Pro- and Antioxidative Effect of Ascorbic Acid on L-Tryptophan in the System Fe^{3+}/Ascorbic Acid/O_2

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L-Tryptophan (Trp) is oxidized in the presence of the Fe^{3+}/L-ascorbic acid/O_2 system in aqueous solution at reaction temperatures of 20 °C. The oxidation is dependent upon pH, increases with increasing L-ascorbic acid concentration, and attains its maximum after 6 h. Increasing the Trp concentration augments its degradation. The differing reaction mechanisms of L-ascorbic acid are presented in two equations. Pro- and antioxidant characteristics of L-ascorbic acid are influenced by Trp in such a way as to shift the equilibrium in favor of prooxidation.

INTRODUCTION

L-Ascorbic acid is used in the food industry as an antioxidant and plays an important role as vitamin C in human nutrition. Mahoney and Graf (1986) have investigated the oxidative characteristics of L-ascorbic acid in model systems. A positive oxidation potential for small concentrations of L-ascorbic acid in the presence of iron (Fe^{3+}) or copper (Cu^{2+}) ions was observed. Udenfriend et al. (1954) utilized this property to hydroxylate aromatics using L-ascorbic acid in the presence of an Fe^{2+}/EDTA complex. Uchida and Kawakishi (1988) were successful in selectively oxidizing Trp and L-histidine groups in bovine serum albumin, making use of the self-oxidation of L-ascorbic acid catalyzed by copper. In all of these reaction systems the concentration of L-ascorbic acid was chosen such that exclusively the prooxidative characteristics were expressed. The aim of this work was to oxidize the essential amino acid Trp in the presence of Fe^{3+} ions, L-ascorbic acid, and molecular oxygen. These are preliminary experiments for the study of the stability of Trp in peptide models and in foods against oxidation in the presence of ascorbic acid and Fe^{3+} ions. The rate of oxidation was determined by measuring the amount of Trp recovered. The reaction was studied as a function of pH value, L-ascorbic acid as well as Trp concentrations, and reaction time.

MATERIALS AND METHODS

Reagents. Trp was purchased from Degussa AG (Hanau, Germany) and L-ascorbic acid from E. Merck (Darmstadt, Germany). All other chemicals used were of analytical purity and obtained from E. Merck.

Apparatus. Liquid chromatographic determinations of Trp were carried out with a high-performance liquid chromatograph (HPLC) system from Merck/Hitachi (Darmstadt, Germany). The HPLC system was composed of a Model L-6200 solvent delivery system, a Rheodyne injector with a 20-μL sample loop, and a reversed-phase column Nucleosil120–3C_{18} (125 × 4 mm; Macherey & Nagel, Düren, Germany). A variable-wavelength UV photometric detector (Model L-4000, Merck/Hitachi) set at 219 nm was connected at the column outlet. The chromatographic response was recorded and integrated by using a Merck/Hitachi Model D-2000 integrator. Quantitative determinations were made using external standards.

Procedures. Oxidation of Trp was performed in a 0.1 M citric acid buffer (20 mL), containing 0.12–0.24 mmol of Trp (*D* 0.06), 0.56–2.24 mmol of ascorbic acid (*D* 0.28), 0.10 mmol of Fe^{3+}, and 0.52 mmol of ethylenediaminetetraacetic acid (EDTA). EDTA forms a chelate complex with iron, thereby increasing its solubility, which is especially important at pH values near the isoelectric point. Furthermore, it enhances the oxidative effect of iron/EDTA complexes (Mahoney and Graf, 1986).

Table I. Dependence of Trp Degradation on pH (Reaction Time = 2.5 h; c_{Trp} = 0.24 mmol/20 mL; $c_{asc\ acid}$ = 2.24 mmol/20 mL)

	pH					
	1.5	2.5	3.5	4.5	5.5	6.5
Trp degradation, %	11	15	19	21	22	41

The reactions were carried out for up to 8 h (*D* 1 h); pH values varied between 1.5 (pH values of 1.5–4.5 were adjusted using citrate/hydrochloric acid) and 6.5 (pH values of 5.5–6.5 were adjusted using citrate/sodium hydroxide) (*D* 1), (Merck, 1988). During the reaction the mixture was stirred under atmospheric conditions and kept in the absence of light. The reaction was stopped by adding degassed distilled water 1:50 (v/v) and cooling. Chromatography was performed directly following this; if the reaction solution had to be stored for many hours, then the solution was frozen at −24 °C.

HPLC solvents were filtered through a 0.45-μm pore size filter (Schleicher & Schuell, Dassel, Germany) and degassed with helium before use. The mobile phase was bidistilled water (A) and acetonitrile/water aceotrop (B), each containing 0.1% trifluoroacetic acid (v/v). The gradient program consisted of solution A for 5 min, a linear gradient from 0 to 20% solution B in 25 min and from 20 to 50% B in 10 min, and a linear gradient from 50 to 0% solution B in 10 min. After 10 min of solution A, the next sample was injected. The solvent flow rate was 1 mL/min.

