Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe

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An improved method of oxygen radical absorbance capacity (ORAC) assay has been developed and validated using fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) as the fluorescent probe. Our results demonstrate that fluorescein (FL) is superior to B-phycoerythrin. The oxidized FL products induced by peroxyl radical were identified by LC/MS, and the reaction mechanism was determined to follow a classic hydrogen atom transfer mechanism. In addition, methodological and mechanistic comparison of ORAC$_{FL}$ with other widely used methods was discussed. It is concluded that, unlike other popular methods, the improved ORAC$_{FL}$ assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radical.

Keywords: *Fluorescein; ORAC; TEAC; FRAP; chain-breaking antioxidant; free radical; hydrogen atom transfer; single electron transfer*

INTRODUCTION

There is increasing interest in the use and measurement of antioxidant capacity in the food, pharmaceutical, and cosmetic industries. This interest is derived from the overwhelming evidence of importance of reactive oxygen/nitrogen species (ROS/RON) in aging and pathogenesis (*1−4*). Recently, Cao et al. developed a method called oxygen radical absorbance capacity (ORAC), which measures antioxidant scavenging activity against peroxyl radical induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C (*5, 6*). In this assay, B-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum*, was the chosen fluorescent probe. The loss of fluorescence of B-PE is an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample as compared to that of the blank in which no antioxidant is present. The ORAC assay provides a very unique and complete assessment in which the inhibition time and inhibition degree are measured as the reaction goes to completion. However, the major limitation of the ORAC$_{PE}$ assay is the use of B-PE as the probe. First, B-PE produces inconsistency from lot to lot, which results in variable reactivity to peroxyl radical (*7*). Second, B-PE is not photostable, and after exposure to excitation light for certain time, it can be photobleached. This phenomenon was observed in a 96-well plate reader where the fluorescence signal was found to decline dramatically without the addition of AAPH (unpublished results). Third, we have observed that B-PE interacts with

polyphenols due to the nonspecific protein binding. These disadvantages prompted us to utilize and validate a stable fluorescent probe to replace B-PE. In this paper, we report the use of fluorescein (FL) (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) as the fluorescent probe. The FL oxidized products induced by peroxyl radical have been identified by LC/MS, and the reaction mechanism was determined to proceed as a classic hydrogen atom transfer (HAT) mechanism. Unlike other popular antioxidant activity methods, the improved ORAC$_{FL}$ assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radical.

MATERIALS AND METHODS

Chemicals and Apparatus. All flavonoid compounds and B-PE were purchased from Sigma (St. Louis, MO). Trolox, ascorbic acid, and disodium fluorescein were obtained from Aldrich (Milwaukee, WI). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Various analyzed samples were obtained "in house". All ORAC analyses were performed on a COBAS FARA II centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ; excitation wavelength = 493 nm and emission filter = 515 nm).

Sample Preparation. Ascorbic acid and flavonoids were directly dissolved in acetone/water mixture (50:50, v/v) and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Black tea leaves, blueberry extracts, bilberry extracts, elderberry extracts, red wine extracts, grape skin extracts and grape seed extracts were initially ground in a mechanical mill to produce a fine power. Then 0.5 g of the powders were accurately weighed, and 20 mL of acetone/water (50:50, v/v) extraction solvent was added. The mixture was shaken at 400 rpm at room temperature on an orbital shaker for 1 h. The extracts were centrifuged at 14000 rpm for 15 min, and the supernatant was ready for analysis after appropriate dilution with buffer solution. For liquid samples, a 20-mL aliquot of sample was centrifuged for 15 min, and the supernatant was

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ready for analysis after appropriate dilution. Blood plasma or serum was diluted 100–200-fold with pH 7.4 phosphate buffer before analysis. To measure the ORAC in nonprotein fraction, protein was removed using 0.5 N perchloric acid (1:1; v:v; plasma:acid), the samples were then centrifuged at 14000*g* for 10 min at 4 °C, and the supernatants were removed as the serum nonprotein fractions and appropriately diluted with pH 7.4 phosphate buffer before analysis.

Experimental Conditions. *ORAC Assay.* The COBAS FARA II was programmed to use a two-reagent system. The reaction mode pipetted and transferred the sample (20 μL), phosphate buffer (5 μL, 75 mM, pH 7.4), and main reagent (365 μL FL, 48 nM) into the main reagent wells of their respective cuvette rotor positions. With spinning of the rotor, the reagents were mixed and incubated for 30 s before recording the initial fluorescence (f_0). Fluorescence readings were taken at 0.5 s and then every minute thereafter (f_1, f_2, f_3, ...) for a duration of 30 min. To determine the maximum voltage for the photomultiplier tube, the AAPH reagent was replaced with buffer, and the analysis was run for 10 min. FL and AAPH were prepared with 75 mM phosphate buffer at pH 7.4. FL working solution was preincubated at 37 °C for 15 min before loading into the COBAS reagent rack. The 75 mM phosphate buffer was used as a blank, and 12.5, 25, 50, and 100 μM Trolox were used as standards. A sample of 40 μM Trolox was used as quality control (QC). Samples and Trolox calibration solutions were always analyzed in duplicate in a "forward-then-reverse" order as follows: blank, 12.5 μM Trolox, 25 μM Trolox, 50 μM Trolox, 100 μM Trolox, QC, sample 1 ... sample 1, QC, 100 μM Trolox, 50 μM Trolox, 25 μM Trolox, 12.5 μM Trolox, blank. This arrangement can correct possible errors due to the signal drifting associated with the different positions of the same sample. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as Trolox equivalents as micromole per liter or per gram. The area under curve (AUC) was calculated as

