【審査論文】

Long-term stability of ubiquinol-10 in natural miso without artificial antioxidants

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Abstract

Ubiquinol-10, the reduced form of coenzyme Q_{10}, has higher bioavailability but lower stability than ubiquinone-10, the oxidized form of coenzyme Q_{10}. During the development of ubiquinol-fortified foods, ensuring a long shelf life of the ubiquinol content without sacrificing the flavor of the food is crucial. In this study, we determined the stability of ubiquinol-10 in natural miso, a fermented food made from soybean, salt, and koji, matured at 20–25 °C and stored in a refrigerator for up to three months. Three types of natural miso were developed, differing in their composition and the duration of maturation, using three different forms of ubiquinol-10: raw powder, stabilized powder, and stabilized granules. The ratio of ubiquinol-10 to total coenzyme Q_{10} was more than 90% during the maturation and storage of natural miso under standard conditions, regardless of the ubiquinol form and type of natural miso. Contrary to expectations, the ubiquinol-10 ratio in a more pigmented natural miso seemed to increase during the maturation period, one month after preparation, when an unstabilized bulk ubiquinol powder was used. These results suggest that naturally occurring antioxidants, which are constituents of miso ingredients and are produced during the maturation process of natural miso, could maintain the reduced state of ubiquinol-10, without the addition of artificial antioxidants or the preservation of anaerobic conditions.

Keywords：ubiquinol-10, CoQ_{10}, miso, fortified food, antioxidants

1. Introduction

Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble and biosynthesized molecule involved in the modulation of energy production and oxidoreduction processes in cells and cellular components [1, 2]. Although CoQ_{10} is not an essential nutrient, its content in the body decreases with aging [3]. Therefore, the use of CoQ_{10} supplements to maintain and improve human health, especially cardiovascular health, fertility, and skeletal muscle health, has been extensively investigated in recent decades [4]. One of the remarkable issues with CoQ_{10} supplements is their limited bioavailability owing to their lipophilicity. Ubiquinol-10, the reduced form of CoQ_{10}, is believed to be more efficacious than ubiquinone-10 (the oxidized form) based on the observation of CoQ_{10} uptake using a Caco-2 monolayer system, in which most of the ubiquinone taken up by the cells was reduced to ubiquinol during or following the uptake

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[5]. Moreover, the bioavailability of CoQ_{10} was higher when supplemented with the ubiquinol-10 form than the ubiquinone-10 form, using identical soft gel capsules [6]. In contrast, ubiquinol-10 is less stable than ubiquinone-10 because it is easily oxidized when exposed to air and light. To protect it against oxidization and maintain its reduced state, ubiquinol-10 supplements sold in the market are encapsulated in a colored gelatin capsule with antioxidants, such as vitamin C, ascorbyl palmitate (lipophilic vitamin C derivative), tocopherols, and rosemary extracts [7].

Food fortification is another effective method of taking supplemental CoQ_{10}. By developing several enhanced water-soluble forms, the addition of CoQ_{10} to food during processing is a relatively easy way to prepare fortified foods [8]. Moreover, the CoQ_{10} content in various fortified foods is relatively stable when ubiquinone-10 is used as a CoQ_{10} source, although the redox state is unknown [9]. In the case of ubiquinol-10, maintenance of both the reduced state and structural stability is essential. Granulated, stabilized, and easily emulsified ubiquinol-10 materials have been developed to use ubiquinol-10 in fortified foods. Nevertheless, fortified ubiquinol-10 in foods is gradually oxidized unless they are placed under anaerobic conditions, such as a nitrogen gas-filled state or with oxygen scavengers in the vacuum state.

Miso is a traditional Japanese fermented seasoning prepared from soybeans, salt, and koji, which is a fermented food made from steamed grains and an edible mold *Aspergillus oryzae*. Fermented soy products, including miso, are of interest because of their health benefits, such as anticancer, antidiabetic, antihypertensive, and anti-inflammatory effects [10]. We have focused on miso as a food that can help CoQ_{10} absorption because people who consume more soy products have higher basal serum CoQ_{10} levels than those who consume fewer soy products [11]. Moreover, miso soup enhances the bioavailability of supplemental ubiquinol-10 at least up to 5 h after consumption [12]. On the basis of this information, we hypothesized that miso might be suitable for making ubiquinol-10-fortified food.