This model was chosen to investigate the stability of Trp in peptides against oxidation as a function of the neighboring amino acids. It is desirable to choose an oxidizing system of practical importance, i.e., one having importance in vivo or in foodstuffs. The Udenfriend system fullfills such criteria.

The effects of other reaction parameters were not studied further, but because similar conditions were continually applied, their effects should remain unchanged.

RESULTS AND DISCUSSION

pH Value. An increase in Trp degradation with increasing pH was observed (Table I). A noticeable jump in the Trp degradation from 22 to 41% lies between pH 5.5 and 6.5. The isoelectric pH value for Trp is 5.9 and lies in the pH range for this jump. The net charge at the isoelectric point is zero. The polarity of the molecule is greatest at pH 5.9 due to the distribution of charge. Furthermore, it must be stressed that the reactivity of L-ascorbic acid also depends on the pH value. This dependence may also influence the Trp degradation at

0021-8561/93/1441-2275$04.00/0

Table II. Dependence of Trp Degradation on Ascorbic Acid Concentration (c_{Trp} = 0.24 mmol/20 mL; pH 6.0; c = mmol/20 mL)

ascorbic acid, c	Trp degradation after 3 h		Trp degradation after 6 h	
	%	c	%	c
0.56	49	0.118	56	0.134
0.84	57	0.137	68	0.163
1.12	56	0.134	71	0.170
1.40	65	0.156	74	0.178
1.68	68	0.163	71	0.170
1.96	67	0.160	69	0.165
2.24	44	0.106	51	0.122

various pH values. The reaction of Trp as a function of pH value seems to depend on the model system used (Friedman and Cuq, 1988; Krogull and Fennema, 1987).

L-Ascorbic Acid. Upon increasing L-ascorbic acid concentration, one finds an initial increase in the degradation of Trp (Table II). The maximal Trp degradation (68%) after 3 h of reaction time was measured for an L-ascorbic acid concentration of about 1.7 mmol/20 mL. Increasing the L-ascorbic acid concentration >2 mmol/20 mL leads to a decrease in the Trp degradation. After 6 h of reaction time, the maximal Trp degradation was found for an L-ascorbic acid concentration of 1.40 mmol/20 mL. This reaction behavior cannot be explained simply in terms of the formation of radicals in the Udenfriend system. According to Grinstead (1960), the radicals are formed via

Scheme I

$$Fe^{2+}/EDTA + O_2 + H_2O \rightarrow Fe^{3+}/EDTA + OH^- + HO_2^{\bullet}$$

$$Fe^{2+}/EDTA + HO_2^{\bullet} + H_2O \rightarrow Fe^{3+}/EDTA + OH^- + H_2O_2$$

$$Fe^{2+}/EDTA + H_2O_2 \rightarrow Fe^{3+}/EDTA + OH^- + {}^{\bullet}OH$$

The formation of hydroxyl radicals via a Fenton reaction has been predicted by numerous authors. In this scheme the L-ascorbic acid acts as a reducing agent on Fe^{3+} ions as shown in Scheme II (abbreviations in the following schemes: AH_2, L-ascorbic acid; ·AH, L-monodehydroascorbic acid radical; A, L-dehydroascorbic acid):

Scheme II

$$2Fe^{3+}/EDTA + AH_2 + 2OH^- \rightarrow 2Fe^{2+}/EDTA + A + 2H_2O$$

It is evident from Scheme II that an increase in L-ascorbic acid concentration leads to the formation of more Fe^{2+} which, upon reoxidation, leads to an increase in the number of radicals. A higher radical concentration should cause an increase in the Trp degradation, which was not observed in the experiments. The Trp degradation decreases with increasing L-ascorbic acid (>2 mmol/20 mL). According to Mahoney and Graf (1986), at lower concentrations L-ascorbic acid enhances hydroxyl radical formation, while at higher concentrations it behaves as an antioxidant in that it intercepts radicals. This effect can be explained by considering the reaction from another viewpoint. The reactions in the Udenfriend system can be described by two reactions derived from L-ascorbic acid.

Scheme III describes the prooxidative part of the metal-catalyzed autoxidation of L-ascorbic acid:

Scheme III

$$AH_2 + 2O_2 \rightarrow A + 2HO_2^{\bullet}$$

The reaction between L-ascorbic acid and molecular oxygen under the formation of a hydrogen superoxide radical (see Scheme IV) has been described in the literature (Scarpa et al., 1983). The reduction of Fe^{3+} ions by L-ascorbic acid via a one-electron transfer and formation of oxygen radicals following the reoxidation of the reduced metal ions has also been reported (Xu and Jordan, 1990). The prooxidative character of L-ascorbic acid actually lies in its capacity to reduce oxygen under the formation of superoxide radical anions or hydrogen superoxide radicals. Via a Fenton reaction, hydroxyl radicals are formed which then can hydroxylate organic substrates.

