Elucidation of Factors for Chiral Conversion of α-Lipoic Acid in Dietary Supplement

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Abstract
We elucidated factors for the chiral conversion of α-lipoic acid enantiomers in α-lipoic acid-containing dietary supplements. Samples were cleaned up by the solid-phase dispersive extraction method using Oasis MAX and MCX as the solid-phase gel. The α-lipoic acid enantiomers were reciprocally converted by heating at 180°C, and finally became a racemate. The chiral conversion rate changed depending on sample purity, particularly the presence or absence of coexisting components in the dietary supplements. As candidates for coexisting components, neutral nonionic and highly polar substances were suggested, such as sugars. We found that chiral conversion was promoted by heating in the presence of glucose. Oftentimes, a relatively large amount of S-(–)α-lipoic acid is detected in dietary supplements claiming to contain R-(+)α-lipoic acid on the bottle label. We speculate that the proportion of S-form may have been increased by coexisting components such as glucose when heat treatment is performed during the manufacturing process.

Keywords: α-Lipoic acid; Enantiomer; Chiral conversion; Dietary supplement; LC/UV

1. Introduction
In Japan, α-lipoic acid (Fig. 1), also called thiocic acid, has been used as a medicine, but since 2004, it has also been used as a dietary supplement [1]. α-Lipoic acid is a naturally occurring [2] sulfur-containing compound [3] that is biosynthesized in plants as well as humans and other animals. α-Lipoic acid exists in the mitochondria and acts as a coenzyme in energy-producing reactions in the body, such as the citric acid cycle and amino acid metabolism [4]. As such, it has been used as a dietary supplement mainly for the purpose of weight loss and recovery from fatigue. In addition to its antioxidant activity [5], α-lipoic acid regulates various signal transduction pathways [6-8]. It has also been suggested that α-lipoic acid prevents hypertension and improves insulin resistance [9], as well as improves symptoms of diabetic polyneuropathy [10] and Alzheimer's disease [11].

α-Lipoic acid has an asymmetric carbon in its structure and exists as an enantiomer. Only the R-form is endogenously synthesized [12] and conjugated to a protein. In our previous study [13], a dietary supplement was found to contain a relatively large amount of S-(–)α-lipoic acid even though the product label states that it contains R-(+)α-lipoic acid derived from natural sources. It was speculated that heat treatment and/or coexisting components contributed to the chiral conversion during the manufacturing process [13]. It has been suggested that the R-form and the S-form of α-lipoic acid have different activities, such as different toxicity in rats [14], different anti-inflammatory effects in mice [15], and different enantioselective pharmacokinetics in humans [16]. In addition, a recent research trend survey on α-lipoic acid by text mining techniques showed that research on α-lipoic acid enantiomers, particularly bioavailability and pharmacokinetics studies of enantiomers, has been increasing in number, and research on this subject would continue [17]. In order to evaluate the quality of α-lipoic acid-containing dietary supplements, it is important to determine the enantiomeric excess of α-lipoic acid and elucidate the factors contributing to the chiral conversion.

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Therefore, in this study, we investigated the factors for α-lipoic acid chiral conversion.

![α-lipoic acid structures](image)

**Fig. 1.** Chemical structures of α-lipoic acid enantiomers.

### 2. Experimental

#### 2.1. Materials and reagents

Racemic α-lipoic acid (RS-form; special grade) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). R-(−)-α-lipoic acid (chemical purity: 98%) and S-(−)-α-lipoic acid (chemical purity: 97.0%) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Racemic α-lipoic acid standard stock solution (2,000 μg/mL) was prepared by dissolving racemic α-lipoic acid in ethanol. Working solution (500 μg/mL) was prepared by diluting the stock solution with purified water. In a similar manner, each α-lipoic acid enantiomer (R-form and S-form) standard stock solution (2,000 μg/mL) was prepared with ethanol, and the working solution (50 μg/mL) was prepared by diluting the stock solution with purified water. All standard stock solutions and working solutions were stored at 4°C until use.

Acetonitrile and methanol (both HPLC grade); and ethanol, formic acid, L-glucose (L-Glu), D-xylose (D-Xyl), and D-maltose (D-Mal) (all special grade) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 25% Aqueous ammonia solution (analytical grade); 1-butanol (HPLC grade); and ethyl acetate, n-hexane, phosphoric acid, D-glucose (D-Glu), and D-fructose (D-Fru) and sucrose (Suc) (all special grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other chemicals were of special grade. Water was purified with a Milli-Q Gradient A10 system equipped with an EDS-Pak Polisher (Merck KgA, Darmstadt, Germany).

