for melanin [26] suggests that by the action of ROS on EM1, melanin was formed.

The oxidative products, possessing a free radical similar to that of dopa-melanin, directly resulting from free-radical-mediated

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reaction are demonstrated by EPR [23]. However, OMs did not exhibit NADH oxidation ability, probably owing to the inhibition of the electron transfer properties by the linked peptide chains which is consistent with the results reported by Rosei *et al*. [23].

Comparison of the different solubility behaviors of OMs from EM1 and Trp-Pro-Tyr-Phe-NH_2 with those from Tyr-Pro-Trp-Phe-OH and Tyr-Pro-Trp-OH demonstrates that the terminal carboxylic group plays a major role in solubility, in agreement with the recent finding by Aroca *et al*. [39].

Our results indicated that OMs are also powerful free radical scavengers, coincident with the previous reports about the scavenging ability of melanin established in model systems [40,41]. To assess their capacities to scavenge free radicals, we measured how efficiently they inhibited superoxide anion-inducd reduction of NBT. A comparison of the results obtained from the various OMs studied, OMs from Tyr-Pro-Trp-Phe-OH function more efficiently than that of EM1 as scavenger, probably due to its higher solubility.

In order to assess the modification of amino acid residues during oxidation, hydrolysis of OMs from EM1 was carried out and demonstrated that free radicals predominately attack Tyr and Trp. It should be pointed out that when free Tyr and Trp residues were incubated with AAPH, the formation of chrome can be observed. Rosei *et al*. reported that an indole ring of dopachrome converted by *N*-terminal Tyr can function as the key site for polymeric condensation leading to the formation of melanins [12,13]. In contrast to other melanins arising from peptides bearing *N*-terminal Tyr [23], the OMs from EM1 still have the capacity for binding to μ-opiate receptor as the Tyr is essential for binding. This is consistent with the data shown by amino acid analysis that 43% Tyr residues remain. While the solubility of Tyr-Pro-Trp-Phe-OH is better than EM1, the transformation of Tyr is more complete, consistent with the decrease of the capability of specific receptor binding. In order to ascertain whether Trp can function as another polymeric condensation site, Trp-Pro-Tyr-Phe-OH was synthesized and the physical and chemical properties of its OMs were studied and compared. The OMs obtained from Trp-Pro-Tyr-Phe-OH, a yellowish pigment, showed higher receptor binding affinity than OMs from EM1. It suggests that most Tyr residues remain and Trp acts as a new polymeric condensation site to give rise to a new polymer.

As regarding the possible structure of EM1–melanin, we proposed a probable pathway for the formation of EM1–melanin (Figure 8) and suggested that a different polymeric structure may arise in contrast to those of dopa-melanin and opio-melanin because of the involvement of Trp as condensation site. It is well established that Tyr can form melanin through Mason–Raper pathway that Tyr → dopa → dopachrome → melanin [37], and in the process of forming dopa-melanin, various positions (C2, C3, C4 and C7) of the indole ring derived from Tyr may be involved in polymerization [42]. When the C2 of the indole ring is linked to a peptide chain, such as in the case of enkephalin, this position and its near site (C3) cannot participate in the polymerization process because of the deactivation induced by the electrophilic action of the nearest carboxylic group [40]. For this reason, the polymeric condensation of EM1–melanin is limited to the C4 and C7 of the indole ring derived from Tyr (Figure 8, Scheme 1). On the other hand, combining the data given above, it is revealed that not only Tyr but also Trp are prone to oxidative modification by ROS. It was reported that $HO^{\bullet}$ react with Trp at the C5 carbon or other sites of the indole to form 5-hydroxytryptophan or other $HO^{\bullet}$ adducts [43]. From the above considerations and taking into account their specific properties, we propose that 5-hydroxytryptophan

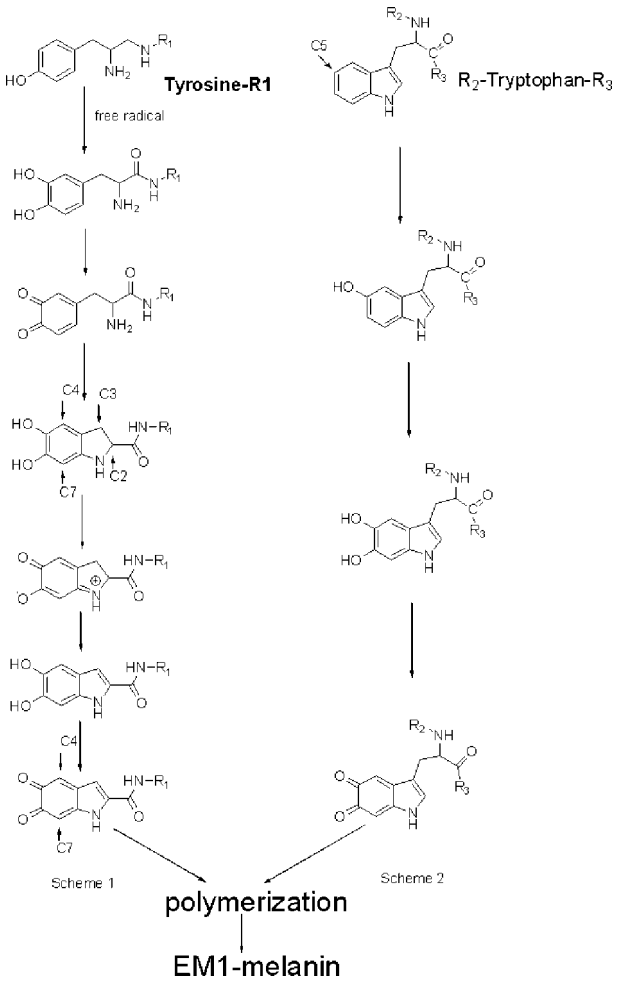

Figure 8. Possible pathways for the formation of EM1–melanin by ROS-mediated oxidation of EM1. $-R_1$, $-R_2$ and $-R_3$ represent Pro-Trp-Phe-NH_2, Tyr-Pro and Phe-NH_2, respectively.

may follow the similar Mason–Raper pathway to form 5,6-dihydroxytryptophan, undergoing further polymerization reaction to form pigments (Figure 8, Scheme 2), which coincide with the observation of Msakos *et al*. [43].

In conclusion, the formation of EM1–melanin by the action of ROS on EM1 is indicative of a possible role of ROS in key transformation of EM1, which, under special conditions, can lead to EM1–melanin without any enzyme intervention. This study may provide new insight to investigate the metabolites of EM1 and promote the pharmacological and clinical use of EM1.

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Nos. 20525206, 20621091 and 20772052), the Specialized Research Fund for the Doctoral Program in Higher Education Institutions of the Ministry of Education of China.

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