In this study, we prepared trial batches of ubiquinol-10-fortified miso products using three kinds of ubiquinol-10: raw ubiquinol-10 powder (QH1), stabilized ubiquinol-10 powder (QH2), and stabilized and easily emulsified ubiquinol-10 granules, named P30. Three types of naturally brewed raw miso, in which microorganisms are alive and undergo fermentation during both maturation and storage periods due to non-heat sterilization, were chosen to make the ubiquinol-10-fortified miso. We determined the levels of reduced and oxidized CoQ_{10} in nine types of miso (three forms of ubiquinol-10 $\times$ three types of miso) during maturation at room temperature and storage in the refrigerator and compared the reduced state of CoQ_{10} in the fortified miso.

2. Materials and Methods

2.1. Ubiquinol-10 samples

This study used three forms of ubiquinol-10 samples (QH1, QH2, and P30) for fortification. QH1 is a raw ubiquinol-10 powder commercially available as Kaneka QH^{TM} (Kaneka Co., Osaka, Japan). The purity of ubiquinol-10 available as Kaneka QH^{TM} was greater than 96%. P30, a granulated form of ubiquinol-10, containing dextrin, gum Arabic, L-ascorbate, and 30% (w/w) ubiquinol-10, was also obtained from Kaneka Co. QH2 is a crystal polymorph of QH1, with high oxidative stability, and it was prepared through the solid-phase conversion method [13]. The changes in the crystal structure of QH1 were

induced by stirring it at a temperature of 40 °C or higher under reduced pressure for 12 h or longer, resulting in the formation of QH2, having high oxidative stability. All ubiquinol-10 samples were stored at 4 °C in heat-sealed moisture-proof bags filled with nitrogen until further use.

2.2. Preparation of ubiquinol-fortified natural miso

Sets of hand-made miso containing boiled soybeans, crude kitchen salt, and koji were obtained from Amekaze Corporation (Osaka, Japan). The type of miso, amounts of raw materials required to make approximately 3 kg of miso, and the maturation period of the three types of miso prepared are shown in Table 1. Ubiquinol-10 (QH1, QH2, or P30) was added at a concentration of approximately 0.1%. For preparation, boiled soybeans were first mashed by hand in a plastic bag. Next, a mixture of crude salt, koji, and ubiquinol-10 powder was added to the mashed soybeans, followed by kneading until the paste became soft yet firm enough to be easily shaped. Then, the paste was shaped into fist-sized balls to remove the air in the paste. Finally, these balls were laid inside a pot, squashed by hand, covered with a thin plastic film, and overlaid with a stone weight. The pot was stored in a well-ventilated, air-conditioned dark room at 20–25 °C during the miso maturation period indicated in Table 1.

Table 1. Ingredients and maturation period of the three types of ubiquinol-fortified natural miso used in this study.

Parameter	White rice miso (WRM)	Rice miso (RM)	Barley miso (BM)
Ingredient (g/batch)			
Boiled soybeans	1200	1500	2000
Crude kitchen salt	180	300	300
Koji (malted rice)	1800	1500	—
Koji (malted barley)	—	—	1000
Ubiquinol-10	3	3	3
Maturation period (month, M)	1	3	6

When the miso matured, it was divided into zipper-locked plastic bags and stored in the refrigerator for up to three months. The colors of the matured white rice miso (WRM), rice miso (RM), and barley miso (BM) were pale yellow-brown, yellow-brown, and dark yellow-brown, respectively (Figure 1).

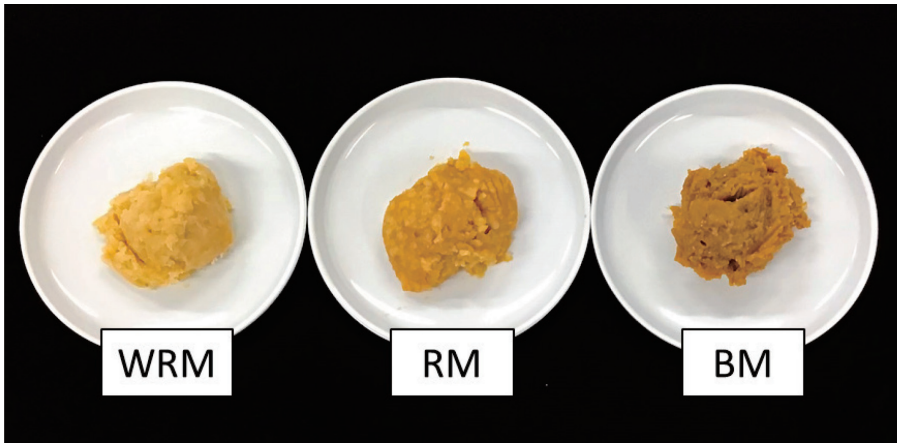

Figure 1: The color of three types of natural miso when matured (WRM, RM, and BM, from left to right). WRM, white rice miso; RM, rice miso; BM, barley miso.