As commercially available dietary supplements containing α-lipoic acid, four products (three capsules and one tablet) manufactured in the United States were purchased. Among them, the one having the highest R-form content (hard capsule type) was used for the subsequent chiral conversion experiments.

Advantec Dismic-25cs cellulose acetate membrane filter (pore diameter: 0.45 μm, filter housing diameter: 25 mm) was from Advantec Toyo Kaisha, Ltd. (Tokyo, Japan).

As the device for solid-phase dispersive extraction (SPDE), @Roka and Captube centrifugation filter units were purchased from Frontier Science Co., Ltd. (Ishikari, Japan). Oasis MAX and MCX solid-phase extraction cartridges (180 mg, particle size 30 μm; Waters Co., Milford, MA, USA) were used. After removing the solid-phase gel from the cartridges, the gel was used for SPDE. The MAX gel was conditioned with methanol and 5% aqueous ammonia solution sequentially, and then suspended in aqueous ammonia solution at the concentration of 50 mg/mL. The MCX gel was conditioned with methanol and purified water sequentially, and then suspended in purified water at the concentration of 50 mg/mL.

For the measurement of glucose concentration in a sample, OneTouch UltraVue manufactured by Johnson & Johnson Co., Ltd. (New Brunswick, NJ, USA) was used together with the glucose kit LFS Quick Sensor. Before using this sensor, a control test was conducted using a known amount of glucose solution, and it was confirmed in advance that the displayed value after measurement was within the permissible range.

#### 2.2. LC/UV apparatus and operating conditions

An LC-10AS pump system equipped with an SPD-10A (Shimadzu Corporation, Kyoto, Japan) was used. LC separation was performed with a CHIRALPAK AD-RH (150 × 4.6 mm i.d., 5 μm; Daicel Corporation, Osaka, Japan). Column temperature was maintained at 40°C. The mobile phase was a mixture of 10 mmol/L phosphoric acid aqueous solution, acetonitrile, and methanol in the ratio of 70:25:13 (v/v/v), and was delivered in the isocratic elution mode at the flow rate of 0.5 mL/min. The UV detection wavelength was set at 220 nm. A 10 μL aliquot of the sample was injected into the system.

#### 2.3. Preparation of sample solution

Three types of samples (A, B, and C) having different purities were prepared. One capsule of the dietary supplement confirmed to contain R-(+)–α-lipoic acid was transferred into a 15 mL plastic centrifuge tube. Ten milliliters of purified water was added, and the centrifuge tube was placed in an ultrasonic bath heated to 40°C to disintegrate the capsule. “Sample A (suspension)” was obtained after the capsule shell disintegrated and the contents were dispersed and suspended after the mixture was left to stand for 30 min. Sample A was centrifuged (2500 × g, 10 min), and the supernatant was filtered using filter paper and a membrane filter to obtain “Sample B (supernatant)”. Next, sample B (1 mL) was cleaned up by SPDE (described below) using Oasis MAX, and the eluate was evaporated to dryness in vacuo and then redissolved in 1 mL of purified water. This was designated as “Sample C (purified solution)”.

SPDE was performed according to our previous method [18–22]. Briefly, a Captube was set on top of @Roka, and a conventional 2.0 mL micro test tube was attached to the
bottom of @Roka. The outline of SPDE using Oasis MAX is as follows. Five hundred microliters of sample solution and 300 µL of an Oasis MAX solid-phase gel suspension (50 mg/mL) were added into the Captube. The solid-phase gel suspension was immediately agitated for 30 s with a vortex mixer to sufficiently disperse the solid-phase gel in the sample solution. The centrifugal filter unit was centrifuged (2500 × g, 30 s), and the filtrate in the micro test tube attached to the bottom of @Roka was discarded together with the micro test tube. To wash the solid-phase gel, 2.5% aqueous ammonia solution (500 µL) was added into the Captube, and the solid-phase gel was dispersed once again. Subsequently, the solvent phase was discarded as described above. The washing step was repeated with water (500 µL) and methanol in a similar manner. Finally, 500 µL of a mixture of 10% aqueous formic acid solution/methanol was added into the Captube, and the solid-phase gel was dispersed, sonicated for 1 min, and then centrifuged (2500 × g, 1 min) once again for elution. The elution operation was repeated two times, and the eluates were combined.

