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Development and Validation of Oxygen Radical Absorbance Capacity Assay for Lipophilic Antioxidants Using Randomly Methylated β-Cyclodextrin as the Solubility Enhancer

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We recently reported the improved oxygen radical absorbance capacity (ORAC) assay using fluorescein (FL) as the fluorescent probe. The current $ORAC_{FL}$ assay is limited in hydrophilic antioxidant due to the aqueous environment of the assay. Lipophilic antioxidants mainly include the vitamin E family and carotenoids, which play a critical role in biological defense systems. In this paper, we expanded the current $ORAC_{FL}$ assay to lipophilic antioxidants. Randomly methylated β-cyclodextrin (RMCD) was introduced as the water solubility enhancer for lipophilic antioxidants. Seven percent RMCD (w/v) in a 50% acetone−H_2O mixture was found to sufficiently solubilize vitamin E compounds and other lipophilic phenolic antioxidants in 75 mM phosphate buffer (pH 7.4). This newly developed ORAC assay (abbbreviated $ORAC_{FL-LIPO}$) was validated through linearity, precision, accuracy, and ruggedness. The validation results demonstrate that the $ORAC_{FL-LIPO}$ assay is reliable and robust. For the first time, by using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid as a standard (1.0), the ORAC values of α-tocopherol, (+)-γ-tocopherol, (+)-δ-tocopherol, α-tocopherol acetate, tocotrienols, 2,6-di-*tert*-butyl-4-methylphenol, and γ-oryzanol were determined to be 0.5 ± 0.02, 0.74 ± 0.03, 1.36 ± 0.14, 0.00, 0.91 ± 0.04, 0.16 ± 0.01, and 3.00 ± 0.26, respectively. The structural information of oxidized α-tocopherol obtained by liquid chromatography/mass spectrometry reveals that the mechanism for the reaction between the vitamin E and the peroxyl radical follows the hydrogen atom transfer mechanism, which is in agreement with the notion that vitamin E is the chain-breaking antioxidant.

KEYWORDS: ORAC; cyclodextrin; LC/MS; lipophilic antioxidants; vitamin E; chain breaking; hydrogen atom transfer

INTRODUCTION

Antioxidants can be physically classified by their solubility into two groups (*1*) (i) hydrophilic antioxidants, such as vitamin C and the majority of polyphenolic compounds, and (ii) lipophilic antioxidants, mainly including vitamin E and carotenoids. Similar to hydrophilic antioxidants, lipophilic antioxidants play an important role in a wide spectrum of biochemical and physiological processes. Of primary interest is their optimal antioxidant activity in vitro and in vivo. Unlike hydrophilic antioxidants, which do not accumulate in the body and are excreted in the urine, lipophilic antioxidants penetrate the lipoprotein cell membrane more easily and therefore reach a higher level of bioavailability (*2*). Although lipophilic antioxidants are highly bioavailable, it is not a trivial task to accurately measure their antioxidant activity in vitro. There are a number of methods available for measuring antioxidant activity such as the oxygen radical absorbance capacity (ORAC) fluorescein (FL) assay from our laboratory; all of these methods are conducted in an aqueous system, as such, none of which are suitable for lipophilic antioxidants. Without knowing the actual effectiveness of the lipophilic antioxidants, consumers can be exposed to unsafe concentrations or ineffective dosages. We overcame this obstacle by introducing randomly methylated β-cyclodextrin (RMCD) as a molecular host to enhance the solubility of lipophilic antioxidants in aqueous solution. Specifically, cyclodextrins (CDs) are cyclic (α-1,4)-linked oligosaccharides of α-D-gluco-pyranose containing a relatively hydrophobic (fatlike) central cavity and hydrophilic (waterlike) outer surface. This property of CD has made it increasingly popular as a vehicle for enhancing the solubility of fat soluble compounds in an aqueous environment in pharmaceutical and food industries (*3*, *4*). In this paper, we will report the development and validation of a new ORAC assay specific for lipophilic antioxidant activity. To our knowledge, it is the first time that vitamin E and other common lipophilic phenolic antioxidants were measured using the ORAC assay.

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MATERIALS AND METHOD

Chemicals and Apparatus. RMCD was purchased from Cyclolab R&D Ltd. (Budapest, Hungary). FL and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were purchased from Aldrich (Milwaukee, WI). 2,2′-Azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). γ-Oryzanol was purchased from TCI America (Portland, OR). Nutriene (tocotrienols) was obtained from Eastman Chemicals Company (Kingsport, TN). 4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a- diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591 C11) was purchased from Molecular Probes, Inc. (Eugene, OR). All other standards were commercially available form Sigma or Aldrich. All ORAC analyses were performed on a COBAS FARA II analyzer (Roche Diagnostic System Inc., Branchburg, NJ) using an excitation wavelength of 493 nm and an emission filter of 515 nm. The photobleaching experiment of BODIPY 581/591 C11 was carried out in a Bio-Tek fluorescence microplate reader FL600 (Bio-Tek, Winooski, VT). The identification of oxidized Trolox and α-tocopherol induced by AAPH was performed by a HP 1100 high-performance liquid chromatography (HPLC) system (Hewlett-Parkard, Palo Alto, CA) coupled with a Finigan LCQ ion trap mass spectroscopic detector (ThermoFinigan, San Jose, CA).

