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ORIGINAL ARTICLE

Formation of CoQ10 reduced form by mixing CoQ10 oxidized form γCD complex and vitamin C in powder

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Abstract We have already reported the enhancement of the stability and bioavailability of coenzyme Q10 (CoQ10) oxidized form by γ-cyclodextrin (γCD) complexation. In a series of the studies, we investigated an easy and economical conversion of CoQ10 oxidized form to its reduced form in complex powder, using inexpensive vitamin C (VC) as the reductant. CoQ10 oxidized form or its γCD complex and VC were physically mixed at the molar ratio of 1:0 to 1:50. The mixtures were stored at 60 °C and 75% RH. The sampling was made at certain interval, and both CoQ10 oxidized and reduced form contents were measured by high performance liquid chromatography (HPLC). The result shows that the conversion ratio to CoQ10 reduced form in γCD complex was significantly higher than that of non-inclusion compound (ca. 80% versus ca. 30% at the maximum). It was also confirmed that CoQ10 reduced form in γCD complex remains as stable as its oxidized form in γCD complex. Free radical scavenging potential of partially reduced CoQ10–γCD complex was assayed with 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Keywords Coenzyme Q10 · Ubiquinone · Ubiquinol · Vitamin C · 1,1-diphenyl-2-picrylhydrazyl

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Introduction

Diseases such as arteriosclerosis, stroke, and diabetes may be caused or worsened by reactive oxygen species increasing the oxidative stress in the blood have become serious problems. To decrease the occurrence of such diseases and complications implicated in oxidative stress, it is beneficial to enhance anti-oxidative potency of the body by supplementing with antioxidants. The fat-soluble antioxidant, vitamin E (VE), which is responsible for protecting the polyunsaturated fatty acids from oxidation by free radicals is widely used for disease prevention. It has also been reported that the coexistence of the reduced form of CoQ10, known as ubiquinol, is important for fully activating the anti-oxidative potency of VE [1]. Intake of ubiquinol itself, has strong anti-oxidative potency, and contributes to strengthen the anti-oxidative potency effectively for the prevention of vascular impairment from ischemic reperfusion, arterial sclerosis and restenosis by it, and complications associated with diabetes.

Both the oxidized form, known as ubiquinone, and ubiquinol are common, however the oxidized form must be converted to the reduced form in order to be active as an antioxidant. When ubiquinone taken orally is converted to ubiquinol in the body by the reducing enzyme, NADPH it then functions as an antioxidant. Elderly or persons under treatment for the diseases mentioned above may have lowered or deterioration of their enzymatic reducing potency and therefore the intake of the ubiquinol could be very beneficial. However, ubiquinol is very unstable and it is difficult to handle since it undergoes auto-oxidation upon contact with air. Various methods for stabilizing ubiquinol have been investigated including: encapsulation by liposome [2, 3], emulsification by surface active agents [3], chelation by EDTA [4] and inclusion by γCD [5]. These

methods require the ubiquinol to be prepared under anoxic conditions with the addition of various stabilizers, and subsequently the resultant formulation products need to be stored in an aluminum bag under anoxic environment with a deoxidant at low temperature.

We have found both stability and bioavailability of CoQ10 are improved by γCD inclusion [6–11] and we launched the ubiquinone-γCD inclusion complex formulated with VC under the trade name of "NANO SUPLI" at the beginning of 2003. In the initial stage of product development, the purpose of concomitant of VC was for beautifying the skin since VC as well as CoQ10 is essential for collagen synthesis. By chance, it was found that the excess amount of VC formulated in this product converts the oxidized form of included CoQ10 to the reduced form partially, which is then stabilized. We then studied the conversion of ubiquinone to ubiquinol in the inclusion complex using VC as the reductant. It was found that significantly more ubiquinol is formed in the γCD inclusion complex compared to non-complexed CoQ10, and it remains stable with little decomposition. In addition, the partially reduced CoQ10–γCD inclusion complex was tested for DPPH radical scavenging activity and the details are reported here.

Materials and methods

Materials

Commercially available CoQ10 under the trade name "Bio Q10" was purchased from MITSUBISHI GAS CHEMICAL COMPANY, INC. It is highly pure CoQ10 oxidized form, ubiquinone. The specific content of CoQ10 is over 99.0% and the analogues are less than 1.0%. CoQ9 or some other geometrics isomers were detected in the CoQ10 according to our MS spectrum evaluation.

