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Biomedicine & Pharmacotherapy 60 (2006) 453–457
BIOMEDICINE & PHARMACOTHERAPY
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Original article

# An in vitro study on the free radical scavenging capacity of ergothioneine: comparison with reduced glutathione, uric acid and trolox

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Available online 14 August 2006

**Abstract**

*Background:* Treatment of oxidative stress-related pathologies is a possible therapeutical strategy for the future. Natural product with antioxidant properties could trigger this goal. The aim of this in vitro study was to assess the antioxidant activity of the natural product ergothioneine (EGT), a compound of plant origin, which is assimilated and conserved by mammals in erythrocytes, kidney, seminal fluid and liver.

*Methods:* We measured the antioxidant activity of EGT as its ability to antagonize the oxidation of α-keto-γ-methiolbutyric acid (KMBA) by hydroxyl radical, peroxyl radicals and peroxynitrite. The results are expressed as total oxyradical scavenging capacity (TOSC) units. Glutathione (GSH), uric acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), the water-soluble analog of vitamin E, were used as the reference antioxidants.

*Results:* EGT was the most active scavenger of free radicals as compared to classic antioxidants as GSH, uric acid and trolox. In particular, the highest antioxidant capacity exhibited by EGT vs. peroxyl radicals (5.53 ± 1.27 units) resulted 25% higher than the value obtained with the reference antioxidant trolox (4.4 ± 0.6 units, $P < 0.01$). The scavenging capacity of EGT towards hydroxyl radicals (0.34 ± 0.09 units) was 60% higher, as compared to uric acid (0.21 ± 0.04 units, $P < 0.001$), which represent the reference antioxidant vs. hydroxyl radicals. Finally, EGT showed the highest antioxidant activity also towards peroxynitrite (5.2 ± 1.0 units), with a scavenging capacity 10% higher than that of uric acid (4.7 ± 0.9 units, $P < 0.05$).

*Conclusions:* This study showed that EGT has potent intrinsic anti-hydroxyl, anti-peroxyl and anti-peroxynitrite radicals antioxidant activity, as compared to classic molecules with antioxidant capacity as GSH, trolox and uric acid. This appears of interest, given the increasing use of non-vitamins cocktails for therapeutical approaches to many oxidative-induced pathologies.
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*Keywords:* Ergothioneine; Free radicals; Hydroxyl radicals; Peroxyl radicals; Peroxynitrite; Reduced glutathione; Uric acid; Antioxidants

## 1. Introduction

Reactive oxygen species (ROS) are ubiquitous reactive derivatives of $O_2$ metabolism, responsible for numerous types of cell damage [1,2]. It has been suggested that a chronic imbalance between formation of ROS and antioxidant defenses characterizes many disease states, such as atherosclerosis and premature aging [2,3].

Within the cardiovascular system, ROS play an essential physiological role in maintaining cardiac and vascular integrity and a pathophysiological role in cardiovascular dysfunction associated with conditions such as hypertension, diabetes, atherosclerosis, ischemia–reperfusion injury, ischemic heart disease, and congestive cardiac failure [4,5]. The assessment of the antioxidant properties of therapeutic molecules and

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doi:10.1016/j.biopha.2006.07.015

their potential use in preventing or limiting the damage induced by free radicals appears to be of particular value.

Dietary ergothioneine (2-mercaptohistidine trimethylbetaine, EGT) (Fig. 1), a compound of plant origin, is a low-molecular-mass thiol, which is assimilated and conserved by mammals and principally present in specific tissues such as erythrocytes, kidney, seminal fluid and liver [6,7]. EGT is a natural chemoprotector against oxidation, including lipid peroxidation [6,8–11]. It has been demonstrated to scavenge singlet oxygen and hydroxyl radicals [12], and to protect in vivo retinal neurons from *N*-methyl-D-aspartate-induced excitotoxicity and to ensure a potent protective effect in PC12 model decreasing $H_2O_2$-induced cell death [13].

Despite these evidences, molecular mechanisms underlying the cytoprotective effects of EGT and its specific antioxidant capability has not been well elucidated. Recently, a highly reproducible method has been developed for the quantitative measurement of the capacity of a molecule or a tissue to neutralize various classes of ROS [14–17]. The total oxyradical scavenging capacity (TOSC) assay is based on the reaction of artificially generated oxyradicals with α-keto-γ-methiolbutyric acid (KMBA), which is completely oxidized to ethylene. In the presence of an antioxidant molecule, competition with KMBA ensues, resulting in a reduction of free radical generation, hence, lower ethylene production.

