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Quenching Mechanisms and Kinetics of Trolox and Ascorbic Acid on the Riboflavin-Photosensitized Oxidation of Tryptophan and Tyrosine

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The effects of 0, 0.3, 0.6, and 0.9 mM Trolox and ascorbic acid on the singlet oxygen oxidation of tryptophan and tyrosine containing 25 ppm of riboflavin were determined by measuring tryptophan and tyrosine concentration by high-performance liquid chromatography analysis. The samples were stored in the a 1000 lx light storage box for 4 h at 30 °C. As the concentration of Trolox and ascorbic acid increased, the degradation of tryptophan and tyrosine decreased significantly at $p < 0.05$. Trolox reduced tryptophan and tyrosine degradation by quenching both singlet oxygen and excited triplet riboflavin, whereas ascorbic acid quenched singlet oxygen only. The total singlet oxygen quenchings of Trolox in the presence of tryptophan and tyrosine were 1.55×10^7 and 1.32×10^7 M^{-1} s^{-1}, respectively. The total singlet oxygen quenchings of ascorbic acid in the presence of tryptophan and tyrosine were 1.16×10^7 and 1.10×10^7 M^{-1} s^{-1}, respectively. Trolox was more effective than ascorbic acid in preventing the degradation of tryptophan and tyrosine.

KEYWORDS: Riboflavin; singlet oxygen; photosensitized oxidation

INTRODUCTION

Riboflavin is a constituent of flavoproteins naturally found in milk. Riboflavin is an active part of coenzymes such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) that catalyze many oxidation−reduction reactions (*1*). Riboflavin helps cells to metabolize carbohydrates, lipids, and proteins and is crucial for the production of biological energy in the electron transport system (*2*). Riboflavin as vitamin B_2 is vital for red blood cell formation, respiration, antibody production, and regulating human growth and reproduction (*3*). A deficiency in riboflavin may affect the metabolisms of glucose, fatty acids, and amino acids.

Riboflavin has complex photochemistry, which arises from its ability to donate or accept a pair of hydrogen atoms. Riboflavin has been known as a good photosensitizer for the formation of singlet oxygen under light, which can be excited by light and react with triplet oxygen (type II) or by substrates (type I) (*4*). Riboflavin has a triene structure and many conjugated double bonds that can easily react with singlet oxygen (*4*). In water matrix, type I reaction of riboflavin is favored due to the low concentration of oxygen and easy oxidation reduction property of riboflavin (*5, 6*). The generation of singlet oxygen from riboflavin was confirmed using electron spin resonance (ESR) and singlet oxygen trapping compounds such as 2,2,6,6-tetramethyl-4-piperidone (*4*). The photosensitizing effect of riboflavin on the oxidation of amino acids, lipids, and vitamins under light has been studied (*8*−*11*). The reaction rate between riboflavin and singlet oxygen was 10^{10} M^{-1} s^{-1} (*12*). The formation of singlet oxygen by riboflavin depends on the availability of oxygen, the concentration of riboflavin, and the presence of oxidizable reactants or quenchers (*13*).

Natural components of foods such as carotenoids and tocopherols have been proved to be effective singlet oxygen quenchers (*14*). Trolox is a water-soluble analogue of α-tocopherol. Trolox is similar in structure to tocopherol with a chroman ring but lacks a hydrophobic phytyl tail (*15*). The phytyl tail of α-tocopherol is substituted with a carboxylic group, which makes Trolox soluble in polar media. The antioxidant activity of Trolox was compared with those of several food grade antioxidants and was proved to be a better antioxidant than α-tocopherol under a wide range of conditions and test systems (*16*). The physical state of the lipid system affects the antioxidant properties of α-tocopherol and Trolox (*17*).

Ascorbic acid acts as a primary and secondary antioxidant. Ascorbic acid has a strong quenching ability for reactive oxygen species such as singlet oxygen and superoxide anion radical by converting their hydroperoxides into stable products (*18*). Yang (*19*) and Jung and others (*20*) reported that the loss of ascorbic acid increased as the added riboflavin content in milk increased under light storage. One hundred percent of ascorbic acid was destroyed after 12 min of light exposure when 6 ppm of riboflavin was added, whereas only 2% was destroyed in the sample to which no riboflavin was added (*20*). The loss of ascorbic acid was significantly reduced when sodium azide, which is a singlet oxygen quencher, was added to the sample.