Sample Preparation. Approximately 0.5 g of sample was dissolved in 20 mL of acetone. An aliquot of sample solution was appropriately diluted with 7% RMCD solvent (w/v) made in a 50% acetone−water mixture (v/v) and was shaken for 1 h at room temperature on an orbital shaker at 400 rpm. The sample solution was ready for analysis after further dilution with 7% RMCD solvent.

Automatic ORAC Assay. The automated ORAC assay was carried out on a COBAS FARA II spectrofluorometric centrifugal analyzer. The procedure was based on a previous report of Ou and co-workers (*5*). With the exception of samples and Trolox standards, which were made in 7% RMCD solvent, all other reagents were prepared at 75 mM phosphate buffer (pH 7.4). In the final assay mixture (0.4 mL total volume), FL (6.3×10^{-8} M) was used as a target of free radical attack and AAPH (1.28×10^{-2} M) was used as a peroxyl radical generator. Seven percent RMCD was used as the blank, and Trolox (12.5, 25, 50, and 100 μM) was used as the control standard. The analyzer was programmed to record the fluorescence of FL every minute after the addition of AAPH. All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample. These results were expressed as micromoles Trolox equivalent (TE).

Competitive Reaction of Trolox and α-Tocopherol with AAPH. A total of 0.5 mL of 0.5 mM Trolox and 0.5 mL of 0.5 mM α-tocopherol (prepared in 7% RMCD) were mixed in a 1.5 mL HPLC sample vial followed by the addition of 20 μL of 640 mM AAPH; the reaction solution was incubated at 37 °C in an autosampler. At every 10 min interval, 5 μL of reaction solution was injected into a Zorbax C18 column (Hewlett-Packard, Palo Alto, CA) (2.1 mm × 150 mm, 3 μm) until the reaction was completed. The mobile phase was 70% methanol with a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm.

Characterization of Oxidized Products of Trolox Induced by AAPH. A mixture of 0.2 mL of 2.0 mM Trolox and 0.8 mL of 640 mM AAPH was incubated at 37 °C for 30 min. The reaction products were separated by a Zorbax C18 column (2.1 mm × 150 mm, 3 μm) at 37 °C with a mobile phase of 70% methanol at a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm. The oxidized products were characterized using a Finnigan LCQ ion trap mass spectrometer equipped with an API chamber and an electrospray ionization (ESI) source. The ionization was negative ion mode, and Aux gas and Sheath gas were set to 72 and 14 units, respectively. An ionization reagent of 1.5 mM ammonium hydroxide was added at a rate of 0.05 mL/min through a Tee device using a secondary HPLC pump before the API chamber. Trolox was used as a standard for calibrating the system.

Characterization of Oxidized Products of α-Tocopherol Induced by AAPH. A mixture of 0.5 mL of 20 mM α-tocopherol (prepared in 7% RMCD) and 0.5 mL of 640 mM AAPH was incubated at 37 °C for 30 min and was extracted with 10 mL of methylene chloride. The methylene chloride phase was separated and washed with 5 mL of deionized water 3 times. The oxidized products of α-tocopherol in the methylene chloride phase were then analyzed by liquid chromatography/mass spectroscopy (LC/MS). With the exception of 100% acetonitrile as the mobile phase, the LC/MS conditions are the same as above.

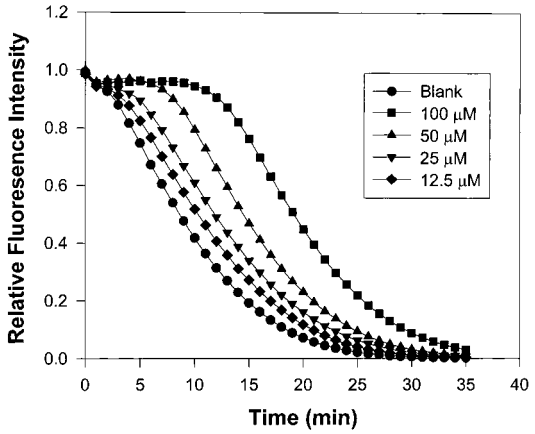

Figure 1. FL fluorescence decay curves induced by AAPH in the presence of α-tocopherol at different concentrations.