2.4. Heating method
R- and S-form α-lipoic acid standard solutions or a sample solution (1 mL each) were transferred into a glass test tube (7 mL volume), respectively. After covering the mouth of the test tube with aluminum foil, the test tube was set in a high-pressure glass tube (15 mL volume; AS ONE Corporation, Osaka, Japan) that was sealed tightly to prevent leakage of liquid to the outside. Furthermore, assuming that the glass test tube breaks due to heating, the high-pressure glass tube was housed in an ultrahigh-pressure stainless steel cell (Stainless-steel cell, Dionex ASE System; Thermo Fisher Scientific, Waltham, MA, USA), which was then placed in an Agilent 6890 gas chromatograph oven (Agilent Technologies, Santa Clara, CA, USA) and heated at 180°C.

2.5. Effect of matrix component in sample (B) on chiral conversion
In order to fractionate the components in sample B, systematic liquid-liquid extraction using organic solvents with different polarities, and SPDE using two types of solid-phase agents (Oasis MAX and MCX) with different ion-exchange properties were carried out. Details are shown in the next subsection. One milliliter of each sample solution obtained by each method was mixed with 1 mL of R-form standard solution. From this, 1 mL was withdrawn as control sample (unheated sample), and the remaining 1 mL was heated for 2 h according to the method described previously.

2.5.1. Systematic liquid-liquid extraction

To 2 mL of sample B, 2 mL of hexane was added, and the mixture was extracted by vortex mixing for 1 min. After centrifugation (2500 × g, 1 min), the hexane phase was separated from the aqueous phase. Subsequently, 2 mL of ethyl acetate was added to the remaining aqueous phase, and liquid-liquid extraction was performed in a similar manner. After separating the ethyl acetate phase, 1-butanol was added to the remaining aqueous phase, and liquid-liquid extraction was performed in a similar manner. The solvents in both the hexane phase and the ethyl acetate phase were dried by nitrogen purging, and the solvent in the 1-butanol phase was evaporated to dryness in vacuo. After that, 2 mL of purified water was added to redissolve each residue and prepare a sample solution. The aqueous phase remaining in the final stage was also used as a sample solution.

2.5.2. Extraction of ionic components by SPDE
SPDE was performed on 1 mL of sample B using Oasis MAX, and the obtained effluent, three types of washing solutions (2.5% aqueous ammonia solution, purified water, and methanol), and eluate (methanol containing 10% formic acid) were evaporated to dryness. One milliliter of purified water was added to redissolve each residue and prepare a sample solution. On the other hand, SPDE was performed using Oasis MCX in a similar manner, and the obtained effluent, washing solutions (2% formic acid aqueous solution, purified water, and methanol), and eluate (methanol containing 5% ammonia) were treated in a similar manner.

2.6. Effect of coexistence of monosaccharides and disaccharides on chiral conversion
To 1 mL of the R-form standard solution was added one of the following solutions: 1 mL of D-Glu aqueous solution (0.1%, 1%, or 10%), 1 mL of D-Glu, D-Fru, or D-Xyl aqueous solution (1%), and 1 mL of D-Mal or Suc aqueous solution (1%). Then, each reaction mixture was heated in a similar manner to that described in section 2.5, and the degree of chiral conversion was measured. In addition, glucose concentration was measured in each sample after heating.

3. Results and discussion
In the LC/UV determination, the absolute calibration curve method was used. The calibration curve range was 5 to 250 µg/mL, and the limit of quantification (LOQ) was set at 5 µg/mL.

3.1. Chiral conversion with heating
The degree of chiral conversion was evaluated on the basis of the enantiomeric excess (ee, %). Figure 2 shows a typical chromatogram of an unheated racemic standard solution (500 µg/mL).
In our previous work, we conducted heating experiments at 38°C, 70°C, and 93°C [21]. In this study as well, heating the R-form and S-form standard solutions at 70°C and 95°C resulted in decreases in peak intensities but no formation of the corresponding enantiomers. When heating was conducted at 180°C for 6 h, the peak intensities decreased and the formation of S-form and R-form, which are the corresponding enantiomers, was confirmed. The ee of the R-form was 92.4 ± 2.64%, and that of the S-form was 81.8 ± 3.84%. From these results, we inferred that the chiral conversion of α-lipoic acid enantiomers occurs under high-temperature conditions.