$$\text{AUC} = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + ... + f_{34}/f_0 + f_{35}/f_0 \tag{1}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*.

The data were analyzed by applying eq 1 in a Microsoft Excel (Microsoft, Redmond, WA) spreadsheet to calculate the AUC. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value (Trolox equivalents) was calculated as

relative ORAC value =
$$[(\text{AUC}_{\text{Sample}} - \text{AUC}_{\text{Blank}})/(\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{Blank}})] \times$$
(molarity of Trolox/molarity of sample) (2)

Characterization of FL Oxidized Products. FL (4.8 $\times$ 10^{-7} M) was incubated at 37 °C for 20 min with AAPH (1.28 $\times$ 10^{-2} M) at 75 mM potassium phosphate buffer (pH 7.4), and the reaction mixture was analyzed by LC/MS. Chromatographic analyses were performed on an HP 1100 series (Hewlett-Packard, Palo Alto, CA) HPLC equipped with an autosampler/injector, binary HPLC pump, column heater, diode array detector, fluorescence detector, and HP ChemStation for data collection and manipulation. Reverse-phase separation was performed on a Zorbax (Hewlett-Packard, Palo Alto, CA) C18 column (2.1 $\times$ 150 mm, 3 μm) at 37 °C. UV detection was recorded at 278 nm, and for fluorescence detection, the excitation wavelength was 491 nm and the emission wavelength was 515 nm. The binary mobile phase consisted of (A) water–acetonitrile–acetic acid (89:9:2) and (B) water–acetonitrile (20:80). The separation was performed using a linear gradient from 0% to 30% B in 30 min. The structural information was obtained using a Finnigan LCQ ion trap mass spectrometer (Thermoquest, San Jose, CA) equipped with an API chamber and an ESI source. The ionization mode was negative mode; Aux gas and Sheath gas were set to 90 and 23
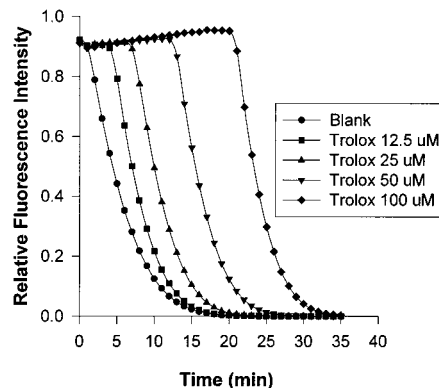
Figure 1. Trolox concentration effect on FL fluorescence decay curve.

Table 1. Summary of Trolox Calibration Curve[a]

run	R^2	slope (*b*)	intercept (*a*)
1	0.9994	2.5368	−2.174
2	0.9993	2.7390	−4.690
3	0.9981	2.6947	−5.109
4	0.9973	2.5291	−3.846
5	0.9928	2.2331	1.361
6	0.9978	2.8868	−3.788
7	0.9981	2.6288	−3.012
8	0.9987	2.5297	−2.589
average	0.9977	2.5846	−2.861
accepted criteria	≥0.9900	na	na

[a] $[Y\,(\mu M) = a + bX$ (net area)]. na, not applicable.

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 by using a secondary HPLC pump before the API chamber. Fluorescein disodium was used as a standard for calibrating the system.

RESULTS

Specificity. The purpose was to demonstrate whether the improved method is specific for antioxidants. This objective can be confirmed by obtaining positive results from a sample containing antioxidants and negative results from a same sample whose antioxidants have been destroyed. The following were chosen for specificity determination: 100 μM gallic acid, 3% blueberry juice, and whole serum. After preincubation with 1.28 $\times$ 10^{-2} M AAPH and Fenton reagent (H$_2$O$_2$ + Fe^{2+}) at 37 °C for 2 h, all three samples were found to have no scavenging activities while showing negative ORAC values. Therefore, the ORAC$_{\text{FL}}$ assay is specific for antioxidants.