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The photodegradation of ascorbic acid by riboflavin photosensitization depends on the light intensity, concentrations of riboflavin, oxygen, and ascorbic acid, temperature, pH of the reaction media, and the presence of other compounds (*20*−*23*). The addition of ascorbic acid greatly decreased amino acid degradation in the solution containing riboflavin. Ascorbic acid at 0.1% showed 20.82% inhibition of total amino acid degradation (*18*). However, the quenching mechanisms and kinetics of Trolox and ascorbic acid on the riboflavin-photosensitized oxidation of amino acids have not been reported.

The objectives of this study were to study (1) the effects of Trolox and ascorbic acid on the photosensitized oxidation of tryptophan and tyrosine and (2) the quenching mechanisms and kinetics of Trolox and ascorbic acid on the riboflavin-photosensitized oxidation of tryptophan and tyrosine.

MATERIALS AND METHODS

Materials. Ascorbic acid (>99.5%), Trolox (>98.0%), riboflavin (≥99.5%), tryptophan, and tyrosine were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile (≥99.9%) and methanol (≥99.9%) were also obtained from Sigma Chemical Co. Serum bottles, Teflon-lined rubber septa, and aluminum caps were purchased from Supelco Inc. (Bellefonte, PA). HPLC grade water (Sigma) was used as solvent for sample preparation. The purity of tryptophan and tyrosine was ≥99.5%.

Sample Preparation and Light Storage. To study the effects of ascorbic acid and Trolox on the degradation of tryptophan and tyrosine in the presence of riboflavin, ascorbic acid or Trolox at 0, 0.3, 0.6, or 0.9 mM was added to 10, 20, 40, and 80 mM tryptophan or tyrosine solution containing 25 ppm of riboflavin. Eight milliliters of the sample was transferred to a 10 mL vial. The sample bottles were stored in duplicate in a light storage box at 30 °C. The concentrations of tryptophan and tyrosine were determined in triplicate after 4 h of storage in the light box.

The light storage box consisted of two rectangular chambers: a glass chamber (60 cm × 30 cm × 50 cm) for sample storage and the wooden box (70 cm × 50 cm × 60 cm) for light sources. The distance from the light sources to the glass chamber was 12 cm. Samples were placed on the wire netting, which was 10 cm above the bottom of the glass chamber. The light sources, four Sylvania 15 W cool white fluorescent lamps (Danvers, MA), were placed on the bottom of the wooden box. The light intensity at the sample level was about 1000 lx.

Determination of Tryptophan and Tyrosine by HPLC. Tryptophan and tyrosine concentrations in the samples were determined by HPLC analysis (Agilent 1100, Santa Clara, CA) equipped with a C-18 Zorbax Eclipse AAA column (3.5 μm, 4.6 × 75 mm) (Agilent Technologies, Santa Clara, CA), a diode array detector (Agilent Technologies), a manual injector (Rheodyne model 7725i, Oak Harbor, WA), and Agilent HPLC software. Samples were filtered with a 0.2 μm membrane filter (Corning Inc., Corning, NY). The injection volume was 20 μL. For the separation of tryptophan and tyrosine the following gradient system was used: 100% A at 0 and 1 min; gradient to 43% A and 57% B at 9.8 min; 100% B at 10 and 12 min; 0% B at 12.5 and 14 min [solvent A, NaH_2PO_4; solvent B, acetonitrile/methanol/water (45:45:10)]. The flow rate of the mobile phase was 1.0 mL/min. Spectral data (210−350 nm) were collected during the whole run. Elution of compounds was monitored at a wavelength of 254 nm for tyrosine and 257 nm for tryptophan. Tryptophan and tyrosine concentrations were calculated using a standard curve.

Statistical Analysis. All of the experiments were done in triplicate. Data were analyzed using the Microsoft Office Excel program. Means were compared using Tukey's studentized range test. A p value of ≤0.05 was considered to be significant.

RESULTS AND DISCUSSION

Reproducibility of Analysis. The coefficients of variation for HPLC analysis of tryptophan and tyrosine for six replicates were 4.7 and 4.9%, respectively (data not shown). The low coefficients of variation were considered to be good and indicated that HPLC analysis was a reproducible method to study oxidation of amino acids in the presence of riboflavin.

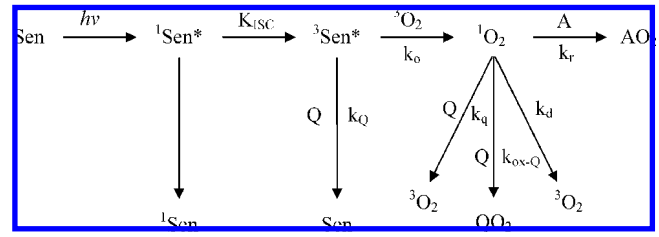

Figure 1. Reactions of photosensitizer with quencher and substrate.