CoQ10 reduced form, ubiquinol, is a gift from MITSUBISHI GAS CHEMICAL COMPANY, INC. and was used as CoQ10 reduced form standard. Reagent grade VC and DPPH were purchased from Wako Pure Chemical Industries, Ltd. The CoQ10–γCD complex was supplied from Wacker Chemie AG under the trade name CAVAMAX® W8 CoQ10. The specific content of CoQ10 was over 20.0%. In fact, the content of CoQ10 was 20.9% and the moisture content was 3.4% in the product (Lot. ES021605). The content of γCD was not analyzed.

Preparation of the sample

CoQ10, and VC were weighed so that their molar ratio was either 1:0, 1:5, 1:10, 1:25, or 1:50. Each sample was then physically mixed well. The CoQ10–γCD inclusion complex was also weighed and physically mixed with VC in the same manner at the same ratio (CoQ10 content was calculated on pure form base). The mixtures were stored at 60 °C and 75% RH under light protection. Sampling was done at certain intervals and residual CoQ10 content was measured.

Measurement of CoQ10 content

Shimadzu HPLC system (LC-2010C) was used for the measurement of CoQ10 content for both oxidized form and reduced forms. Phonomenex HPLC column (Luna 5u C18(2) 100A : 4.6 mm I.D. × 150 mm) was used. Column temperature was set at 40 °C. Only *N*,*N*-dimethylformamide (DMF) was used as mobile phase with the flow rate of 0.8 mL/min. CoQ10 was detected using UV detector at 280 nm. The ubiquinone conversion ratio to ubiquinol was calculated using the following equation.

Conversion ratio to CoQ10 (%) = (Quantity of CoQ10 reduced form/Quantity of CoQ10 oxidized plus reduced forms) × 100.

Mass spectrum of CoQ10

Shimadzu LCMS system (LCMS-2020) was used for the detection of CoQ10 mass spectrum for both oxidized form and reduced form. Phonomenex HPLC column (Luna 5u C18(2) 100A: 4.6 mm I.D. × 150 mm) was used. Column temperature was set at 35 °C. The mobile phase was used a mixture of acetonitrile and isopropanol (8:7) containing 0.5% formic acid and 0.1% trisodium citrate aqueous solution (1 mg/mL), with the flow rate of 0.2 mL/min.

The mass spectrometer fitted with an electro spray ionisation (ESI) source was used for analysis. It was operated in the positive ion mode with following parameters: probe voltage +4.50 kV (+ESI), nebulizer gas flow 1.5 L/min, drying gas flow 15.0 L/min, block heater 200 °C, DL temperature 250 °C.

Measurement of DPPH radical scavenging activity

The samples of CoQ10 mixed with various amounts of VC were stored for 0, 7, 14 and 60 days. Then the included VC was removed by extraction with ion exchange water. The resultant sample was dried to powder and used for measurement. The sample was then dissolved in DMF and diluted stepwise. 1 mL was taken from each diluted sample and mixed with 1 mL of 0.3 mM DPPH ethanol solution using a vortex mixer.

The mixed solution was left 30 min at room temperature in a dark spot and light absorption was measured at 525 nm using Shimadzu absorption spectrophotometer (UV mini-1240).

1 mL of DPPH ethanol solution mixed with 1 mL of DMF solution was used as the "control" and 1 mL of ethanol mixed with 1 mL of sample solution was used as the "blank". Both are analyzed in the same way. The "blank" solution was prepared without DPPH in order to exclude the influence of absorbance with the sample at 525 nm.

DPPH radical scavenging activity was rated with the following equation. DPPH has a free radical, and its maximum absorption is 525 nm in ethanol solution. The radical was scavenged by a proton with antioxidant. Accordingly its absorption in 525 nm was decreased.

Radical scavenging activity (%) = (1 − ((Absorbance of sample plus DPPH solution − Absorbance of "blank" solution)/Absorbance of "control" solution)) × 100.

Result and discussion

CoQ10 oxidized form γCD inclusion complex was physically mixed with an excess volume of VC (50 times in molar quantity), stored at 60 °C and 75%RH under light protection for 60 days and analyzed with HPLC. As shown in Fig. 1, substantive amount of CoQ10 reduced form converted from CoQ10 oxidized form was observed.