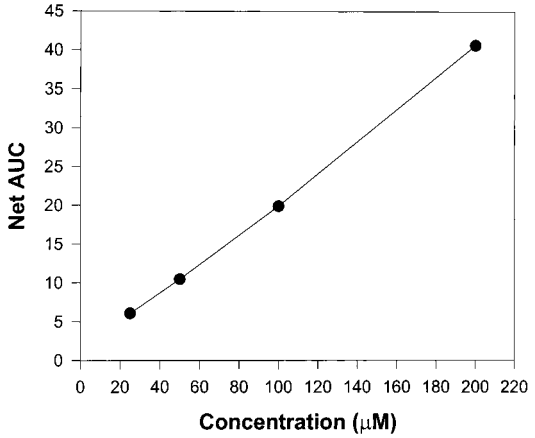

Figure 2. Regression of net AUC of α-tocopherol on different concentrations of α-tocopherol. The net AUC = $AUC_{sample} - AUC_{blank}$; the AUC was calculated by the equation previously described by Ou et al. (see *5*).

Photobleaching Experiment of BODIPY 581/591 C11. BODIPY 581/591 C11 was dissolved in a 1:9 butyronitrile and octane mixture (v/v) to give a 7.9×10^{-7} M solution; 200 μL of the BODIPY solution was then added into a 96 well polyproplene plate. The photostability of BODIPY was evaluated by monitoring the change of fluorescence intensity over a 1 h period. The fluorescence reading was taken every minute by a Bio-Tek fluorescence microplate reader FL600 (Bio-Tek, Winooski, VT) equipped with an excitation filter for 545/40 nm and an emission filter for 620/40 nm.

RESULTS

Method Validation. *(a) Linearity.* The correlation between the net area under the curve (AUC) for the antioxidant and its concentration was evaluated using four pure compounds. **Figure 1** illustrates the FL fluorescence decay curves in the presence of α-tocopherol and AAPH. **Figure 2** depicts the linear response between the concentration and the net AUC for α-tocopherol. **Table 1** summarizes the net AUCs corresponding to the different concentrations and the linear coefficient (r^2) for the pure compounds.

(b) Limit of Quantitation (LOQ) and Limit of Detection (LOD). The LOQ is the lowest concentration on the calibration curve, while the LOD is the lowest amount of antioxidant that

Table 1. Net AUC vs Concentration[a]

compd	concn (μM)	net area	r^2
γ-oryzanol	25	28.94	
	12.5	15.87	0.9979
	6.25	8.51	
	3.125	4.32	
γ-tocopherol	100	28.45	
	50	14.83	0.9971
	25	7.78	
δ-tocopherol	75	36.11	
	50	27.52	
	25	15.56	0.9668
	12.5	8.34	
	6.25	4.46	
α-tocopherol	200	40.67	
	100	19.89	
	50	10.45	0.9990
	25	6.07	

[a] Regression equation is expressed as Y (net area) = kX (concentration) + intercept.

Table 2. Precision and Accuracy of the $ORAC_{FL-LIPO}$ Assay

vitamin E	QC1	QC2	QC3
nominal concn (μM)	40	80	160
	Run 1		
intramean (μM)	41.69	84.48	175.40
SD[a]	2.87	2.12	5.67
% RSD[b]	8.79	2.52	3.23
% REC[c]	104.25	105.60	109.62
N	4	4	4
	Run 2		
intramean (μM)	42.74	92.01	171.24
SD	2.65	5.12	10.86
% RSD	6.20	5.57	6.34
% REC	106.86	115.01	107.03
N	4	4	4
	Run 3		
intramean	39.0425	85.8525	167.21
SD	5.46	6.54	2.61
% RSD	13.99	7.62	1.56
% REC	97.60	107.31	104.50
N	4	4	4
	pooled runs		
intermean (μM)	38.16	87.45	171.28
SD	3.66	6.89	6.38
% RSD	9.66	5.24	3.71
% REC	95.39	109.31	107.05
N	12	12	12

[a] Standard deviation. [b] Relative standard deviation. [c] Recovery.

can be detected. In our experiment, the LOQ and LOD were determined to be 12.5 and 5.0 μM, respectively.

(c) Precision and Accuracy. **Table 2** summarizes the precision and accuracy of the ORAC assay using α-tocopherol as a candidate compound. The precision, which is expressed as relative standard deviation (%RSD) for all quality control concentrations, was within $\pm$15%. The accuracy of the method varies from 97.60 to 115.01% within individual batches and from 95.39 to 107.05% between all of the batches.

(d) Ruggedness. To determine the reproducibility of the method, a ruggedness experiment was performed. Using two COBAS FARA II analyzers, 12.5 μM γ-oryzanol was analyzed for 50 days. Results are shown in **Figure 3**.

ORAC Values for Pure Lipophilic Antioxidants. Table 3 lists the results for common lipophilic phenolic antioxidants, which were referenced as the TEs. γ-Oryzanol possesses the highest value of 3.0, while vitamin E acetate shows no antioxidant activity.