Determination of Quenching Mechanisms and Kinetics of Trolox and Ascorbic Acid in Riboflavin-Photosensitized Oxidation. Riboflavin is a water-soluble photosensitizer for singlet oxygen formation (*24*).The formation of oxidized amino acid products by singlet oxygen oxidation, in the presence of riboflavin under light, is shown in **Figure 1**. When riboflavin (Sen) absorbs light energy, it first becomes an excited singlet sensitizer (^{1}Sen*) and then becomes an excited triplet sensitizer (^{3}Sen*) by an intersystem crossing (K_{ISC}) mechanism. The energy of excited triplet sensitizer is transferred to ordinary triplet oxygen ($^3O_2$) to produce singlet oxygen ($^1O_2$) by a triplet−triplet annihilation mechanism. Singlet oxygen reacts with substrates (A) such as amino acids in foods to form oxidized products (AO_2), or singlet oxygen is quenched physically or chemically by quenchers (Q) or may undergo natural decay. The quenching mechanism of the photosensitized singlet oxygen oxidation can be determined by measuring total quenching, physical quenching, and chemical quenching.

The steady-state kinetic equation for singlet oxygen oxidation in the presence of riboflavin is as follows (*25*):

$$\left\{\frac{d[AO_2]}{dt}\right\}^{-1} = K^{-1}\left(1+\frac{k_Q[Q]}{k_O[3O_2]}\right)\times \left[1+\frac{(k_Q+k_{ox\text{-}Q})(Q)+k_d}{k_r}x[A]^{-1}\right]$$

where $[AO_2]$ = the concentration of amino acids oxidized products; K = the rate of singlet oxygen formation (the quantum yield of intersystem crossing); k_r = the reaction rate constant of amino acid with singlet oxygen; A = substrate; k_q = the reaction rate constant of physical singlet oxygen quenching; $k_{ox\text{-}Q}$ = the reaction rate constant of chemical singlet oxygen quenching; [Q] = quencher (Trolox or ascorbic acid); and $k+_d$ = the decay rate of singlet oxygen in a solvent.

In this study, the substrate [A] is either tryptophan or tyrosine and the quencher [Q] is either Trolox or ascorbic acid.

If quencher reduced the photosensitized oxidation of amino acids by only singlet oxygen quenching, the steady-state kinetic equation for singlet oxygen formation is simplified due to $k_q[Q] = 0$ as follows (*12, 25, 26*):

$$\left\{\frac{d[AO_2]}{dt}\right\} = K^{-1}\left[1+\frac{(k_Q+k_{ox\text{-}Q})(Q)+k_d}{k_r}x[A]^{-1}\right]$$

The plot of $[AO_2]^{-1}$ versus $[A]^{-1}$ at various quencher concentrations gives constant y-intercept equal to K^{-1} and slope equal to$K^{-1}x[(k_Q + k_{ox\text{-}Q})(Q) + k_d/k_r]$. The ratio of slope to y-intercept gives$[(k_Q + k_{ox\text{-}Q})(Q) + k_d/k_r]$, which is independent of quencher concentration. When S/I is plotted against [Q], it would result in a line with an intercept of k_d/k_r and a slope of $(k_Q + k_{ox\text{-}Q})/k_r$.

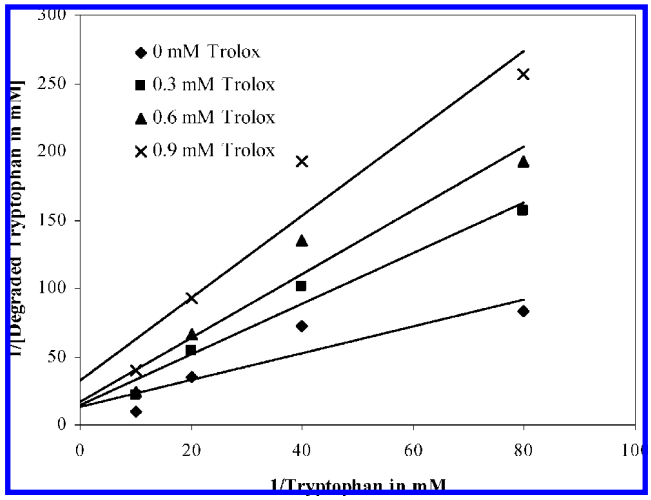

Figure 2. Effects of 0, 0.3, 0.6, and 0.9 mM Trolox on the degradation of tryptophan in aqueous solution containing 25 ppm of riboflavin under light at 30 °C.