CoQ10 was determined sensitivity on using the ESI source in positive mode. Then, main product ions of CoQ10 reduced form and its CoQ10 oxidized form were identified each $m/z = 887.8$ $[M + Na]^+$ and $m/z = 885.8$ $[M + Na]^+$ (Fig. 2).

We then evaluated the differences of the conversion ratio from CoQ10 oxidized form to CoQ10 reduced form between the CoQ10/VC physical mixtures and CoQ10-γCD inclusion complex/VC physical mixtures. For the evaluation totally ten test samples were prepared. Five test samples are the physical mixtures of CoQ10 and VC with different molecular ratios, 1:0, 1:5, 1:10, 1:25 and 1:50. And five test samples are the physical mixtures of CoQ10–γCD inclusion complex and VC with different molecular ratios, 1:0, 1:5, 1:10, 1:25 and 1:50. The conversion ratios of CoQ10 reduced form were measured after storing at 60 °C and 75% RH under light protection for 0, 7, 14 and 60 days.

As shown in Fig. 3, it was observed that the conversion ratio is significantly higher when the CoQ10–γCD inclusion complex used than non-complexed CoQ10, and the ratios get higher with increasing amounts of VC. This result may be due to increased contact between CoQ10 and VC at the molecular level. CoQ10 before inclusion is apt to coagulate, while after inclusion the CoQ10 disperses, which leads to an increase in contact area. We recently reported the simulated structure of the CoQ10–γCD inclusion complex. In the investigation, NMR, LC and

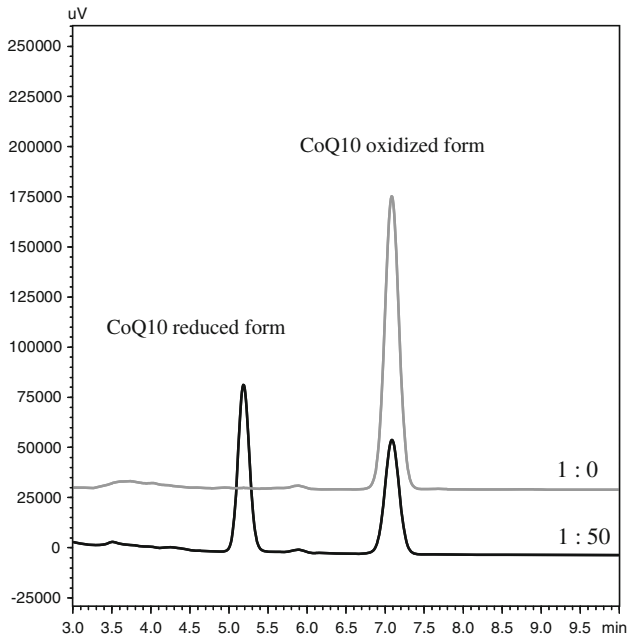

Fig. 1 LC chromatogram of CoQ10 samples at 280 nm; CoQ10 reduced form formation after addition of 50 times moles of VC to CoQ10 oxidized form γCD inclusion complex and 60 days storage at 60 °C and 75% RH

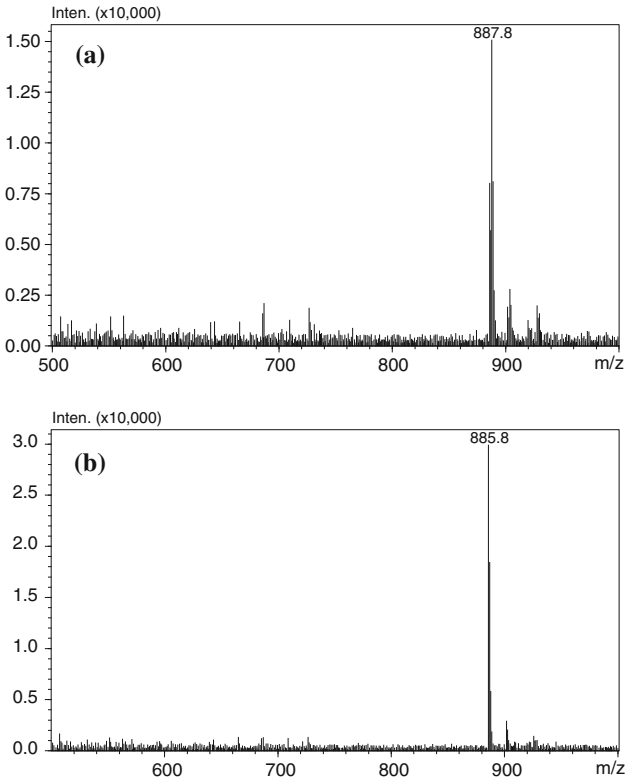

Fig. 2 Mass spectrums of (**a**) CoQ10 reduced form and (**b**) CoQ10 oxidized form