Linearity. The linear relationship between net area and antioxidant concentration was evaluated using Trolox, black tea leaves, blueberry extracts, and grape skin extracts at different concentrations. Figure 1 illustrates the FL fluorescence decay curves in the presence of Trolox and AAPH. Table 1 summarizes the correlation coefficient, slope, and intercept of the Trolox standard curve. Table 2 shows the net areas corresponding to the different concentrations of black tea leaves, blueberry extracts, and grape skin extracts and the calculated ORAC values. All analyzed samples in the various forms demonstrate a good linear relationship between net area and concentration. Trolox was used as a calibration standard. The limit of quantitation and the limit of detection are 12.5 and 5 μM Trolox equivalents, respectively. An acceptable correlation of coefficient (r^2) was ≥0.99.

Table 2. Net Area Corresponding to Different Concentrations of Extracts from Tea, Blueberry, and Grape Skins

natural products	concn(mg/L)	net area	$ORAC_{FL}{}^a$
black tea leaves	8	5.92	1586
	16	10.81	1566
	32	21.51	1629
blueberry extracts	5	5.73	2441
	10	11.32	2635
	20	22.98	2792
grape skin extracts	1.2	8.34	15675
	2.4	15.63	15521
	4.8	29.89	14714

[a] ORAC values are expressed as Trolox equivalents per gram on dry basis. The relative standard deviation (RSD) for average value of each sample was less than 15%.

Table 3. Precision and Accuracy of Quality Control (QC) Samples[a]

	QC1	QC2	QC3
nominal Trolox concn (μM)	20.00	40.00	75.00
run 1			
intra-mean (μM)	18.21	41.81	74.79
SD	1.26	3.51	5.49
% RSD	6.90	8.40	7.34
% REC	91.05	100.05	99.72
n	4	4	4
run 2			
intra-mean (μM)	21.33	42.79	76.18
SD	1.58	3.92	6.12
% RSD	7.41	9.16	8.03
% REC	106.65	107.03	101.57
n	4	4	4
run 3			
intra-mean (μM)	21.45	41.35	76.21
SD	1.37	3.21	5.19
% RSD	6.39	7.76	6.81
% REC	107.25	103.35	101.61
n	4	4	4
pooled runs			
inter-mean (μM)	20.33	41.98	75.72
SD	1.59	0.74	0.81
% RSD	7.82	1.76	1.16
% REC	101.65	104.95	100.96
n	12	12	12

[a] % RSD, relative standard deviation. % REC, relative recovery.

Precision and Accuracy. Table 3 summarizes the precision and accuracy of the $ORAC_{FL}$ assay. The precision, which is expressed as relative standard deviation (% RSD) for all quality control samples, was within ±15%. The accuracy of the method varies from 91 to 107% within individual batches and from 101 to 105% between all the batches.

Ruggedness. The reproducibility of $ORAC_{FL}$ was evaluated by a ruggedness study, which was performed by analyzing 20 μM gallic acid using two COBAS FARA II analyzers day to day. Results are shown in Figure 2.

Comparison of FL with B-PE. We performed analyses for grape seed extracts (GSE) in the absence of AAPH by using both B-PE and FL. Figure 3a shows a strong protein binding between B-PE and GSE that occurred instantly. The degree of binding appears to be inversely proportional to GSE concentration. No interaction was observed from FL and GSE matrix within 35 min (Figure 3b). Figures 4 and 5 represent the FL and B-PE fluorescence decay curves, respectively, and as shown, B-PE fluorescence declined more rapidly than did FL fluorescence. Various samples were analyzed, and the results are summarized in Tables 4−6. The FL yields a consistently higher ORAC value as compared to B-PE (∼1.6−3.5-fold).
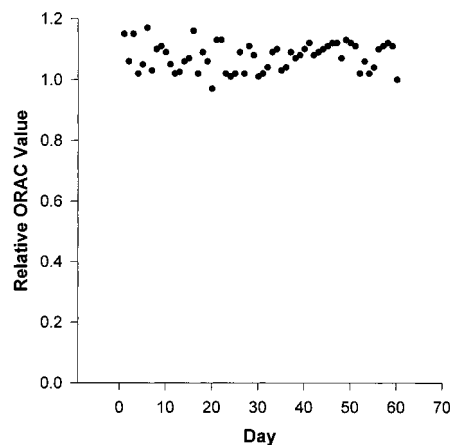

Figure 2. Ruggedness of ORAC method determined by 20 μM gallic acid. The relative ORAC values were obtained from two COBAS FARA II analyzers over several different days.
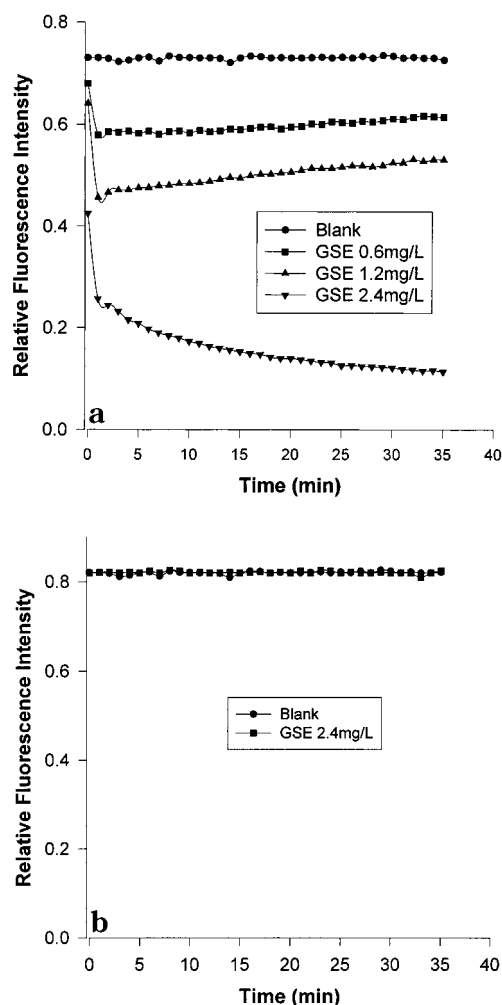