In the case when there is only excited triplet sensitizer quenching, then the equation becomes

$$\left\{\frac{\mathrm{d}[\mathrm{AO}_2]}{\mathrm{d}t}\right\} = K^{-1}\left(1 + \frac{k_\mathrm{Q}[\mathrm{Q}]}{k_\mathrm{Q}[{}^3\mathrm{O}_2]}\right)\left[1 + \frac{k_\mathrm{d}}{k_\mathrm{r}}x[\mathrm{A}]^{-1}\right]$$

The plot of $[\mathrm{AO}_2]^{-1}$ versus $[\mathrm{A}]^{-1}$ at various quencher concentrations gives y-intercept equal to $K^{-1}(k_\mathrm{o}[{}^3\mathrm{O}_2] + k_\mathrm{Q}[\mathrm{Q}]/k_\mathrm{o}[{}^3\mathrm{O}_2])$. The slope of this plot is equal to $K^{-1}(k_\mathrm{d}(k_\mathrm{o}[{}^3\mathrm{O}_2] + k_\mathrm{Q}[\mathrm{Q}])/k_\mathrm{r}k_\mathrm{o}[{}^3O_2])$. The ratio of slope to the y-intercept of this plot is $k_\mathrm{d}/k_\mathrm{r}$, which is independent of the quencher concentration.

When both triplet sensitizer and singlet oxygen are quenched, then y-intercept is equal to $K^{-1}(k_\mathrm{o}[{}^3\mathrm{O}_2] + k_\mathrm{Q}[\mathrm{Q}]/k_\mathrm{o}[{}^3O_2])$ and slope is equal to $K^{-1}((k_\mathrm{o}[{}^3\mathrm{O}_2] + k_Q[\mathrm{Q}])((k_\mathrm{q} + k_{\mathrm{ox\text{-}Q}})[\mathrm{Q}] + k_d/k_rk_o[{}^3O_2])$. The ratio of slope to the y-intercept is $\{(k_\mathrm{q}+k_{\mathrm{ox\text{-}Q}})[\mathrm{Q}] + k_\mathrm{d}/k_r\}$. All of these terms are dependent on the quencher concentrations.

When no quencher is added in a model system, then the equation becomes (*20, 25, 27*) $\{\mathrm{d}[\mathrm{AO}_2]/\mathrm{d}t\}^{-1} = k^{-1}(1 + k_\mathrm{d}/k_\mathrm{r}\, x[\mathrm{A}]^{-1})$. The ratio of slope to y-intercept of the plot $[\mathrm{AO}_2]^{-1}$ versus $[\mathrm{A}]^{-1}$ is equal to $k_\mathrm{d}/k_\mathrm{r}$. The reaction rate constant k_r, between substrate and singlet oxygen, can be determined if the singlet oxygen decay rate k_d is known (*25*). The singlet oxygen decay rate in water has been reported as $2.5 \times 10^5\ \mathrm{s}^{-1}$ (*28, 29*).

Effect of Trolox on the Riboflavin-Photosensitized Oxidation of Tryptophan and Tyrosine. The reciprocal plots of degraded tryptophan and tyrosine concentrations against their initial concentrations in the presence of various concentrations of Trolox after storage in light for 4 h are shown in **Figures 2** and **4**.

The plot of slope/intercept for tryptophan against various Trolox concentrations is shown in **Figure 3**. A regression line $Y = 0.09217x + 1.484$ ($R^2 = 0.98$) is obtained. The intercept of the regression line would give $k_\mathrm{d}/k_\mathrm{r}$, and the slope of this line gives $[(k_\mathrm{Q} + k_{\mathrm{ox\text{-}Q}})/k_\mathrm{r}]$ (*9, 30*). Using the singlet oxygen decay rate in water, $k_\mathrm{d} = 2.5 \times 10^5\ \mathrm{s}^{-1}$ (*29*), k_r can be calculated as $k_\mathrm{r} = \mathrm{k_d}/\mathrm{intercept} = 2.5 \times 10^5\ \mathrm{s}^{-1}/1.48\ \mathrm{mM} = 1.68 \times 10^5\ \mathrm{mM}^{-1}\ \mathrm{s}^{-1} = 1.68 \times 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. The slope 0.092 equals $[(k_\mathrm{Q} + k_{\mathrm{ox\text{-}Q}})/k_\mathrm{r}]$. Therefore, the total singlet oxygen quenching rate $(k_\mathrm{Q} + \mathrm{k_{ox\text{-}Q}})$ of Trolox during the photooxidation of tryptophan in the presence of riboflavin is $(0.092 \times 1.68) \times 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1} = 1.55 \times 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$.