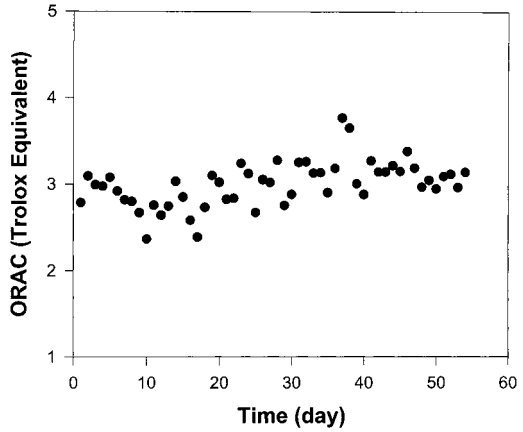

Figure 3. Ruggedness of the $ORAC_{FL-LIPO}$ assay. An amount of 12.5 μM γ-oryzanol was analyzed using two COBAS FARA II analyzers for 55 days.

Table 3. Relative ORAC Values of Pure Compounds ($n \geq 4$)

compds	ORAC
Trolox	1.0
α-tocopherol	0.50 $\pm$ 0.02
α-tocopherol acetate	0.00
(+)-γ-tocopherol	0.74 $\pm$ 0.03
(+)-δ-tocopherol	1.36 $\pm$ 0.14
nutriene (tocotrienols)	0.91 $\pm$ 0.04
2,2,5,7,8-pentamethyl-6-chromanol	1.02 $\pm$ 0.11
2-*tert*-butyl-4-methylphenol	0.41 $\pm$ 0.01
BHT	0.16 $\pm$ 0.01
γ-oryzanol	3.00 $\pm$ 0.26

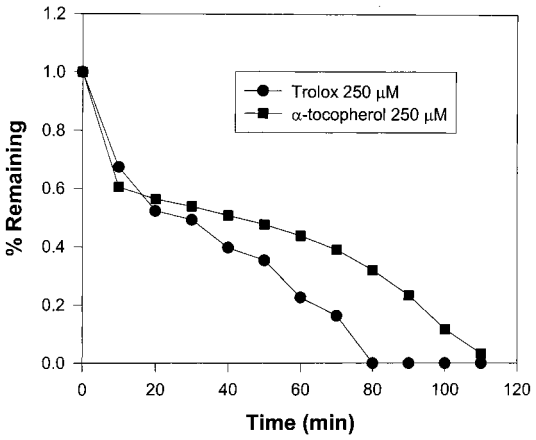

Figure 4. Kinetic curves for Trolox and α-tocopherol in the presence of AAPH. The reaction mixture contains 0.25 mM Trolox, 0.25 mM α-tocopherol, and 12.8 mM AAPH in RMCD (7% w/v) solution. The mixture was incubated at 37 °C in a HPLC autosampler, and 5 μL mixture was analyzed by HPLC at every 10 min interval until the reaction was completed.

Competitive Reaction between Trolox and α-Tocopherol with AAPH. The competitive reaction between Trolox and α-tocopherol with AAPH was examined, and the kinetic curves for Trolox and α-tocopherol were illustrated in **Figure 4**. It is evident that the reaction rate of Trolox with AAPH is much faster than that of α-tocopherol with AAPH.

Mechanistic Studies. The mechanisms for peroxidation of Trolox and α-tocopherol can be elucidated based on their

