Journal of Food and Drug Analysis, Vol. 16, No. 6, 2008, Pages 46-53 藥物食品分析 第十六卷 第六期

The Protective Effect of Amino Acids on the Copper(II)–Catalyzed Autoxidation of Ascorbic Acid

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(Received: April 2, 2008; Accepted: April 23, 2008)

ABSTRACT

Protection of ascorbic acid (AA: vitamin C) from Cu(II)-catalyzed autoxidation is an important aspect of antioxidant chemistry. The autoxidation of AA in the absence and presence of Cu(II) ions was investigated in aerated solution at room temperature and I = 0.1 ionic strength (KNO_3). Effects of eight biochemically important amino acids (alanine, glycine, aspartic acid, asparagine, glutamic acid, glutamine, phenylalanine, and histidine) on this system were studied. The concentration of unoxidized AA remaining in solution was measured with the CUPRAC (cupric ion reducing antioxidant capacity) spectrophotometric method without interference from the amino acids tested. The autoxidation rate constants of AA decreased with increasing amino acid concentration for fixed Cu, and increased with Cu(II) for fixed amino acid concentrations. Catalytic autoxidation of AA was inhibited by stable Cu-amino acid complexes; histidine, having the highest conditional stability constant for its Cu-complex, showed the strongest inhibitive effect. Since amino acids are food and drug compatible compounds, they can be used in commercial products to increase the stability of vitamin C.

Key words: ascorbic acid, vitamin C, autoxidation, copper(II) catalysis, amino acids, protection, antioxidants

INTRODUCTION

Ascorbic acid (AA: vitamin C) is a water-soluble antioxidant vitamin that participates in many biochemical reactions. AA exists in nature in the reduced (L-ascorbic acid) and oxidized (dehydroascorbic acid) forms. AA is rather unstable with respect to light, air oxygen, other oxidants, alkalis and transition metals. One of its main biochemical roles is the decrease of oxidation hazards on the cell metabolism[(1)]. It may be added as supplement to various food and vitamin products, and its stability in such environments depends on food composition, pH, and oxygen content[(2)].

Traces of transition metal ions like Cu(II) and Fe(III) may act as catalysts in the autoxidation of ascorbic acid. Because of its biochemical importance in the food and pharmaceutical industries, Cu(II)-catalyzed oxidation of AA by molecular oxygen has been extensively studied[(3-5)]. It has been found that the catalytic efficiency of Cu(II) complexes on AA autoxidation depends on the nature of the ligands and on the coordination geometry of the metal ion[(6)]. Complexation of Cu(II) and Fe(III) by certain ligands such as EDTA, CDTA, NTA, citrate, acetate, tartrate, phthalate, bishistidine and glutathione modifies catalytic activity[(3,4,7-10)]. Stability of the cupric or ferric chelate was shown by Khan and Martell to be linearly correlated to its catalytic activity in AA oxidation[(3,4)].

Amino acids are the building blocks of proteins. Proteins are synthesized in the organism from amino acids, and converted into 20 structurally different amino acids upon hydrolysis. Amino acids may undergo various changes in metabolism, and may convert into raw materials for the synthesis of other essentially important substances, e.g. haemoglobin, the oxygen transport iron-protein of red blood cells[(11,12)]. Since amino acids show a strong potential for binding transition metal ions like Cu(II) and Fe(III), they may act as synergic agents or protective antioxidants in systems bearing AA. Although Cu(II)-amino acid prototype compounds have been studied for understanding the mechanism of copper-biopolymer binding[(13)], there are relatively few reports in literature for synergic combinations of antioxidants, especially metal sequestering type antioxidants that prevent oxidative degradation reactions. Since AA may act as a reducing agent toward Fe(III) and Cu(II), the lower oxidation states of these metal ions thus formed may give Fenton reactions with hydrogen peroxide[(14)], producing the highly reactive hydroxyl radical ($^{\cdot}OH$) resulting in tissue

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damage. Therefore, AA may act as a mediator of lipid peroxidation through redox cycling of transition metal ions[15]. Thus, it is biochemically important to see the potential AA-stabilizing effect of amino acids.

This study aims to investigate the copper-binding (therefore AA-protective) roles of eight biochemically important amino acids (i.e. alanine, glycine, aspartic acid, asparagine, glutamic acid, glutamine, phenylalanine, and histidine) in aerated solution at pH 4.5, room temperature, and constant ionic strength. Phenylalanine and histidine are essential, whereas glycine and glutamine are conditionally essential (i.e. to be supplied exogeneously to specific populations) amino acids. Alanine and glycine are osmolytes of quantitative importance in cell volume regulation. Both amino acids, and additionally aspartic acid, asparagine, and glutamic acid take part in neurotransmission in the mammalian nervous system[16-18]. The concentration of AA remaining in solution as a function of time was measured with the cupric-neocuproine spectrophotometric reagent[19]. This reagent is the chromogenic oxidant of the CUPRAC assay of total antioxidant capacity (TAC)[20,21], arising not only from AA but from certain other vitamins (e.g. vitamin E), antioxidant phenolics (e.g. phenolic and hydroxycinnamic acids), and flavonoids. This method practically met no interference from the tested amino acids and other UV-Vis absorbing compounds in AA determinations.