Scheme IV

$$AH_2 + O_2 \rightarrow {}^{\bullet}AH + HO_2^{\bullet}$$

$$Fe^{3+} + AH_2 \rightarrow Fe^{2+} + {}^{\bullet}AH + H^+$$

$$Fe^{2+} + O_2 + H_2O \rightarrow Fe^{3+} + HO_2^{\bullet} + OH^-$$

$$2\,{}^{\bullet}AH \rightarrow AH_2 + A$$

$$AH_2 + 2O_2 \rightarrow 2HO_2^{\bullet} + A$$

Hydrogen superoxide radicals formed from the reduction of oxygen and the hydroxyl radicals formed therefrom (see Scheme I) represent the actual oxidizing agents for the Trp substrate. Because Trp degradation initially increases with increasing L-ascorbic acid concentration, more radicals must be forming according to Scheme III.

Scheme V describes the antioxidative function of L-ascorbic acid, where, as in the first reaction, it itself is oxidized to L-dehydroascorbic acid.

Scheme V

$$AH_2 + HO_2^{\bullet} \rightarrow {}^{\bullet}AH + H_2O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

$$AH_2 + {}^{\bullet}OH \rightarrow {}^{\bullet}AH + H_2O$$

$$Fe^{3+} + AH_2 \rightarrow Fe^{2+} + {}^{\bullet}AH + H^+$$

$$2\,{}^{\bullet}AH \rightarrow AH_2 + A$$

$$3AH_2 + 2HO_2^{\bullet} \rightarrow 3A + 4H_2O$$

Interception of the radicals by L-ascorbic acid is a reaction competing with the oxidation of Trp. The probability that a hydrogen superoxide radical or hydroxyl radical formed is reduced by L-ascorbic acid increases with increasing L-ascorbic acid concentration or L-ascorbic acid:Trp ratio. At ratios greater than 7:1, Trp degradation diminishes considerably. This means that, despite an increasing concentration of radicals, fewer Trp molecules are being oxidized.

Table III. Dependence of Trp Degradation on Trp Concentration ($c_{asc\ acid}$ = 1.12 mmol/20 mL; pH 6.0; c = mmol/20 mL)

	Trp degradation after			
	3 h		6 h	
Trp, c_0	%	c	%	c
0.12	67	0.08	81	0.10
0.18	63	0.11	78	0.14
0.24	56	0.14	71	0.17

Table IV. Dependence of Trp Degradation on Reaction Time (c_{Trp} = 0.15 mmol/20 mL; $c_{asc\ acid}$ = 1.20 mmol/20 mL; pH 6.0; c = mmol/20 mL)

reaction time, h	Trp degradation	
	%	c
1	29	0.044
2	44	0.066
3	62	0.093
4	71	0.107
5	76	0.114
6	79	0.119
7	82	0.123
8	83	0.125

L-Tryptophan. The dependence of the degradation of Trp on its initial concentration was studied for 3- and 6-h reaction times (Table III). An increase in Trp concentration for equal L-ascorbic acid concentrations leads to enhanced Trp degradation, when measured in millimoles per 20 mL; the amount of Trp degraded expressed in percent, in contrast, decreases. A high Trp concentration checks the reaction of L-ascorbic acid, due to the increased probability of Trp reacting with the hydrogen superoxide radicals. L-Ascorbic acid not used up in Scheme V reacts according to Scheme III, thereby producing more radicals, so that the millimoles per 20 mL of Trp degraded increased. The degradation of Trp expressed in percent decreases with increasing Trp concentration. This is due to the fact that the L-ascorbic acid is used up in Scheme III and then the radical concentration is ever increasing toward a maximal value.

Reaction Time. Studies of the reaction time were done, and again these describe the influence of the Trp concentration on Scheme V. At the onset of the reaction the Trp concentration is greatest, keeping Scheme V of L-ascorbic acid in check, so that the largest radical concentration via Scheme III is built in the first hour (Table IV). Increasing amounts of L-ascorbic acid are used up in Scheme V upon prolonged Trp degradation, leading to a decrease in the amount of Trp degraded per hour. Forty-four micromoles of Trp are oxidized in the first hour, 125 μmol in 8 h.

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Received for review January 14, 1993. Revised manuscript received August 11, 1993. Accepted August 19, 1993.[⊛]

[⊛] Abstract published in *Advance ACS Abstracts,* October 1, 1993.