Next, in a preliminary experiment, it was found that α-lipoic acid in supplements is more easily chiral converted than standard products. Therefore, considering the possibility that the coexisting components in the supplement act catalytically on the progress of the chiral conversion, the following three types of samples (A, B, and C) having different purities were prepared using the supplement aqueous solution (suspension). That is, sample A was suspended state without any purification treatment, sample B was the supernatant solution of the sample A by filtration in order to remove insoluble components, and sample C was the cleaned up solution of sample B by SPDE using Oasis MAX. As a result, when sample A (suspension), sample B (supernatant), and sample C (purified solution) described in section 2.3 were heated at 180°C, chiral conversion occurred in all three samples. In this experiment, sample A and sample B were almost racemized after 6 h (ee, sample A: 2.37%, sample B: 0.487%), whereas sample C had 23.4% ee, showing clearly little racemization despite heating at the same temperature. Figure 3 shows the chromatograms of sample A before and after heating.

Figure 4 shows the time courses of ee when the R-form and S-form standard solutions of α-lipoic acid and samples A, B, and C were heated at 180°C. The ee of R-(+)-α-lipoic acid standard solution that has no coexisting components was 92.4%, indicating minimal chiral conversion.
components in the suspension.Conventionally, gelatin, pullulan, carrageenan, starch, gum arabic, hydroxypropyl methylcellulose and the like have been used as film-forming agents for capsules. However, detailed information on them could not be obtained from the labels of the supplements used. Even if the constituents are unknown, we thought that capsule outer skin itself has a possibility to exert a chiral conversion. Therefore, after taking out the contents from the capsule, chiral conversion experiment using only the outer skin was carried out. As a result, little effect was observed on the chiral conversion. From these results, we suggested that coexisting water-soluble components derived from the supplement matrix promoted the chiral conversion of α-lipoic acid.

3.2. Effect of components in sample matrix on chiral conversion

3.2.1. Effect of polar components in sample B

The above experimental results led us to formulate the hypothesis that coexisting water-soluble components might have affected the chiral conversion. To verify this hypothesis, the components of sample B obtained by systematic liquid-liquid extraction using organic solvents (hexane, ethyl acetate, and 1-butanol) were added to the R-form standard solution, and the mixture was heated and examined for the effect of the components on the chiral conversion. The hexane and ethyl acetate extracts showed around 90% ee (89.9 and 90.2%, respectively), the 1-butanol extract gave 81.7% ee, and the aqueous phase gave 64.8% ee. From these results, we speculated that among the components derived from the supplement matrix, the most polar water-soluble components promoted the chiral conversion of α-lipoic acid.

3.2.2. Effect of ionic components in sample B

From the experimental results in subsection 3.2.1, we suggested that the components extracted with a highly polar solvent (water) may affect the chiral conversion. Ionic substances and highly polar neutral substances were considered candidate components. Therefore, in order to identify the ionic substances, we examined in detail the eluate and so on obtained by SPDE using Oasis MAX and MCX. As Oasis MAX is capable of anion exchange and hydrophobic interaction, we inferred that cationic substances and highly polar neutral substances are present in the effluent and the washing solutions treated with Oasis MAX, and the eluate contains anionic substances and highly hydrophobic substances. On the other hand, as Oasis MCX is capable of cation exchange and hydrophobic interaction, we inferred that anionic substances and highly polar neutral substances are present in the effluent and the washing solutions treated with MCX, and the eluate contains cationic substances and highly hydrophobic substances.

Each of the respective liquid phases obtained from SPDE using Oasis MAX and MCX was added to R-form standard solution, and the solution was heated for 2 h. From the experimental results shown in Fig. 4, we confirmed that chiral conversion took place even after a short heating time, and it was necessary to examine candidate substances in order to determine the factors for chiral conversion. Therefore, in order to acquire experimental results as quickly as possible, the heating time was set to 2 h in the experiments in this section. We found that the chiral conversion rate (decrease in ee) was highest for the effluent (load) obtained from SPDE using Oasis MAX and MCX (Fig. 5). From these results, we speculated that matrix-derived neutral nonionic and highly polar substances in the dietary supplement promoted the chiral conversion of α-lipoic acid.