2.3. Determination of reduced and oxidized CoQ_{10} contents in the miso

About ten grams of the miso sample was transferred into a 50 mL conical tube and stored at -80 °C until used. Quantitative analysis of reduced (ubiquinol-10) and oxidized CoQ_{10} contents in miso during maturation and storage processes was performed by Kaneka Techno Research Co., Ltd., using liquid chromatography with tandem mass spectrometry (LC/MS/MS) [14, 15]. About 50 mg of miso sample was homogenized with 2 mL 2-propanol and centrifuged at 1500 × g for 10 min. Then, 40 µL of the supernatant was diluted with 160 µL of 2-propanol, 250 µL of methanol, and 50 µL of internal standard solution (50 ng/mL of oxidized CoQ_9, in 2-propanol), filtered through a polytetrafluoroethylene membrane filter; and used as the sample for LC/MS/MS analysis using a reversed-phase octadecyl-silica column and a QTRAP5500 (AB Sciex, Framingham, MA, USA) system. Both reduced and oxidized forms of CoQ_{10} were quantified by calculating the peak area in the LC-MS/MS analysis. The total CoQ_{10} (µg/g miso) content was determined as the sum of the reduced and oxidized CoQ_{10} contents. Moreover, the value of the reduced ratio of CoQ_{10} (%) was determined as the proportion of the reduced form (ubiquinol-10) to total CoQ_{10}.

3. Results and Discussion

The total CoQ_{10} content (displayed as bar graphs) and the reduced ratio of CoQ_{10} (displayed as line graphs) of the WRM, RM, and BM are shown in Figures 2a, 2b, and 2c, respectively. Throughout the maturation and storage periods, the total CoQ_{10} content was between 800 and 1400 µg/g miso in the three types of miso. Although the quantity of CoQ_{10} appeared to change, it did not decrease in a time-dependent manner. For example, CoQ_{10} content was the highest after maturation and storage for one month in the WRM using QH1, QH2, and P30 (Figure 2a). Since we did not take multiple samples to determine the CoQ_{10} at each point, we could not evaluate the stability of the chemical structure of CoQ_{10} in miso with statistical analysis from this result. Therefore, we can only guess that these variations might be a result of the uneven dispersion of CoQ_{10} in natural miso and measurement error during sampling and determination and that CoQ_{10} is relatively stable in miso throughout maturation and storage, similar to other CoQ_{10}-fortified foods as described by Pravest et al. [9].

The reduced ratio of CoQ_{10} in all types of miso prepared was maintained at more than 90%, irrespective of the ubiquinol-10 samples and the miso types. However, we did not determine the reduced ratio from multiple samples per measurement to ascertain the stability of the CoQ_{10} chemical structure. Therefore, we could not discuss the data statistically. Nevertheless, comparing the ubiquinol forms in the same miso type, P30 seemed to be more stable in the reduced state than QH1 and QH2, throughout the maturation and storage stage in WRM (Figure 2a). A similar trend was observed in the early maturation stage (1M) in RM and BM. However, the reduced ratio of CoQ_{10} in RM and BM containing QH1 and QH2 seemed to increase and approach that in the P30-containing RM and BM during the later maturation stage (Figures 2b and 2c). Remarkably, in BM, a high CoQ_{10} reduced ratio was maintained up to three months after storage, even when the unstabilized form, QH1, was used (Figure 2c). In contrast, ubiquinol-10 (reduced CoQ_{10}) seemed to be oxidized faintly in the QH1-containing WRM (Figure 2a). These results suggest that the reduced state of ubiquinol-10 is very stable in miso and better protected