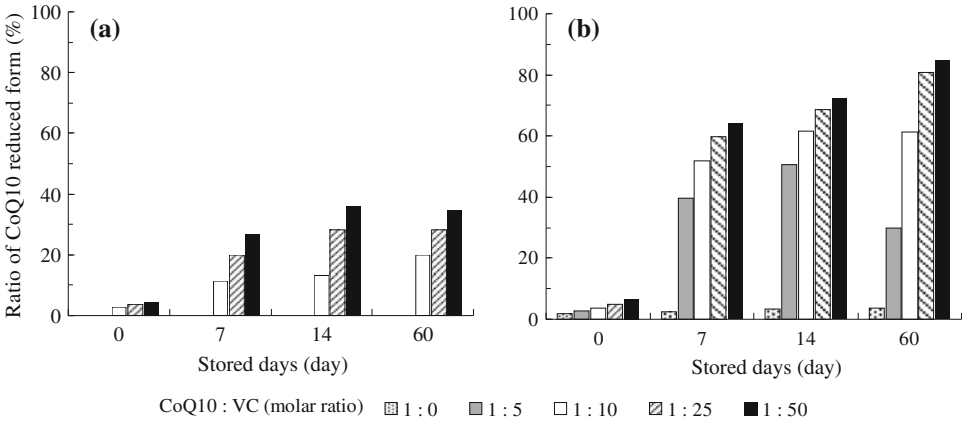

Fig. 3 CoQ10 reduced form ratio in (**a**) CoQ10 samples and (**b**) CoQ10-γCD inclusion complex samples

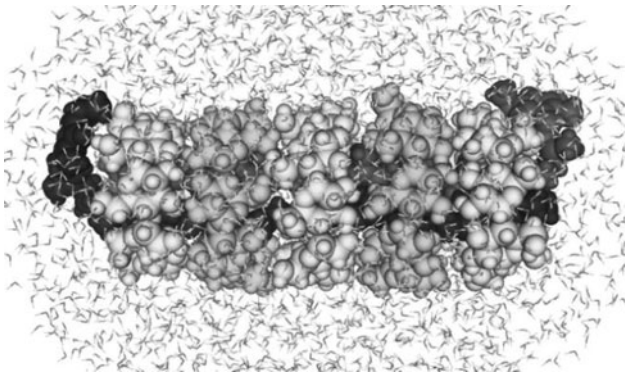

Fig. 4 Estimated configuration of CoQ10–γCD inclusion complex in water

DSC analyses on the CoQ10–γCD complex, confirmed formation of the inclusion complex with γCD/CoQ10 ratio of 2.5. Three different kinds of complex models that correspond to the ratio were then constructed and molecular mechanics and molecular dynamics calculations were carried out to provide several complex structures. In view of the stabilization energy, we concluded the structure that two molecules of CoQ10 and five molecules of γCD are involved in the inclusion, and that the isoprenoid chain of CoQ10 is shrouded in the γCD cavity while the quinone part is positioned outside [12]. The simulated structure is shown in Fig. 4. From this simulation result, it is estimated that the quinone part can convert to quinol by reductant. Then, the converted reduced form of CoQ10 in inclusion complex remains stable owing to the hydrogen bond between the hydroxyl group on quinol and the secondary hydroxyl group of γCD (Fig. 5). If the quinine part is positioned inside of the γCD cavity, the conversion to its reduced form will be inhibited. Therefore, this conversion study of CoQ10 reduced form is thought to be supportive evidence of our structural investigation study.

