Studies on the antioxidant activity of ergothioneine

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Ergothioneine (EGT), a thiohistidine betaine, exists in various fungi, plants, and animals. Mushrooms contain plenty of EGT, especially golden oyster mushrooms. Animals are unable to biosynthesize EGT, so they take in EGT from their diet. In humans, EGT have been reported to accumulate in various cells and tissues including erythrocytes, bone marrow, liver, kidney, seminal fluid, and the lens and cornea of the eye, at high concentrations (100 μM to 2 mM). EGT concentrations correspond to about one fifth of the glutathione (GSH) concentrations in human body. EGT and GSH were known to strong biological antioxidants. Compared with GSH, there are only a limited number of reports about the redox mechanism of EGT *in vivo* and *in vitro*.

The purpose of this study was to clarify the oxidation mechanism of EGT from the chemical reactions with various oxidants. In addition, both the absorption and metabolism of EGT *in vivo* were investigated. Also, the effects of EGT under the exercise oxidative stress were preliminary carried out *in vivo*.

1. Oxidation mechanism of EGT (in vitro)

To elucidate the antioxidant mechanism of EGT *in vitro*, the stoichiometry of EGT and hydrogen peroxide (H_2O_2), and oxidation products of EGT were investigated by instrumental analysis. EGT (1 mM) was oxidized by hydrogen peroxide (0–3 mM) in water at 37 °C for 24 or 48 h. From this morel reactions, it is indicated that one molecule of EGT could reduce two molecules of hydrogen peroxide in water. Thus, EGT can reduce twice as much hydrogen peroxide as ascorbic acid and tocopherol.

Next, the oxidation products of EGT were analyzed by LC-MS and NMR. In the reaction mixture of EGT and H_2O_2, ion peaks were detected at *m/z* 95, 154, 198 by LC-MS. These peaks were not observed in the sample immediately after starting the reaction. Therefore, a product with *m/z* 198 was expected to be the major EGT

oxidation product in after reaction with hydrogen peroxide. For identification of the structure of the EGT oxidation product, the fraction with the ion peak at *m/z* 198 was collected repeatedly and analyzed by ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC. These results demonstrated that the major EGT oxidation product in the aqueous condition was determined to hercynine (histidine betaine).

We propose a reaction mechanism for generation of hercynine *in vitro* as follows. First, one molecule of ERGO reacts with two molecules of hydrogen peroxide, and ergothioneine sulfinic acid is formed as an unstable intermediate in aqueous medium. Ergothioneine sulfinic acid is rapidly hydrolyzed to hercynine and sulfurous acid.

Hercynine was identified as an oxidation product of EGT. However, in order to investigate the possibility of further oxidation of hercynine, hercynine was reacted with $Ca(ClO)_2$ and analyzed by LC-MS/MS, high resolution accurate mass spectrometer (HRAM). After reaction for 30 min, an unknown peak was observed at *m/z* 157, and this product is unstable in aqueous solution. Therefore, hercynine was considered to be oxidized by $Ca(ClO)_2$, and the oxidation product of hercynine would be further decomposed in water.

2. Metabolism of EGT and hercynine (in vivo)

EGT and hercynine are substrates of organic cation transporter 1 (OCTN1). However, it has been reported that transport of hercynine was 25-fold lower than transport of EGT in HEK 293 cells. There are no reports about metabolism of orally ingested hercynine *in vivo*. The metabolism of EGT and hercynine were compared *in vivo*. Mice were fed with EGT or hercynine in low or high concentrations. After administration periods, their concentrations in tissues were measured by LC-MS analyses. In liver, kidney, heart and plasma, EGT levels of the EGT-taking group were higher than ones of the non-taking group, but hercynine was not detected in any tissues. EGT would be accumulated in tissues, but hercynine would not. No peaks of EGT and hercynine were detected in feces of all mice. These results suggested that the orally ingested EGT and hercynine seemed to be absorbed in the small intestine with high

absorption rates. Hercynine was detected only in the urine of the high-hercynine group. Therefore, hercynine may be absorbed into the intestinal tract, then hercynine was assumed to be changed to certain compounds through the oxidation and metabolism *in vivo*.

3. Effects of EGT under the exercise oxidative stress (in vivo)

It is expected that EGT would be oxidized in the body and be formed to hercynine in the group given EGT, but hercynine was not detected in any of the tissues. So, we performed an experiment using mice given exercise oxidative stress as follows: mice given EGT for two weeks were run on a treadmill machine. After running test, the mice were sacrificed, and the tissues were harvested. The level of AMPK α , HEL, EGT and hercynine were measured in muscle. The EGT-taking group tended to run longer than the control group, and the level of AMPKα was slightly higher. The EGT concentrations in muscle were increased by the intake of EGT but hercynine was not detected. These experimental conditions were thought to be insufficient for applying the appropriate oxidative stress to mice. The other experimental conditions or methods which could be applying more stronger oxidative stress to mice should be necessary.

To summarize this doctoral dissertation, we showed the antioxidant mechanism of EGT against hydrogen peroxide *in vitro*, demonstrating that one molecule of EGT reduces two molecules of hydrogen peroxide. First, we found that EGT was changes to hercynine as the main oxidation product. It was also shown that hercynine is further oxidized to P1 by $Ca(ClO)_2$, which is a stronger oxidant than hydrogen peroxide. These results suggested that EGT was shown to be a more efficient antioxidant than other food-derived antioxidants (ascorbates or tocopherols). Furthermore, hercynine, the oxidation product of EGT could be degraded to small molecules (P1 and so on) with other chemical oxidants. That means, hercynine would be expected to be a further antioxidant. In addition, EGT and hercynine administration experiments *in vivo* confirmed the high accumulation of EGT in tissues. In addition, unlike EGT, hercynine

did not accumulate in tissues. It was considered that hercynine might be undergoing oxidation or some metabolisms in the body.

These results suggest that EGT is an efficient antioxidant that reacts well against hydrogen peroxide, a major reactive oxygen species in the body. Furthermore, because hercynine, the oxidation product of EGT, can reduce $Ca(ClO)_2$, it was estimated that ingestion of EGT could scavenge the two types of ROS present in the body. We conclude that the daily intake of EGT is significant for biological defense against oxidative stress.