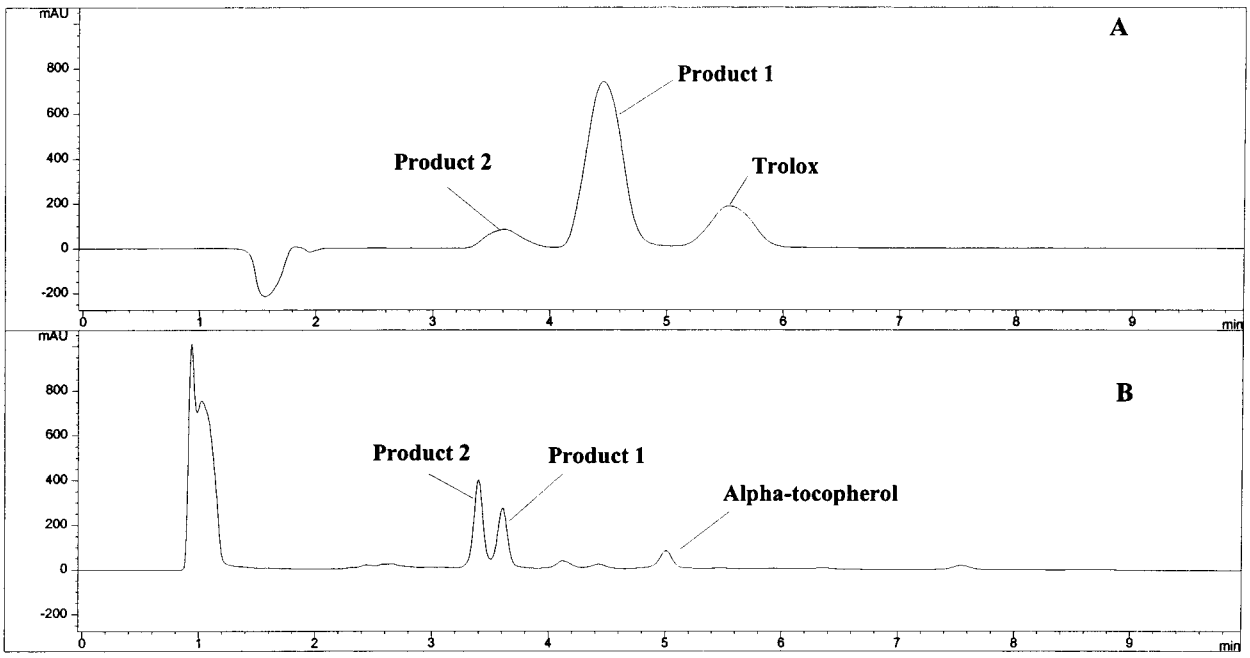

Figure 5. (**a**) HPLC chromatogram for the Trolox oxidation profile induced by AAPH. The mixture contained 0.4 mM Trolox and 512 mM AAPH in 75 mM phosphate buffer (pH 7.4) and was incubated at 37 °C for 30 min. The reaction products were separated by a Zorbax C18 column (2.1 mm × 150 mm, 3 μm) at 37 °C with a mobile phase of 70% methanol at a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm. The oxidized products were characterized by using a Finnigan LCQ ion trap mass spectrometer equipped with an API chamber and an ESI source. (**b**) HPLC chromatogram for the α-tocopherol oxidation profile induced by AAPH. A mixture of 0.5 mL of 20 mM α-tocopherol (prepared in 7% RMCD) and 0.5 mL of 640 mM AAPH was incubated at 37 °C for 30 min and was extracted with 10 mL of methylene chloride. The methylene chloride phase was separated and washed with 5 mL of DI water 3 times. The oxidized products of α-tocopherol in the methylene chloride phase were then analyzed by LC/MS. With the exception of 100% acetonitrile as the mobile phase, the LC/MS conditions are the same as **a**.

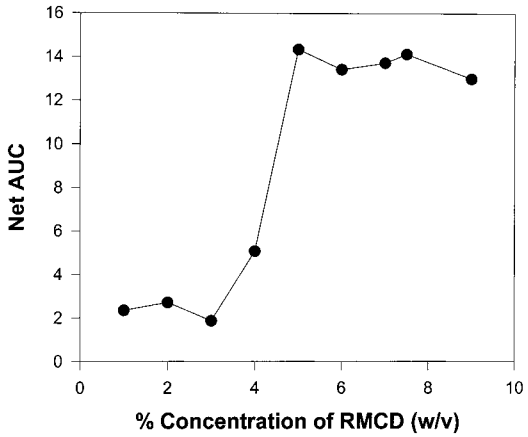

Figure 6. Effect of RMCD concentration (w/v) on the net AUC of 50 μM α-tocopherol.

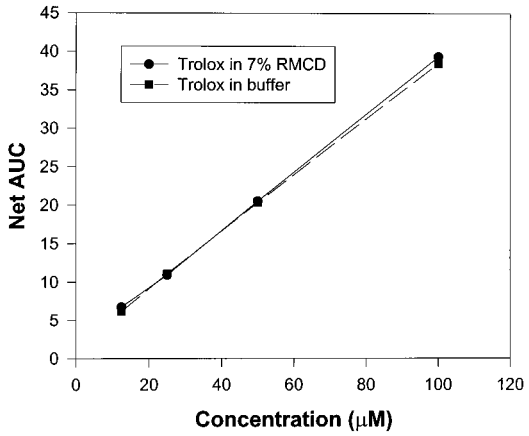

Figure 7. Regression of net AUC of Trolox on different concentrations of Trolox in the presence/absence of RMCD (7% w/v). Each data point represents an average of six separate measurements.

oxidized products obtained by LC/MS. As shown in **Figure 5a**,**b**, both Trolox and α-tocopherol were oxidized into two major products with 16 and 32 mass units increase. **Figure 8** illustrates the proposed structures for oxidized products and the oxidation mechanism.

DISCUSSIONS

The ORAC assay was originally developed by Cao and coworkers (*6*) and was significantly improved by Ou et al. (*5*). However, the improved ORAC assay does not address the issue of lipophilic antioxidants because the assay is performed in aqueous solution. The application of CD for solubilization of lipophilic compounds has been extensively studied, particularly with the carotene/fatty acids−CD interaction (*3*, *4*, *7*). CDs are a group of naturally occurring cage molecules, which are built up from α-D-glucose units. Depending on the number of glucose moieties in the ring (6, 7, or 8), they are named α-, β-, and γ-cyclodextrin. CDs are doughnut-shaped and can bind a wide variety of organic "guest" compounds inside their apolar cavities in aqueous solution. The main driving force for this binding is hydrophobic interactions. Recently, Szente et al. studied the solubilization of carotene and fatty acids in aqueous solution, and their results indicated that the solubilizing power of CD derivatives are methylated-β-cyclodextrin ≫ hydroxypropylated-β-cyclodextrin = branched-β-cyclodextrin (*4*). The use of 10−40% methylated-β-cyclodextrin results in the 1000-fold enhancements of the aqueous solubility of lipophilic compounds. In light of Szente's work, we employed RMCD in the ORAC