CoQ10 reduced form, ubiquinol, is known as an important antioxidant in living body. In order to confirm the antioxidant activity enhancement corresponding the increase of conversion ratio of ubiquinol, free radical

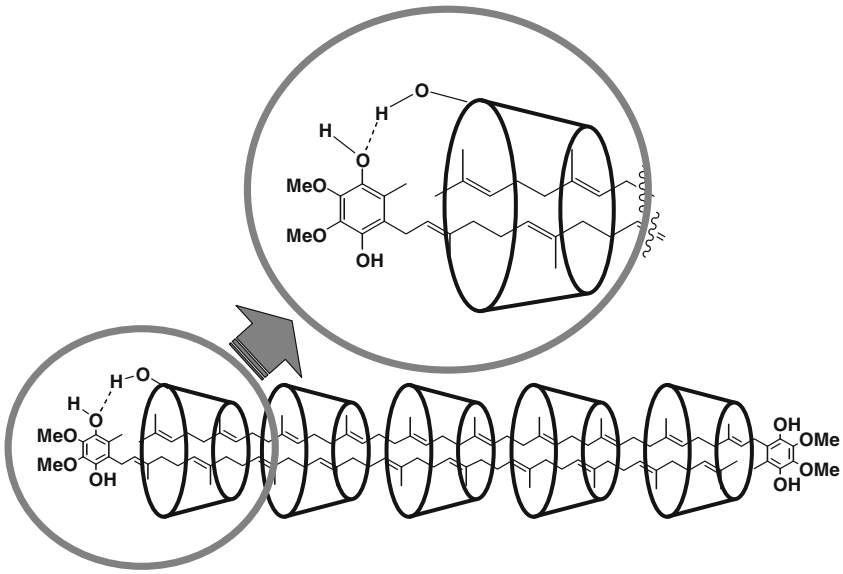

Fig. 5 Stabilization of CoQ10 reduced form by γCD

scavenging potential of partially reduced CoQ10–γCD complex was assayed with DPPH using ten samples obtained after the treatment with VC for 60 days. All samples were washed with water to remove VC in order to avoid the influence of VC for the measurement of DPPH radical scavenging activity. The complete removal of VC was confirmed with HPLC for each sample. The CoQ10 reduced form and oxidized form contents were also measured.

Figure 6 shows the total DPPH radical scavenging activity per one mole of CoQ10 (oxidized form plus reduced form). As anticipated the radical scavenging activity was correlated with the conversion ratio of CoQ10 reduced form. Higher radical scavenging activities were observed from the samples having higher contents of CoQ10 reduced form.

Conclusion

It has become apparent from various aspects that the intake of ubiquinol is beneficial for certain people, whose reducing capacity in the body is lowered, for example, the patients of diseases such as arteriosclerosis, stroke, diabetes and for the elderly. However, there have been many problems for the actual usage of ubiquinol. Commercially available ubiquinol is far more expensive than ubiquinone and it is required that it be kept and handled under oxygen-free conditions. We have established a simple and economical way for producing ubiquinol the concomitant use of ubiquinone–γCD inclusion complex with VC. CoQ10 is used not only as a nutritional supplement, but also for cosmetics. In cosmetic use, it's stability in the formulation and poor percutaneous absorption remains to be solved. Percutaneous absorbability was compared between non-complexed CoQ10 and CoQ10–γCD inclusion complex using three dimensionally cultured epidermis models. It was found that the CoQ10–γCD inclusion complex is superior to the former.

Ubiquinol is reported to be more absorbed percutaneously than ubiquinone or the water-soluble form [13]. Therefore, it is expected that CoQ10 partially converted to the reduced form by this method may increase subcutaneous absorption than the ubiquinone.

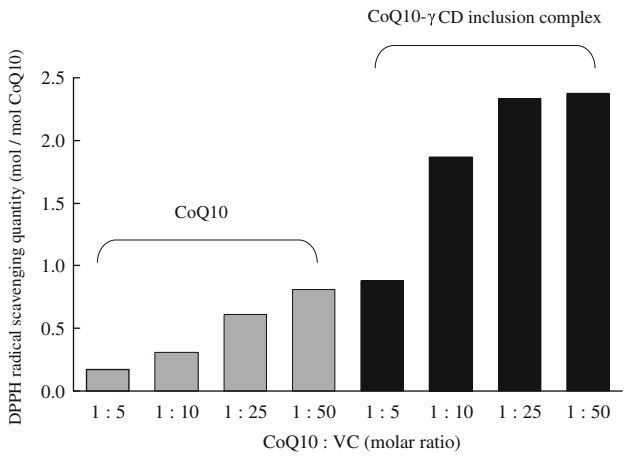

Fig. 6 DPPH radical scavenging quantity per 1 mol of CoQ10 (sum of oxidized form and reduced form)

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