The plot of slope/intercept for tyrosine against various Trolox concentrations is shown in **Figure 5**. A regression line $Y = 0.07067x + 1.327$ ($R^2 = 0.84$) is obtained. The intercept of the regression line would give $k_\mathrm{d}/k_\mathrm{r}$ and the slope of this line gives $[(k_\mathrm{Q} + k_{\mathrm{ox\text{-}Q}})/k_\mathrm{r}]$ (*9, 30*). Using the singlet oxygen decay rate in water, $k_\mathrm{d} = 2.5 \times 10^5\ \mathrm{s}^{-1}$ (*29*), k_r can be calculated as $k_\mathrm{r} = k_\mathrm{d}/\mathrm{intercept} = 2.5 \times 10^5\ \mathrm{s}^{-1}/1.32\ \mathrm{mM} = 1.89 \times 10^5\ \mathrm{mM}^{-1}\ \mathrm{s}^{-1} = 1.89 \times 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. The slope 0.070 equals $[(k_\mathrm{Q}+k_{\mathrm{ox\text{-}Q}})/k_\mathrm{r}]$. Therefore, the total singlet oxygen quenching rate $(k_\mathrm{Q} + k_{\mathrm{ox\text{-}Q}})$ of Trolox during photooxidation of tyrosine in the presence of riboflavin is $0.070 \times 1.89 \times 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1} = 1.32 \times 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$.

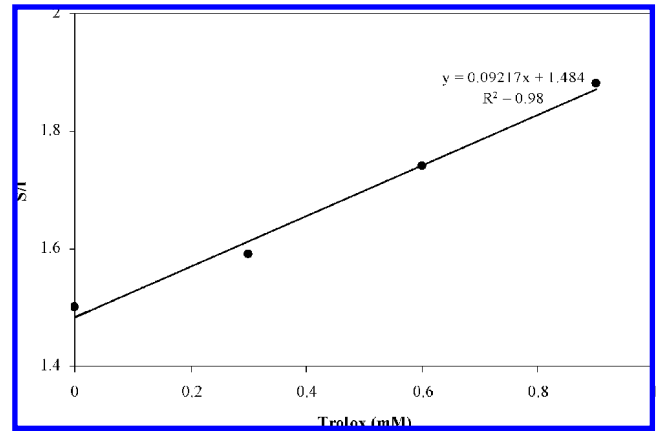

Figure 3. Relationship of slope/intercept in **Figure 2** to the concentrations of Trolox.

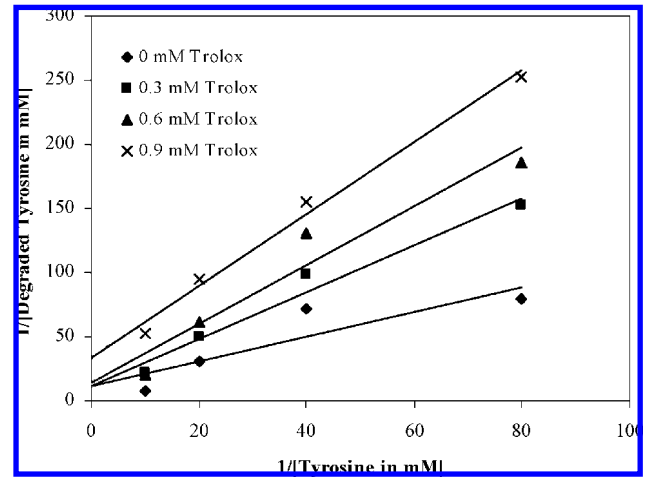

Figure 4. Effects of 0, 0.3, 0.6, and 0.9 mM Trolox on the degradation of tyrosine in aqueous solution containing 25 ppm of riboflavin under light at 30 °C.

As the Trolox concentration increased, the concentrations of oxidized tryptophan and tyrosine decreased. No significant differences in the intercepts of reciprocal plots in **Figures 2** and **4** were observed for 0, 0.3, and 0.6 mM concentrations of Trolox. This indicates that at these concentrations Trolox quenched singlet oxygen only (*25*). However, the intercept of 0.9 mM concentration Trolox is significantly different from those of the other concentrations (**Table 1**). This clearly indicates that beyond 0.6 mM concentration, Trolox quenched both singlet oxygen and excited triplet sensitizer. The same trend was observed for tyrosine also. The reaction rate of singlet oxygen (K_r) with tyrosine was higher than that with tryptophan. The present values for Trolox (K_r) are in good agreement with the estimated values. The rate constant of Trolox with singlet oxygen is higher in the presence of tryptophan than in the presence of tyrosine (**Table 3**).