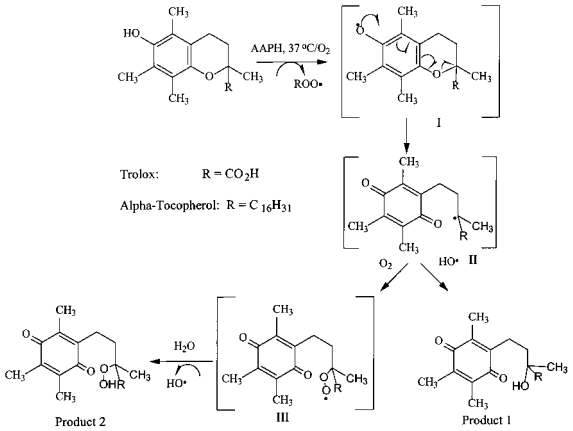

Figure 8. Proposed oxidation mechanism for Trolox and α-tocopherol in the presence of AAPH.

assay for lipophilic antioxidants. As demonstrated in the following sections, RMCD was determined to be an ideal solubility enhancer for the studied antioxidants, and for the first time, the antioxidant values for tocopherols, tocotrienols, and other common lipophilic antioxidants were obtained using the ORAC assay.

Effect of RMCD Concentration on the Solubility of Vitamin E in Aqueous Solution. Figure 6 shows the net AUC of 50 μM α-tocopherol at different RMCD concentrations (w/v) in 75 mM phosphate buffer solution (pH 7.4). The net AUC of α-tocopherol increases with the increase of RMCD concentration and reaches a plateau after 4% RMCD. The plateau indicates that α-tocopherol is completely soluble in 75 mM phosphate buffer solution. This conclusion was further confirmed by the HPLC studies (data not shown). Taking into account the concentration variations, 7% RMCD in 50% acetone solution was chosen for sample preparation.

Effect of RMCD on the ORAC Value of Trolox. Lipophilic antioxidants, such as the vitamin E family and oryzanol, consist of a long aliphatic tail (≥16 carbons) and a hydrophilic phenol group on the head. The tail can fit into a CD cavity allowing the hydrophilic phenol group to remain in aqueous solution. Therefore, the reactivity of the headgroup is not inhibited by CD complexation. RMCD itself consists of hydroxyl and methoxyl functional groups and is doughnut-shaped with an open moiety; therefore, RMCD does not possess any antioxidant activity nor does it prevent the complexed antioxidant molecule from functioning as antioxidant. To confirm this, the ORAC values of Trolox were examined in the presence (or absence) of 7% RMCD. **Figure 7** shows the linear curves of the net AUC against the Trolox concentrations in phosphate buffer and 7% RMCD solution, respectively. It becomes obvious that the two curves are almost superimposed, suggesting that RMCD is inert in the ORAC assay.

Difference in ORAC Value between α-Tocopherol and Trolox. Because of the structural similarity, Trolox is expected to possess a similar ORAC value to that of α-tocopherol. However, our results reveal that the ORAC value of α-tocopherol is about 50% less than that of Trolox. The difference in ORAC value prompted us to investigate the reaction kinetics and mechanisms of Trolox/α-tocopherol with AAPH. **Figure 4** shows the competitive reaction kinetic curves between Trolox and α-tocopherol in the presence of AAPH. As shown, the rate of Trolox with AAPH tends to be much faster than that of α-tocopherol with AAPH. This observation is in agreement with their ORAC values. At this point, we suggest that the inductive effect on the phenol group derived from the long ($C_{16}H_{31}$) aliphatic tail of α-tocopherol, an electron-donating group, may be the cause of the lower ORAC value. A remotely related analogue is that the higher alkanoic acid has lower acidity than that of acetic acid, because the alkyl groups manifest a small but significant electron donation to the carboxyl carbon (*8*). We further measured the ORAC value for α-tocopherol acetate, a popular ingredient in vitamin E supplements. Our result indicated that α-tocopherol acetate does not possess any antioxidant capacity under current experimental conditions. This result provides additional evidence to support our conclusion that the phenol group is an essential group for radical trapping antioxidant activity.

Mechanistic Studies on the Vitamin E Antioxidants. We examined the oxidized products of α-tocopherol and Trolox with AAPH by LC/MS and identified two oxidized products for each compound. **Figure 5a**,**b** shows the oxidation profiles for Trolox and α-tocopherol induced by AAPH obtained by HPLC, respectively. **Figure 8** illustrates the proposed reaction mechanisms for Trolox and α-tocopherol in the presence of AAPH. As shown, the reaction was initiated by the formation of phenoxyl radical **I** due to a hydrogen atom being abstracted from the phenol group by the peroxyl radical. Phenoxyl radical **I** can further undergo intramolecular arrangement to form intermediate **II**, a tertiary carbyl radical. In the presence of O_2, intermediate **II** is peroxidized to yield intermediate **III**, a peroxyl radical that may abstract a hydrogen from the water molecule to yield product **2** and a highly reactive hydroxyl radical (HO•). Meanwhile, the intermediate **II** can couple with the generated HO• to produce product **1**.