Figure 3. (a) Relative fluorescence versus time (minutes) of reaction: blank and grape seed extracts (GSE) at various concentrations using B-PE as the fluorescent probe. (b) Blank and GSE at 2.4 mg/L using FL as the fluorescent probe.

Mechanistic Studies. The mechanisms for peroxidation of FL can be elucidated based on FL oxidized products. As shown in Figure 6, FL was oxidized into three minor fluorescent products (FL1, FL2, FL3) and one major nonfluorescent product (FL4) with a maximum absorption at 278 nm. Figure 7 illustrates the proposed structures of FL oxidized products and the

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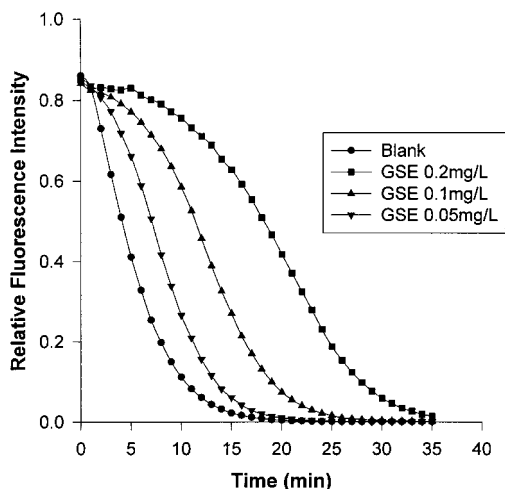
Figure 4. FL fluorescence decay curve induced by AAPH in the presence of grape seed extract (GSE) at different concentrations.
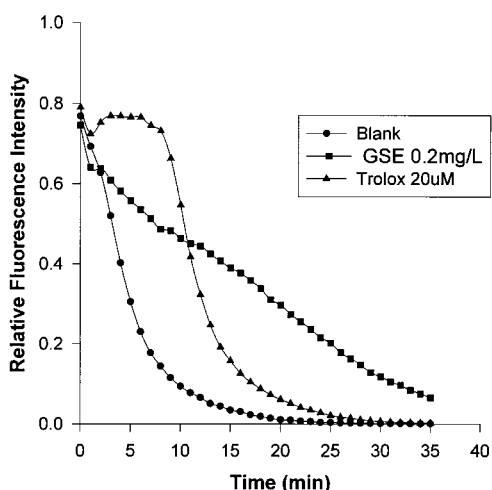
Figure 5. B-PE fluorescence decay curve induced by AAPH in the presence of 4 mg/mL grape seed extract (GSE).

Table 4. Relative ORAC Values of Pure Chemicals with Antioxidant Activity[a]

compounds	$ORAC_{FL}$	$ORAC_{PE}$	ratio
caffeic acid	4.37 ± 0.24	1.40 ± 0.09	3.12
chlorogenic acid	3.14 ± 0.19	1.90 ± 0.12	1.65
quercetrin	6.47 ± 0.29	2.70 ± 0.18	2.39
genistein	5.93 ± 0.45	2.3 ± 0.16	2.58
glutathione	0.62 ± 0.02	0.32 ± 0.01	1.94
rutin	6.01 ± 0.25	1.95 ± 0.21	3.08
quercetin	7.28 ± 0.22	2.07 ± 0.05	3.52
catechin	6.76 ± 0.22	2.57 ± 0.18	2.63
vitamin C	0.95 ± 0.02	0.43 ± 0.03	2.21

[a] ORAC values are expressed as relative Trolox equivalent calculated based on eq 2 ($n > 3$).

oxidation scheme. The chromatographic and mass spectroscopic data for the oxidized products are summarized in Table 7.