Table 1. Intercepts and Slopes from Regression Lines in **Figures 3** and **5** for Determining the Quenching Rate of Trolox on Singlet Oxygen Oxidation of Tryptophan and Tyrosine

amino acid	concn (mM)	intercept[a] (*I*, 1/mM)	slope (*S*)	*S*/*I* (mM)
tryptophan	0	12.10a	18.20	1.50
	0.3	12.24a	19.53	1.59
	0.6	13.32a	23.25	1.74
	0.9	29.92b	56.32	1.88
tyrosine	0	13.30a	17.30	1.30
	0.3	12.82a	18.21	1.42
	0.6	15.00a	21.46	1.43
	0.9	31.30b	51.65	1.65

[a] Numbers with different letters are significantly different at $\alpha = 0.05$.

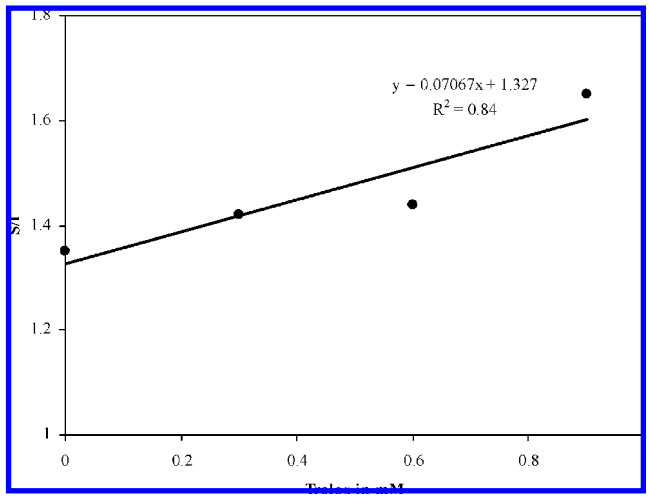

Figure 5. Relationship of slope/intercept in **Figure 4** to the concentrations of Trolox.

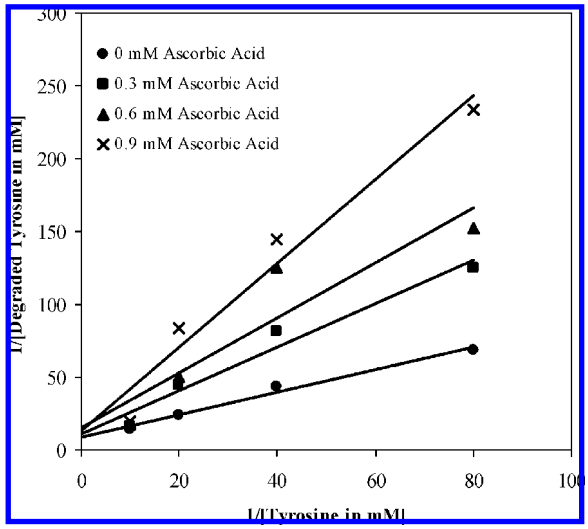

Figure 6. Effects of 0, 0.3, 0.6, and 0.9 mM ascorbic acid on the degradation of tryptophan in aqueous solution containing 25 ppm of riboflavin under light at 30 °C.

Effect of Ascorbic Acid on the Riboflavin-Photosensitized Oxidation of Tryptophan and Tyrosine. The reciprocal plots of degraded tryptophan and tyrosine concentrations against their initial concentrations in the presence of various concentrations of ascorbic acid after storage in light for 4 h are shown in **Figures 6** and **8**.

The plot of slope/intercept for tryptophan against various ascorbic acid concentrations is shown in **Figure 7**. A regression

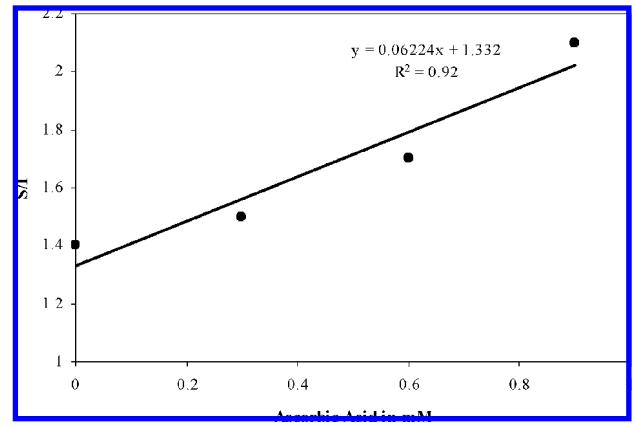

Figure 7. Relationship of slope/intercept in **Figure 6** to the concentrations of ascorbic acid.