Structure−Activity Relationship. Table 3 summarizes the ORAC values for some common lipophilic antioxidants. The ORAC values for the three tocopherols are significantly different. The ORAC values of α-, γ-, and δ-tocopherol are 0.50, 0.74, and 1.36, respectively. The difference in ORAC value can be attributed to the steric effect of the ortho methyl groups. The number of methyl groups ortho to the phenol group of tocopherol decreases from two (α), one (γ), and zero (δ). A similar trend was observed for 2-*tert*-butyl-4-methylphenol (0.41 ± 0.01) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.16 ± 0.01). Apparently, the steric factor has a significant impact on the antioxidant activity; the less steric hindrance results in higher ORAC values. Our findings, however, are in contrast with the theoretical calculations by Wright and co-workers who calculated the O−H bond dissociation energies (BDE) for α- (75.78 kcal/mol), γ- (79.57 kcal/mol), and δ-tocopherol (81.43 kcal/mol) (*9*). On the basis of the BDE, they suggested that α-tocopherol has the highest antioxidant activity among the three. In fact, the ORAC value is a kinetic parameter while BDE relates to thermodynamics of a reaction. Therefore, BDE and ORAC values may not necessarily have the same trend. From our results, steric hindrance plays a significant negative role in antioxidant activity. γ-Oryzanol was found to possess a much higher ORAC value than that of any vitamin E antioxidant. In support of our result, a recent study by Godber and co-worker found that γ-oryzanol has a much higher antioxidant activity than tocopherols in protecting a cholesterol oxidation system accelerated by AAPH (*10*). The significantly greater ORAC value of γ-oryzanol can be attributed to the electronic contribution of an ortho methoxyl group and the larger π-conjugation system, involving 11 atoms, as compared to vitamin E with only 8 such atoms. A recent report of Mulder and co-workers on coenzyme $Q_{10}H_2$ reveals that the hydrogen atom abstraction is surprisingly easy from intramolecularly hydrogen-bonded meth-

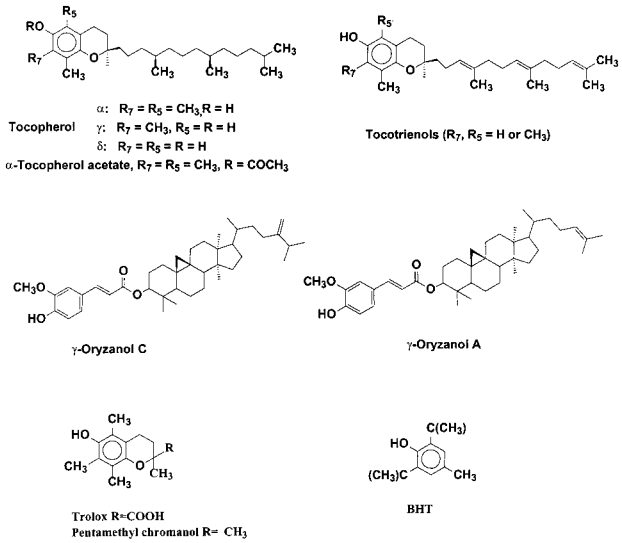

Figure 9. Structures of studied lipophilic antioxidants.

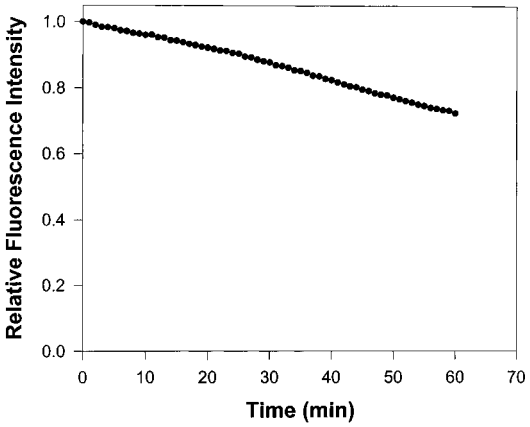

Figure 10. Fluorescence decay curve of BODIPY 581/591 C11 under microplate reader conditions (Bio-Tek FL 600). Solvent = butyronitrile/octane (1:9); temperature = 41 °C; concentration of BODIPY = 7.9×10^{-7} M.

oxyphenols (*11*). γ-Oryzanol possesses such a structural feature, and thus, it exhibits strong antioxidant activity. **Figure 9** lists the molecular structures of lipophilic antioxidants studied in this paper.