DISCUSSION

The original $ORAC_{PE}$ was based largely on Glazer's work (*8*) in which B-PE was utilized as the fluorescent probe. The reason for choosing B-PE as the fluorescent probe is due to B-PE's distinct excitation and emission wavelengths, high fluorescence yield, sensitivity to ROS, and water solubility (*9*). Later, the developers of the

Table 5. $ORAC_{FL}$ and $ORAC_{PE}$ Values for Biological Fluids and Beverages[a]

sample	$ORAC_{FL}$	$ORAC_{PE}$	$ORAC_{FL}$/ $ORAC_{PE}$
urine	1542 ± 178	926 ± 133	1.67
whole serum	7780 ± 467	3383 ± 278	2.30
serum (protein free)	347 ± 5.63	186 ± 9.11	1.87
blueberry juice	23748 ± 1555	7511 ± 683	3.16
bilberry juice	34659 ± 2069	12507 ± 893	2.77
grape juice	31441 ± 1821	12124 ± 912	2.59
raspberry juice	54034 ± 2863	23056 ± 1800	2.34
black tea	17267 ± 441	8714 ± 213	1.89

[a] ORAC values are expressed as micromol of Trolox equivalent per liter ($n > 3$).

Table 6. $ORAC_{FL}$ and $ORAC_{PE}$ of Various Natural Product Extracts[a]

| sample	$ORAC_{FL}$	$ORAC_{PE}$	$ORAC_{FL}$/ $ORAC_{PE}$
bilberry	2646 ± 190	1283 ± 144	2.06
elderberry	2221 ± 164	1174 ± 182	1.89
red wine extract	6942 ± 669	2194 ± 105	3.16
grape seeds extract A	11889 ± 234	3516 ± 135	3.38
grape seeds extract B	11681 ± 923	2989 ± 368	1.89

[a] $ORAC_{FL}$ and $ORAC_{PE}$ values are expressed as micromol of Trolox equivalents per gram ($n > 3$).

**Table 7. Ion Trap Mass Data for FL and Its Major Oxidized Products[a]**

compound	RT (min)	λ_{max}	$[M - 1]^-$	MS^2	MS^3	MS^4
fluorescein	30.4	493	331.1	287.2	259.3	
					269.2	243.3
FL1	24.4	493	661.0	617.1	573.3	545.2
					589.0	
FL2	28.8	493	375.0	331.1	287.2	259.2
FL3	26.4	345	349.0	305.0	261.1	233.1
FL4	3.1	278	221.3		204.2	187.1

[a] See Figure 6 for the HPLC separation of these products and Figure 7 for the proposed structures of FL oxidized products. FL4 is an unidentified product.

$ORAC_{PE}$ reported that different PEs, such as B-PE and R-PE, were found to possess different fluorescence intensity and reactivity to peroxyl radical; these differences even existed in the same PE with different lots. Hence, they suggested that PE of a single lot number be used for a planned project (*7*). Since the commercial available B-PE is only approximately 30% pure, the inconsistency of PE from lot to lot is very likely attributed to the isolation process from *P. cruentum.* Initially, we adapted the $ORAC_{PE}$ method to measure antioxidant activity in our laboratory. In addition to the inherent variability, we found that B-PE interacted with polyphenols (Figure 3a), a major class of antioxidants from natural products. The interaction between B-PE and polyphenols is caused by nonspecific protein binding. Complexing of polyphenols with protein has been known and studied for decades (*10–12*). The most important mechanism seems to involve hydrophobic interactions and also hydrogen bonding (*13, 14*). The nonspecific protein binding causes falsely low ORAC values as demonstrated in Tables 4–6 where $ORAC_{PE}$ values are consistently lower than those of $ORAC_{FL}$. It is noted that the ratio between two values varies from 1.5 to 3.1, indicating that different compounds have different affinities to PE. Another disadvantage of B-PE is photoinstability. The $ORAC_{PE}$ method has been criticized for the lack of accessibility because of the rare availability of FARA COBAS II analyzer. We attempted