**Fig. 5.** Comparisons of ee after mixing R-(+)-α-lipoic acid standard solution with SPDE extracts and heating at 180°C for 2 h. [A] Fractions from Oasis MAX, and [B] fractions from Oasis MCX. Each fraction of [A]; Control: Average ee of each extract before heating, Load: effluent form MAX, Wash 1: 2.5% aqueous ammonia solution, Wash 2: purified water, Wash 3: methanol, Elute: 10% formic acid in methanol. Each fraction of [B]; Control: Average ee of each extract before heating, Load: effluent form MCX, Wash 1: 2% formic acid aqueous solution, Wash 2: purified water, Wash 3: methanol, Elute: 5% ammonia in methanol.
3.3. Effect of sugars on chiral conversion

Some labels on dietary supplement bottles state that the ingredients include such saccharides as cornstarch, dextrin, and reduced maltose starch syrup, aside from α-lipoic acid. From this, we strongly suspected that saccharides are involved in the chiral conversion of α-lipoic acid as neutral nonionic and highly polar substances. Therefore, we examined monosaccharides and disaccharides, which are essential nutrients in food.

Chiral conversion occurred when 1% D-Glu aqueous solution was added to the R-form standard solution and the solution was heated at 180°C for 2 h. Similarly, chiral conversion occurred when 1% l-Glu aqueous solution was added, but the conversion proceeded more slowly than when the D-form was added (ee, D-Glu: 69.0%, l-Glu: 76.9%). Therefore, we inferred that stereochemical structure of Glu may affect the chiral conversion of α-lipoic acid.

Next, the effect of different Glu concentrations (0.1, 1, 10%) on the chiral conversion was examined. Chiral conversion proceeded more rapidly when 1% D-Glu aqueous solution was used than when 0.1% D-Glu aqueous solution was used. However, when 10% D-Glu aqueous solution was used, the sample solution turned brown, and no α-lipoic acid peak was detected. We speculated that such side reactions as caramelization and/or the Maillard reaction occurred when the solution containing 10% D-Glu aqueous solution was heated, suppressing the chiral conversion.

Sugars other than Glu were also examined in the same manner. 1% D-Fru aqueous solution resulted in 84.6% ee, and 1% D-Xyl aqueous solution, 71.1% ee. D-Fru is a ketose, whereas D-Glu and D-Xyl are aldoses. Furthermore, the chiral conversion also proceeded with 1% D-Mal aqueous solution and 1% Suc aqueous solution, both of which are disaccharides (D-Mal: 68.8% ee, Suc: 65.3% ee). D-Mal is composed of two molecules of α-D-Glu, and Suc is made up of one Glu unit and one Fru unit linked together by an acetal oxygen bridge between C1 of α-D-Glu and C2 of β-D-Fru. We hypothesized that both disaccharides were hydrolyzed by heating, resulting in the production of D-Glu, and accordingly, chiral conversion proceeded (Table 1).

In order to test this hypothesis, we conducted an experiment to determine whether disaccharides (Suc and D-Mal) were hydrolyzed by heating (180°C) to produce Glu. In the blank sample, Glu concentration in the sample before heating was 26.5 mg/dL; however, it increased to 70.5 mg/dL after heating. An increase in Glu concentration (151 mg/dL) was confirmed in the heated sample containing 1% Suc aqueous solution. On the other hand, a much greater increase in Glu concentration (245 mg/dL) was detected in the heated sample containing 1% D-Mal aqueous solution.

From these results, we confirmed that a portion of disaccharides (Suc, D-Mal, etc.) present in dietary supplement is hydrolyzed by heating to produce D-Glu.

4. Conclusions

We clarified that S(-) and R(+)-α-lipoic acid enantiomers are converted by heating at 180°C. The coexistence of reducing sugars, such as Glu, Fru, and Xyl, promoted the chiral conversion. Moreover, Glu was produced by the decomposition of disaccharides (Mal and Suc) present in dietary supplement by heating. From these results, we inferred that chiral conversion from R(+)-α-lipoic acid might occur in a sample from which a relatively high proportion of S(-)-α-lipoic acid was detected, by heating in the manufacturing process.

Conflict of interest

There are no financial or other relations that could lead to a conflict of interest.

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