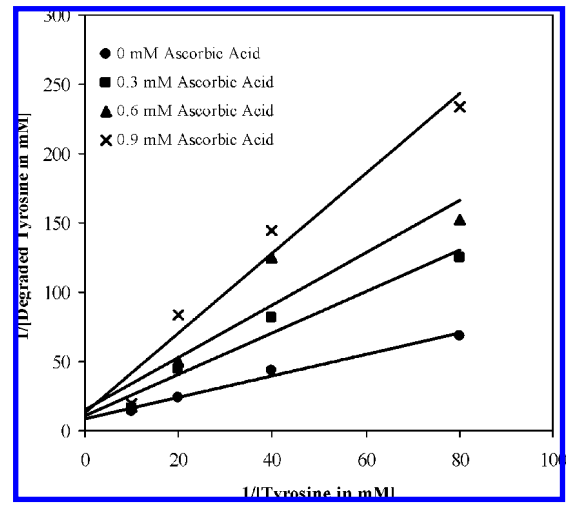

Figure 8. Effects of 0, 0.3, 0.6, and 0.9 mM ascorbic acid on the degradation of tyrosine in aqueous solution containing 25 ppm of riboflavin under light at 30 °C.

line $Y = 0.06224x + 1.332$ ($R^2 = 0.92$) is obtained. The intercept of the regression line would give k_d/k_r, and the slope of this line gives $[(k_Q + k_{ox\text{-}Q})/k_r]$ (*9, 30*). Using the singlet oxygen decay rate in water, $k_d = 2.5 \times 10^5$ s^{-1} (*29*), k_r can be calculated as $k_r = k_d$/intercept $= 2.5 \times 10^5$ s^{-1}/1.33 mM $= 1.88 \times 10^5$ mM^{-1} s^{-1} $= 1.88 \times 10^8$ M^{-1} s^{-1}. The slope 0.062 equals $[(k_Q + k_{ox\text{-}Q})/k_r]$. Therefore, the total singlet oxygen quenching rate ($k_Q + k_{ox\text{-}Q}$) of ascorbic acid during photooxidation of tryptophan in the presence of riboflavin is $(0.062 \times 1.88) \times 10^8$ M^{-1} s^{-1} $= 1.16 \times 10^7$ M^{-1} s^{-1}.

The plot of slope/intercept for tyrosine against various ascorbic acid concentrations is shown in **Figure 9**. A regression line $Y = 0.0533x + 1.207$ ($R^2 = 0.92$) is obtained. The intercept of the regression line would give k_d/k_r, and the slope of this line gives $(k_Q + k_{ox\text{-}Q})/k_r$ (*9, 30*). Using the singlet oxygen decay rate in water, $k_d = 2.5 \times 10^5$ s^{-1} (*29*), k_r can be calculated as $k_r = k_d$/intercept $= 2.5 \times 10^5$ s^{-1}/1.20 mM $= 2.08 \times 10^5$ mM^{-1} s^{-1} $= 2.08 \times 10^8$ M^{-1} s^{-1}. The slope 0.0533 equals $(k_Q + k_{ox\text{-}Q})/k_r$. Therefore, the total singlet oxygen quenching rate ($k_Q + k_{ox\text{-}Q}$) of ascorbic acid during photooxidation of tyrosine in the presence of riboflavin is $0.0533 \times 2.08 \times 10^8$ M^{-1} s^{-1} $= 1.10 \times 10^7$ M^{-1} s^{-1}.

As the ascorbic acid concentration increased, the concentrations of oxidized tryptophan and tyrosine decreased. The slopes in the reciprocal plots in **Figures 6** and **8** increased significantly as the concentration of ascorbic acid increased from 0 to 0.9

Table 2. Intercepts and Slopes from Regression Lines in **Figures 7** and **9** for Determining the Quenching Rate of Ascorbic Acid on Singlet Oxygen Oxidation of Tryptophan and Tyrosine

amino acid	concn (mM)	intercept[a] (I, 1/mM)	slope (S)	S/I (mM)
tryptophan	0	10.10a	14.26	1.41
	0.3	12.02a	19.14	1.59
	0.6	13.30a	23.04	1.73
	0.9	14.01a	30.21	2.15
tyrosine	0	11.07a	13.39	1.21
	0.3	12.64a	18.45	1.46
	0.6	14.50a	21.75	1.50
	0.9	14.62a	27.34	1.87

[a] Numbers with different letters are significantly different at $\alpha = 0.05$.