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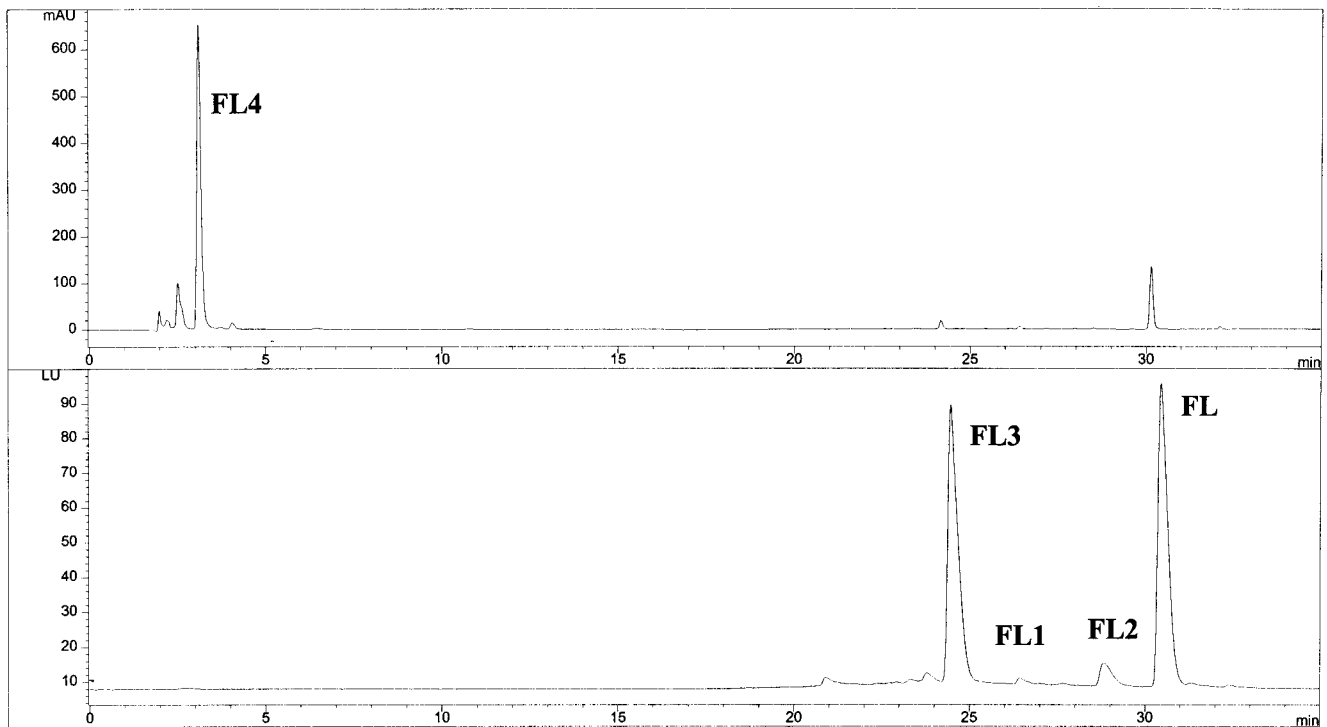
Figure 6. HPLC output monitored at 278 nm (top) and fluorescence at 493 nm excitation and 515 emission of fluorescein (bottom) and its oxidized products in the presence of AAPH.
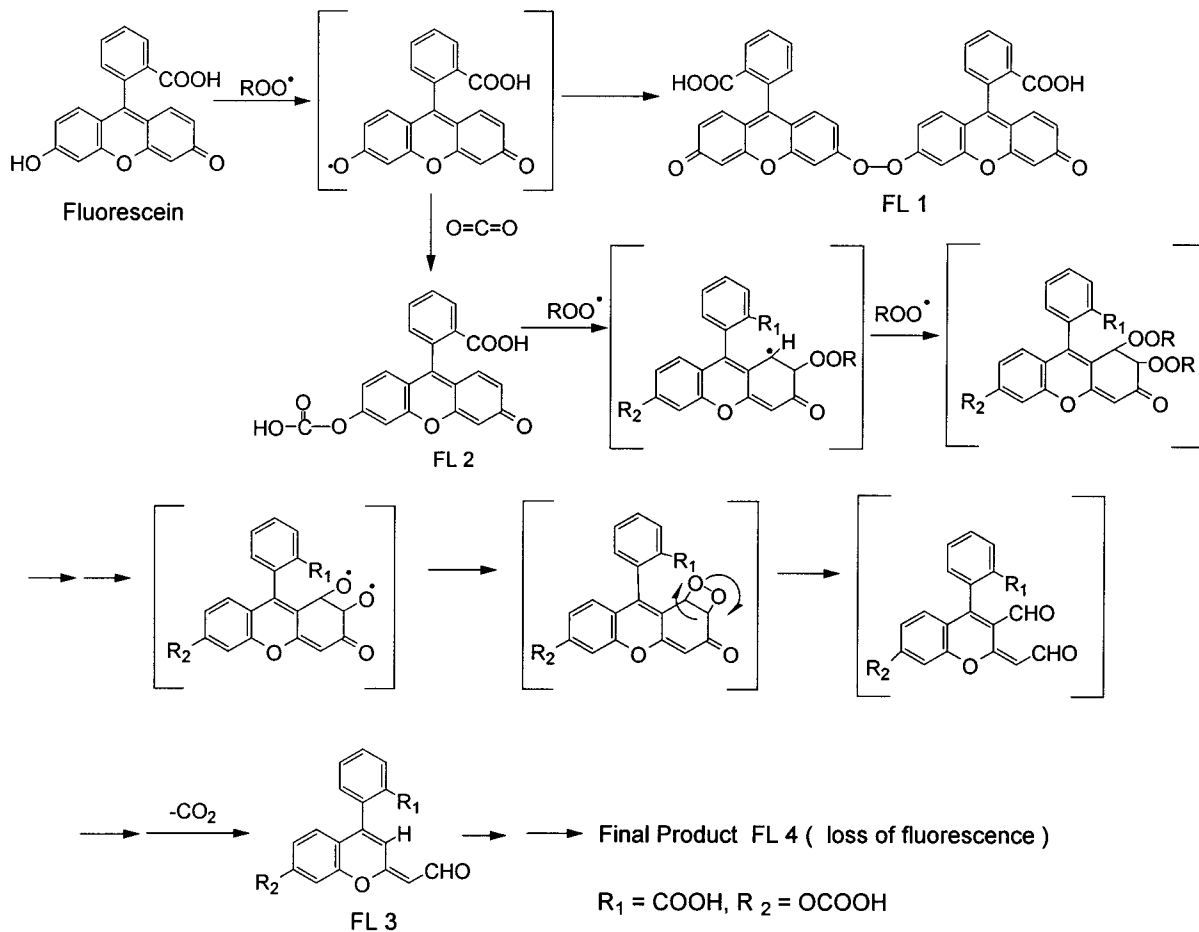
Figure 7. Proposed FL oxidation pathway in the presence of AAPH.