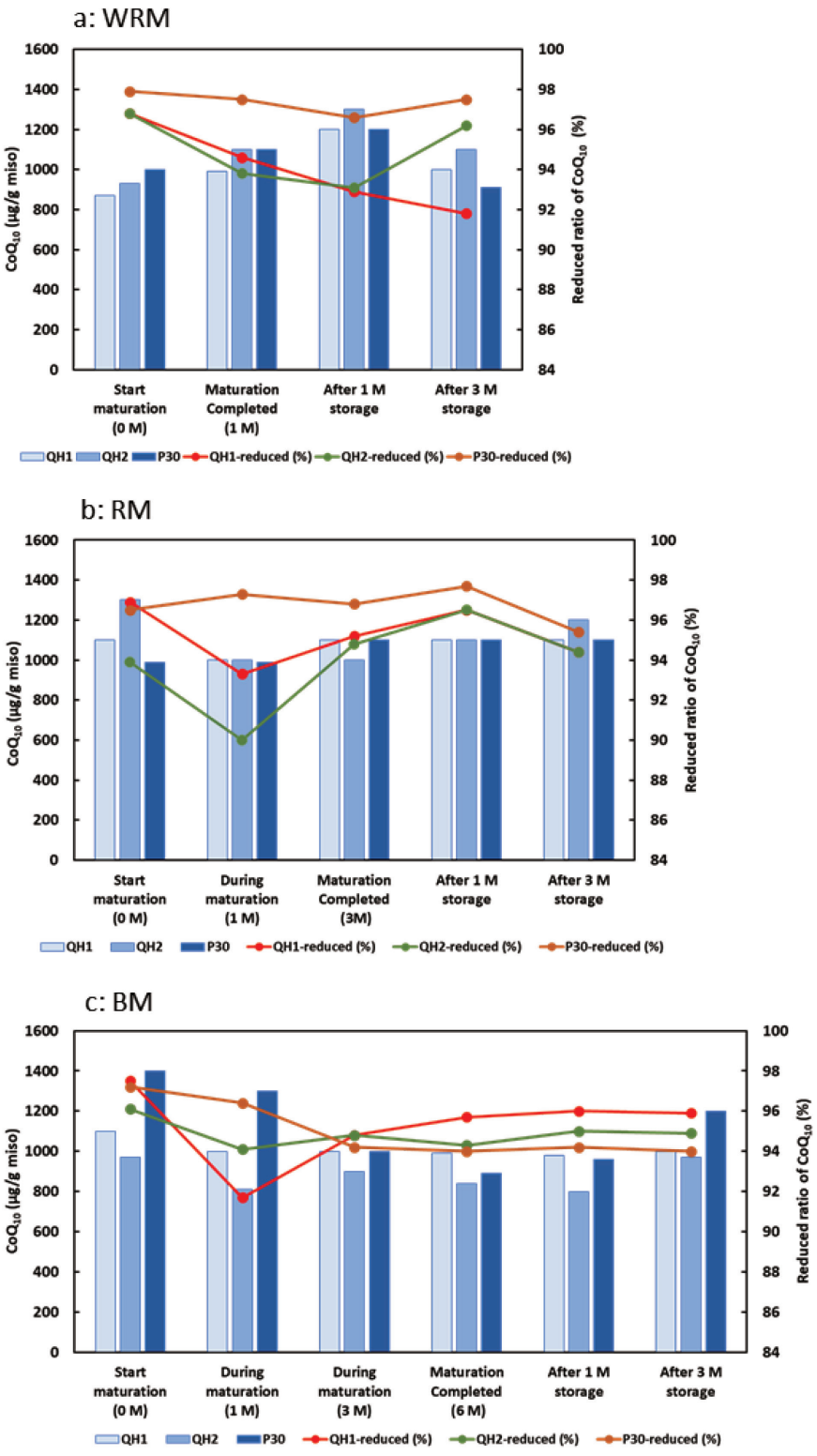

Figure 2: CoQ_{10} content (bar graphs) and the ratio of the reduced form of CoQ_{10} (line graphs) in natural miso during maturation and storage. (a) White rice miso (WRM), (b) rice miso (RM), (c) barley miso (BM). Note: M means month in the figure.

from oxidization in BM than in WRM when reductant-free QH1 is used.

Bulk ubiquinol-10 is unstable and easily oxidized in atmospheric oxygen, and more than 75% of ubiquinol-10 is oxidized three months after storage at room temperature [16]. In foods, the reduced

ratio of CoQ_{10} is varied, ranging from 4.9% to 98.1% [17]. The food with the highest reduced ratio of CoQ_{10} was canned tuna, which can be maintained in vacuo until the can is opened. Other foods with a higher reduced CoQ_{10} ratio are vegetables and fruits rich in the antioxidant vitamin C, such as parsley, Japanese radish, lotus root, orange, grapefruit, and persimmon. Therefore, when making ubiquinol-10-fortified foods, we must consider that they should be kept under anaerobic conditions or with artificial antioxidants to prevent the oxidization of fortified ubiquinol-10. Takahashi developed a system to prevent oxidation using a combination of a reducing agent and a proper oxidizing enzyme, such as glucose and glucose oxidase [18]. However, the reduced ratio of CoQ_{10} in the ubiquinol-10-fortified miso was maintained at more than 90% throughout maturation and storage (Figure 2) without maintaining anaerobic conditions or adding artificial antioxidants. Additionally, the reduced ratio of endogenous CoQ_{10} in miso is maintained at a highly reduced state (99.5% [12] and 96.1% [17], respectively) as well, suggesting the existence of naturally arising antioxidants potent enough to maintain the reduced state of both endogenous CoQ_{10} and added ubiquinol-10 in miso.