Table 3. Reaction Rate Constants of Singlet Oxygen and Tryptophan and Tyrosine (K_r) and Quenching Rates of Tryptophan and Tyrosine

quencher	amino acid	K_r ($\times 10^8$ M^{-1} s^{-1})	quenching rate ($\times 10^7$ M^{-1} s^{-1})
Trolox	tryptophan	1.68	1.55
	tyrosine	1.89	1.32
ascorbic acid	tryptophan	1.88	1.16
	tyrosine	2.08	1.10

mM (**Table 2**). However, the y-intercepts of the plots were not significantly different ($p < 0.05$). This clearly indicates that ascorbic acid quenched singlet oxygen only (*25*). Jung and others (*26*) found that the rate of reaction of ascorbic acid with singlet oxygen produced by photosensitization with 6 ppm of methylene blue was 5.77×10^8 M^{-1} s^{-1} in a potassium phosphate buffer at pH 6 and 20 °C. The solution was stored for 3 min in light at intensity of 5500 lx. Yang (*19*) reported a reaction rate for ascorbic acid with singlet oxygen of 3.08×10^8 M^{-1} s^{-1} in an aqueous solution at pH 7.0 and 25 °C. The samples were stored in light at an intensity of 4000 lx for 1 h, and the sensitizer was FD&C Red No. 3 at 40 ppm. King and Min (*9*) found that the reaction rate of ascorbic acid with singlet oxygen produced by photosensitization with 15 ppm of riboflavin was 2.23×10^7 M^{-1} s^{-1}. This result of King and Min (*9*) was the same as our quenching rate, whereas the result of Jung and others (*20*) was 27 times and the rate of Yang (*19*) was 14 times our quenching rate for ascorbic acid with singlet oxygen. Jung and others (*20*) used a light intensity 1500 lx higher than ours, and this could have resulted in greater oxidation of ascorbic acid. Also, different sensitizers can give different reaction rates for singlet oxygen and our solution contained tryptophan and tyrosine which competes with ascorbic acid with singlet oxygen. Ascorbic acid is less effective than Trolox in quenching singlet oxygen during oxidation of both tryptophan and tyrosine. This may be because of higher rates of degradation of ascorbic acid and also because ascorbic acid quenched singlet oxygen only, whereas Trolox quenched both singlet oxygen and excited triplet sensitizer.

The rate constants obtained experimentally may be affected by a number of factors such as the temperature, the pH, the ionic strength of solvent systems, and the ratio of organic solvents in the aqueous mixture (*14*). The types of sensitizer for singlet oxygen generation may also affect the quenching rate constant (*7*).

In conclusion, riboflavin produces singlet oxygen under light. Trolox and ascorbic acid acted as singlet oxygen quenchers and can protect tryptophan and tyrosine. However, the quenching mechanisms are different between Trolox and ascorbic acid.

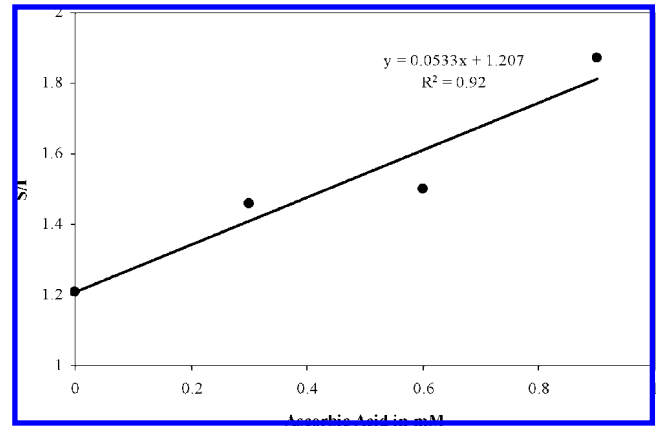

Figure 9. Relationship of slope/intercept in **Figure 8** to the concentrations of ascorbic acid.

Trolox quenched both singlet oxygen and excited triplet riboflavin under light, whereas ascorbic acid quenched singlet oxygen only. The singlet oxygen quenching rates of Trolox in the presence of tryptophan and tyrosine were 1.55×10^7 and 1.32×10^7 M^{-1} s^{-1}, respectively (**Table 3**). The singlet oxygen quenching rates of ascorbic acid in the presence of tryptophan and tyrosine were 1.15×10^7 and 1.06×10^7 M^{-1} s^{-1}, respectively. Singlet oxygen quencher alone could not completely protect tryptophan and tyrosine. Trolox, which is a quencher of both singlet oxygen and excited triplet riboflavin, can better protect tryptophan and tyrosine.

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