to adapt the ORAC$_{PE}$ method to a 96-well plate reader. Unfortunately, the fluorescence of B-PE was found to drop dramatically in a short period of time in the absence of AAPH. Moreover, being a protein isolated from *P. cruentum*, B-PE is costly. In general, 75% of the cost of ORAC$_{PE}$ is for B-PE. Clearly, in terms of the

criteria for method validation and cost-effectiveness, B-PE is less than ideal as a fluorescent probe. In contrast to PE, FL and its derivatives are the most used fluorescent probes for labeling and sensing biomolecules (*15*). Recently, Nagano et al. successfully utilized the FL based fluorescent probes to detect $^1O_2$ and NO$^•$ in vivo (*16, 17*). FL ($pK_a = 6.4$) is a synthetic compound with high quantum yield of fluorescence at pH > 7.0 ($\phi = 0.78$) and long wavelengths (492/515 nm, excitation/ emission). As compared to PE, FL is extremely inexpensive. Importantly, FL does not interact with other compounds as shown in Figure 3b. Moreover, FL is very stable in a 96-well plate reader without photobleaching; this advantage makes the ORAC$_{FL}$ method more accessible to other researchers (in preparation). It is necessary to point out that the fluorescence intensity of FL is pH sensitive. When pH drops below 7, its intensity decreases greatly. However, the ORAC$_{FL}$ assay is very sensitive; samples always need to be diluted greatly with 75 mM phosphate buffer at pH 7.4 before analysis. Considering the extreme situation in which pure acetic acid is analyzed, the pH only dropped slightly from 7.4 to 7.35 based on our experimental data and theoretical calculation. Therefore, the pH sensitivity of FL does not affect the ORAC$_{FL}$ assay.

Although the FDA still has no regulations concerning standardization in the nutraceutical and food supplement industry, there are good scientific and business reasons for validating assay procedures even in the absence of regulatory or compendia requirements. In the present study, the ORAC$_{FL}$ procedure has been validated through specificity, linearity, precision and accuracy, and ruggedness. The results from validation experiments clearly demonstrate that the ORAC$_{FL}$ assay is specific for antioxidants and is sensitive, precise, and robust within accepted criteria.

Elucidation of oxidation mechanisms involved in the ORAC$_{FL}$ assay is an important part of method validation. In general, the antioxidant reactions involve multiple steps including the initiation, propagation, branching, and termination of free radicals. This whole process is termed a chain reaction. The antioxidants therefore fall into two mechanistic groups: those which inhibit or retard the formation of free radicals from their unstable precursors (initiation) are called the "preventive" antioxidants, and those which interrupt the radical chain reaction (propagation and branching) are the "chain-breaking" antioxidants. The chain-breaking antioxidants are the most studied antioxidants, and the mechanism follows the HAT mechanism. The driving force for HAT is the formation of a delocalized stable radical that does not continue the chain reaction or continues it with only a low efficiency. Specifically, a chain-breaking antioxidant (AH) donates its labile hydrogen atom to ROO$^•$ much more rapidly than ROO$^•$ reacts with substrate. The radical A$^•$ is stable and is not able to continue the autoxidation of the chain. The HAT mechanism has been extensive studied and has been widely accepted as the predominate mechanism for autoxidation initiated by oxygen radicals (*18, 19*). B-PE is multi-subunit protein with the structure of $(\alpha\beta)_6\gamma$ (*8*); it is very difficult to determine the reaction mechanisms when B-PE is used as the probe. While FL is a small organic molecule with simple structural skeleton, the oxidation mechanisms can be elucidated based on its oxidized products characterized by LC/MS.

As illustrated in Figure 7, the first step of FL oxidation involves one hydrogen of the phenol group being abstracted by a peroxyl radical, forming a stable FL phenoxyl radical (FLO$^•$) that readily undergoes dimerization to form a dimeric FL1 with m/z 661. Alternatively, FLO$^•$ can attack trace amounts of CO_2 in the buffer solution to yield FL2 with m/z 375.0. Besides hydrogen abstraction, ROO$^•$ can also add the reactive conjugated C−C double bond to form a stable delocalized radical that further reacts with ROO$^•$ to form the endoperoxide intermediate, followed by decomposition to yield FL3 with m/z 349.0. FL4, a major oxidative product with m/z 221.3, appears to be derived from FL3 due to the further oxidation. FL4 does not possess fluorescent emission at 495/515 nm. It is clear that the oxidative mechanism for the ORAC$_{FL}$ assay follows the HAT mechanism. Therefore, the ORAC$_{FL}$ assay directly measures the antioxidant activity of chain-breaking antioxidants against peroxyl radical.