Comparison of the $ORAC_{FL-LIPO}$ Assay with Other Related Methods. So far, only a few methods have been developed to measure lipophilic antioxidant activity. For example, Pulido et al. used a modified ferric-reducing/antioxidant power assay (FRAP) to measure the antioxidant activity of carotenoids (*12*); Acosta et al. modified the Trolox equivalent antioxidant capacity assay (TEAC) to measure the antioxidant activity of lipophilic vitamins (*13*). The drawbacks of using FRAP and TEAC for antioxidant activity measurement have been extensively discussed in our previous paper (*5*). Recently, Naguib developed a fluorometric method for lipophilic antioxidant activity, in which BODIPY 581/591 or BODIPY 665/676 was utilized as the fluorescent probe (*14*). Aldini et al. further modified Naguib's method to measure the lipid compartment of plasma (*15*). However, as shown in **Figure 10**, the BODIBY dye was found to be photobleached after it was exposed to excitation light. The photobleaching is likely caused by the cis/trans isomerization of the olefinic double bond in the presence of light (**Figure 11**). Therefore, the BODIBY-based method is not suitable for quantitation of antioxidant capacity.

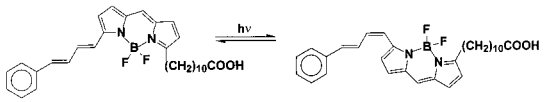

Figure 11. Possible photobleaching mechanism for BODIPY 581/591 after it was exposed to excitation light.

CONCLUSION

Using RMCD as the solubility enhancer, we successfully developed a validated ORAC assay for lipophilic antioxidants ($ORAC_{FL-LIPO}$). The $ORAC_{FL-LIPO}$ method was determined to be robust, reliable, and sensitive. We have also demonstrated that the phenol group is the key functional group for antioxidant activity, and the steric hindrance around the phenol group decreases ORAC values of tocopherols and other phenolic lipophilic antioxidants. The stereoelectronic effect derived from the long ($C_{16}H_{31}$) aliphatic tail of α-tocopherol also causes the lower ORAC value than that of Trolox. The oxidized Trolox and α-tocopherol have been identified using LC/MS, and the reaction mechanism was determined to follow a classic hydrogen atom transfer (HAT) mechanism. It is necessary to point out that the $ORAC_{FL-LIPO}$ assay does not measure the antioxidant activity of carotenoids and polyunsaturated fatty acids, since chemically carotenoids and fatty acids are not the chain-breaking antioxidants. Instead, they may act as the singlet oxygen scavengers and therefore follow a different reaction mechanism.

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LITERATURE CITED

(1) Halliwell, B. Antioxidants in human health and diseases. *Annu. Rev. Nutr.* **1996**, *16*, 33−50.
(2) Burton, G. W.; Ingold, K. U. Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.* **1986**, *19*, 194−201.
(3) Pfitzner, I.; Francz, P. I.; Biesalski, H. K. Carotenoid: methyl-beta-cyclodextrin formulations: an improved method for supplementation of cultured cells. *BBA Gen. Subjects* **2000**, *1474*, 163−168.
(4) Szente, L.; Mikuni, K.; Hashimoto, H.; Szejtli, J. Stabilization and solubilization of lipophilic natural colorants with cyclodextrins. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1998**, *32*, 81−89.
(5) Ou, B.; Hampsh-Woodill, M.; Prior, R. Development and validation of an improved Oxygen Radical Absorption Capacity assay using fluorescein as a fluorescent Probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.
(6) Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants *Free Radical Biol. Med.* **1993**, *14*, 303−311.
(7) Szente, L.; Szejtli, J.; Szeman, J.; Kato, L. Fatty-acid cyclodextrin complexes − properties and applications *J. Inclusion Phenom. Mol. Recognit. Chem.* **1993**, *16*, 339−354.
(8) Streitwieser, A.; Heathcock, C. H. Carboxylic Acids. In *Introduction to Organic Chemistry*; Macmillan Publishing Co., Inc.: New York, 1976; p 429.
(9) Wright, J. S.; Carpenter, D. J.; McKay, D. J.; Ingold, K. U. Theoretical calculation of substituent effects on the O−H bond strength of phenolic antioxidants related to vitamin E. *J. Am. Chem. Soc.* **1997**, *119*, 4245−4252.
(10) Xu, Z.; Hua, N.; Godber, J. S. Antioxidant activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2,2′-azobis(2-methylpropi-

onamidine) dihydrochloride. *J Agric. Food Chem.* **2001**, *49*, 2077−2081.
(11) de Heer, M. I.; Mulder, P.; Korth, H.-G.; Ingold, K. U.; Lusztyk, J. Hydrogen atom abstraction kinetics from intramolecularly hydrogen bonded ubiquinol-10 and other (poly)methoxy phenols *J. Am. Chem. Soc*. **2000**, *122*, 2355−2360.
(12) Pulido, R.; Bravo, L.; Saura-Calixo, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396−3402.
(13) Arnao, M. B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem.* **2001**, *73*, 239−244.
(14) Naguib, Y. M. A. Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* **2000**, *48*, 1150−1154.
(15) Aldini, G.; Yeum, K.−J.; Russell, R. M.; Krinsky, N. I. A method to measure the oxidizability of both the aqueous and lipid compartments of plasma *Free Radical Biol. Med*. **2001**, *31*, 1043−1050.

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