Miso is rich in compounds with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, including the polyphenols in soybean and their metabolites that are produced during the fermentation of miso [19, 20]. Furthermore, three research groups reported that melanoidins, which are brown and heterogeneous amino-carbonyl compounds generated non-enzymatically via the Maillard reaction between sugars and amino acids, might be another type of potent antioxidant in miso [21-23]. Interestingly, Iwaya et al. reported a positive correlation between DPPH radical-scavenging activity and the degree of pigmentation, guiding the amount of melanoidin produced, which increases during the maturation process in miso [21]. Shimohashi also reported the exact correlation between DPPH radical-scavenging activity and the amount of artificial amino-carbonyl products produced from D-glucose and L-lysine [22]. From these observations, the darkest miso, BM, can be richer in natural antioxidants than the palest miso, WRM. The result showing that the reduced CoQ_{10} ratio in BM is maintained, but decreases in WRM, during the storage process of QH1-fortified miso after maturation (red polygonal lines in Figures 2a and 2c) supports the above possibility. Hence, for the preparation of ubiquinol-fortified WRM, whose endogenous antioxidant content might be diminutive, P30 containing artificial vitamin C (an antioxidant) is superior to QH1 in maintaining a high reduced ratio of CoQ_{10}.

This study had some limitations. First, we did not perform multiple samplings of the miso samples to determine the reduced and oxidized CoQ_{10}. It hindered a significant test and might lower the authenticity of the data. We performed a single sampling because the extraction of CoQ_{10} while maintaining the redox state is time-consuming and requires expert skills and techniques. Furthermore, initially, we did not anticipate the potent stability of fortified ubiquinol-10 in miso. We only expected to find differences in the stability of ubiquinol-10 among QH1, QH2, and P30 in the fortified miso preparation. Second, we did not determine the antioxidant capacity, including the DPPH radical-scavenging activity, in WRM, RM, and BM. Since the amount of endogenous CoQ_{10} in miso is less than 0.4 % of the fortified ubiquinol-10, we did not prepare unfortified WRM, RM, and BM as controls. Therefore, we could not directly determine the endogenous antioxidant capacity. Third, it is not easy to identify compounds that maintain the reduced status of CoQ_{10} in natural miso because multiple antioxidant

compounds are produced during the maturation of miso [23]. Future studies developing ubiquinol-10-fortified foods should investigate the endogenous CoQ_{10} amount and the antioxidant capacity of the foods and perform multiple sampling to enable a significant data test.

4. Conclusions

In conclusion, we successfully prepared ubiquinol-10-fortified natural miso with a stable ubiquinol-10 structure (reduced form of CoQ_{10}). Furthermore, compounds with strong reducibility in natural miso, formed during the maturation process, are associated with the extreme stability of ubiquinol-10 in natural miso.

Data Availability

The data used to support the findings of this study are provided in the manuscript.

Conflicts of Interest

T. S. had no personal or financial conflicts of interest with Kaneka Co. T. Y. and K. H. are employees of Kaneka Co. Extraction and quantitative analysis of reduced (ubiquinol-10) and oxidized CoQ_{10} contents in miso was performed with R&D expenses from Kaneka Co. In addition, Kaneka Co. is applying for patents in Japan for the findings of this study (P2020-159771, 2020-159772). All other expenses for this research, including English proofreading, were funded by the Grants-in-Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Science (Grant Number JP19K11797).

Authors' Contributions

Conceptualization, T. S.; Methodology, T. S. and T. Y.; Investigation, T. S. and T. Y.; Resources, T. Y. and K. H.; Data curation, T. S.; Writing–Original Draft Preparation, T. S.; Writing–review & editing, T. S., T. Y., and K. H.; Visualization, T. S.; Funding Acquisition: T. S.

Funding Statement

This study was supported in part by the Grants-in-Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Science (Grant Number JP19K11797).

Acknowledgements

We thank Mr. Minoru TOYODA of Amekaze Co. for providing boiled soybeans, crude kitchen salt, and koji for natural miso preparation. We also thank Ms. Momoko ISHIDA, Ms. Miho KOBAYASHI, Ms. Misaki SAKATA, Ms. Momoka SUGIURA, Ms. Shoka HIKICHI, and Ms. Yu-uri YAMANE of Wayo Women's University for their help with the preparation of ubiquinol-10-fortified natural miso. Lastly, we thank Editage (www. editage.com) for English language editing.

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（2022年11月15日受理）