Besides the ORAC$_{FL}$ assay, a number of other methods for measuring antioxidant activity have been reported over recent years. Among them, FRAP (ferric reducing/antioxidant power) (*20*) and TEAC (Trolox equivalent antioxidant capacity) (*21*) have gained popularity because they are simple and speedy. For example, TEAC and FRAP fix the reaction time at 4 and 6 min, respectively. Although several review articles on the comparison of different methods have been published (*22, 23*), little attention has been paid to basic chemical principles involved in these methods. The lack of mechanistic understanding of antioxidant will bring confusion to this important field. Therefore, we compare ORAC$_{FL}$ with other methods from a mechanistic point of view. FRAP and TEAC are the single electron-transfer mechanism instead of HAT mechanism (*20, 24*). As a result, neither TEAC nor FRAP actually measures chain-breaking antioxidant activity or preventive antioxidant activity. Specifically, the FRAP assay depends on the reduction of a ferric tripyridyltriazine [Fe(III)−(TPTZ)$_2$] complex to the ferrous tripyridyltriazine [Fe(II)−(TPTZ)$_2$] by an antioxidant at the nonphysiological condition with low pH of 3.6. On the other hand, if the FRAP assay is used to assess in vivo antioxidant status, Fe(II) can interact with H_2O_2 to produce HO$^•$, the most harmful ROS. The original TEAC assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation. This has been criticized because the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical and the added hydrogen peroxide could oxidize antioxidants before the measurement. In the improved TEAC, ABTS$^{+•}$ is pregenerated by potassium persulfate (*25*). However, very similar to FRAP, any trace ions contained in biological fluids and natural products can inevitably reduce ABTS$^{+•}$ to ABTS, causing falsely higher results. The standard redox potential of Fe(II)/Fe(III) is 0.77 V, and that of ABTS/ABTS$^{+•}$ is 0.68 V (*26*). It must be pointed out that no oxygen radical is involved in either FRAP or TEAC (ABTS$^{+•}$ is not a ROS). Therefore, FRAP/TEAC results do not necessarily reflect antioxidant activities. This conclusion can be further confirmed by the fact that FRAP does not measure the thiol antioxidants, such as glutathione. In addition, FRAP and TEAC presumably rely on the hypothesis that the redox reactions proceed so fast that all reactions are complete within a short

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period of time; in fact, this is not always true. For example, Pulido and co-workers recently examined the FRAP assay of dietary polyphenols in water and methanol (*27*). The UV−vis absorption of Fe(II)(TPTZ)$_2$ was slowly increasing even after several hours of reaction time. The polyphenols with such behaviors include caffeic acid, tannic acid, ferulic acid, and quercetin. Clearly, FRAP and TEAC only partially measure the reducing capability based upon Fe(III) and ABTS$^{+•}$, respectively, which is not relevant to antioxidant activity mechanistically and physiologically.

In summary, an improved ORAC assay (ORAC$_{FL}$) using FL as the fluorescent probe has been developed and validated. The validation results demonstrate that the ORAC$_{FL}$ method is robust. As compared to B-PE, FL does not interact with antioxidant samples. Meanwhile, FL shows an excellent photostability so that the ORAC$_{FL}$ assay can be transferred to a 96-well plate reader. Furthermore, the use of FL substantially reduces the cost of experiment. Therefore we suggest that FL be considered as a standard to evaluate chain-breaking antioxidant activity. On the basis of the FL oxidized products, the mechanism of FL oxidation induced by peroxyl radical is determined to follow the HAT mechanism. In contrast, the popular FRAP and TEAC follow a single electron-transfer mechanism. Hence, only the ORAC$_{FL}$ assay directly estimates the chain-breaking antioxidant activity, while the FRAP and TEAC assays actually measure the specific oxidant-reducing power not equivalent to antioxidant activity. However the ORAC$_{FL}$ assay cannot be considered a "total antioxidant activity assay" since the assay is performed in aqueous solution. Therefore, the ORAC$_{FL}$ assay primarily measures hydrophilic antioxidant activity against peroxyl radical. In fact, it is impossible to measure total antioxidant activity using only a single assay. To elucidate a full profile of antioxidant activity against various ROS/RNS, such as O$_2^{-•}$, HO$^•$, and NO$^•$, the development of different methods specific for each ROS/RNS is needed.

ABBREVIATIONS USED

FL, fluorescein; B-PE, B-phycoerythrin; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ORAC, oxygen radical absorbance capacity; GSE, grape seed extracts; HAT, hydrogen atom transfer; TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power;

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