The purpose of this study was to assess the antioxidant scavenging capacity of EGT and to compare its efficiency in neutralizing hydroxyl radicals, peroxyl radicals, and peroxynitrite with that of some classical antioxidants such as reduced glutathione (GSH), uric acid and trolox, the water-soluble analog of vitamin E.

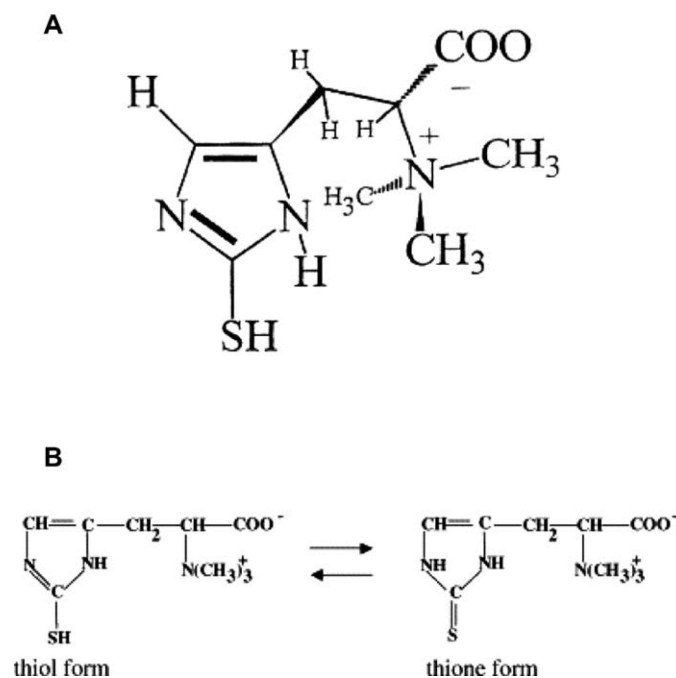


Fig. 1. The structure of EGT and its tautomeric thione form.

## 2. Methods

### 2.1. Chemicals

L-Ergothioneine ($M_w = 229.3$) was purchased by OXIS International (Japan) and stored at –20° C until usage. For the assay an aliquot of the compound was dissolved in sterile water and the solution was diluted to desired concentrations immediately before use. The antioxidant capacity of EGT was obtained by analyzing the substance starting from a stock of 100 mM and using different dilutions (5–120 μM).

Ascorbic acid, KMBA, GSH, and uric acid, 2,2′-azo-bis-amidinopropane (ABAP), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. TOSC assay

Peroxyl radicals were generated by thermal homolysis of 20 mM ABAP at 35 °C in 100 mM potassium phosphate buffer, pH 7.4. Hydroxyl radicals were generated at 35 °C by the iron plus ascorbate-driven Fenton reaction (1.8 μM $Fe^{3+}$, 3.6 μM EDTA, and 180 μM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4). Peroxynitrite was generated from the decomposition of SIN-1 in the presence of 0.2 mM KMBA, 100 mM potassium phosphate buffer, pH 7.4, and 0.1 mM DTPA, at 35 °C. The concentration of SIN-1 was varied to achieve an ethylene yield equivalent to the iron–ascorbate and ABAP systems. Reactions with 0.2 mM KMBA were carried out in 10 ml vials sealed with gas-tight Mininert® valves (Supelco, Bellefonte, PA) in a final volume of 1 ml. Ethylene production was measured by gas-chromatographic analysis of 200 μl aliquots taken from the head space of vials at timed intervals during the course of the reaction. Analyses were performed with a Hewlett-Packard gas chromatograph (HP 6890 Series, Andoven, MA) equipped with a Supelco SPB-1 capillary column and a flame ionization detector (FID). The oven, injection and FID temperatures were respectively, 35, 160 and 220 °C. Helium was the carrier gas (at a flow rate of 1 ml/min); a split ratio of 20:1 was used. Total ethylene formation was quantified from the area under the kinetic curves that best define the experimental points obtained for control reactions and after addition of EGT during the reaction. TOSC values were quantified from the equation $TOSC = 100 - (SA/CA \times 100)$, where SA and CA are the integrated areas for sample and control reaction, respectively. A TOSC value of 0 corresponds to a sample with no scavenging capacity (i.e. no inhibition of ethylene formation when compared to the control reaction, SA∫CA = 1) [14–17].

The linearity of dose–response curve between EGT concentration (in μg) and the antioxidant (TOSC value) response was tested and good correlation coefficients (generally greater than 0.9) were obtained at the different doses used to test the validity of our experiments (Fig. 2). Each experiment was performed in duplicate to account for the intrinsic variability of

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