MATERIALS AND METHODS

I. *Chemicals and Instruments*

All chemicals were of analytical reagent grade and were supplied by E. Merck (AG, Darmstadt, Germany). L-ascorbic acid (mentioned as ascorbic acid in the text) was used without purification. Cu(II)-Nc (neocuproine) reagent was prepared from $CuCl_2$ and neocuproine (2,9-dimethyl-1,10-phenanthroline) and used for AA assay as described elsewhere[19]. In investigating Cu-catalyzed autoxidation of AA, amino acids (*i.e.*, alanine, glycine, aspartic acid, asparagine, glutamic acid, glutamine, phenylalanine, and histidine) were used as possible copper chelators. These Cu-amino acid chelates were prepared in 0.01 M acetate buffer at pH 4.5, prepared according to Hsieh and Harris[22]. Deionized distilled water was used throughout.

The CUPRAC spectrophotometric determination of AA was carried out using a mixture of Cu(II) and neocuproine as chromogenic oxidant[19]. The pH of the medium was 7.0 (adjusted with ammonium acetate buffer), and AA was determined by measuring the absorbance of the reduction product, Cu(I)-neocuproine, at 450 nm against a reagent blank.

The absorbances were measured and spectra taken with an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Germany) using a pair of matched quartz cuvettes of 1 cm thickness. The pH measurements were made using a Metrohm 632 Digital pH- meter (CH- 9100 Herisau, Switzerland) with a combined glass electrode. The ionic strength of the reaction medium was maintained at I = 0.1 (KNO_3) in order to keep the activity coefficients of the relevant species constant during the kinetic process. All experiments were carried out at 25°C with a thermostated system (± 0.5°C) which contained an immersion circulator (Thermomix 1419 B. Braun model, AG Melsungen, Germany).

II. *Sample Preparation and Kinetic Measurements*

All stock solutions were prepared daily. 7.5×10^{-3} M Nc solution in 96% (v/v) ethanol, 1.0×10^{-2} M $CuCl_2$ aqueous solution and 1 M ammonium acetate aqueous solution were used in the determination of AA with Cu(II)- Nc reagent.

The stock solutions of 1.57×10^{-4} M of copper(II) nitrate, 5.67×10^{-3} M of AA, 1.0×10^{-2} M acetate buffer (at pH 4.5) and 1.0×10^{-2} M of the corresponding amino acids were prepared in water. In 100 mL flasks of kinetic measurement, the final concentrations of constituents in the mixtures were 0.1 M KNO_3, 0.0015 M acetate buffer, 1.0×10^{-4} - 2.0×10^{-3} M amino acid, 0.785×10^{-7} - 6.28×10^{-7} M copper(II) nitrate, and lastly added 5.67×10^{-4} M ascorbic acid. Reaction was timed with a chronometer when AA was added to the sample solution. The sample solution flask was placed in a water bath thermostated at 25°C. During this time a stream of air (flow rate = 60 L/h) was passed through the flask and the solution was saturated with oxygen. The air was presaturated with water vapor passed through a wash-bottle. Since the rate of reaction was slow compared to the rate of dissolution of oxygen, the reacting solution was considered saturated with oxygen at all time. After the addition of ascorbic acid, an aliquot of 0.6 mL was taken every 5 min for spectrophotometric measurement, and pH of the sample solution was controlled at the desired level. The rate of oxidation was measured for 30 min by quantifying the residual AA during the course of oxidation. All tests were made in triplicate, and the results were presented as arithmetic means.

Uncatalyzed and cupric amino acid chelate–catalyzed AA (5.67×10^{-4} M) oxidations were investigated in mixture solutions containing Cu(II) at 0, 0.785, 1.57, 3.14, 4.71, and 6.28×10^{-7} M, and amino acid at 0.1, 0.5, 1.0, and 2.0×10^{-3} M concentrations. Note that histidine was an exception showing the strongest inhibitive effect on Cu-catalyzed autoxidation of AA, therefore concentrations below 5.0×10^{-4} M were studied for this amino acid. The conditions of temperature, pH, and ionic strength for these mixtures were as described for all solutions.

III. *Procedure for AA Determination*

AA was determined by measuring the absorbance

of the reduction product (*i.e*., Cu(I)-neocuproine) of the CUPRAC reagent at 450 nm against a reagent blank[19]. One mL $CuCl_2$ solution was placed in a test tube, and then 1 mL of Nc, 1 mL ammonium acetate to bring the final pH to 7, 1.4 mL of water, and 0.6 mL AA solutions were added by mixing in this order. After two minutes, the absorbance at 450 nm was recorded against a reagent blank. All solutions in which copper-catalyzed autoxidation of AA in the absence or presence of amino acid chelators were followed were analyzed for ascorbic acid in this manner. In the linear range of measurement where Beer's law was valid, the 450-nm absorbances were proportional to the concentrations of AA remaining in solution.

RESULTS

I. *Theoretical Considerations*

Acidity (pK_a) values of ascorbic acid are 4.1 and 11.79[23]. Basically, the undissociated (H_2A) and mono-anionic (HA^-) forms of ascorbic acid predominated in solutions in the pH range of 3.2 - 4.5. Cupric nitrate was used as the autoxidation catalyst because among the inorganic anions, copper (II) forms the least stable complex with the relatively weak Lewis base, nitrate. On the other hand, sulfate and chloride form more stable complexes with copper, though all of them are relatively weak.

Oxidation of ascorbic acid in the presence of an excess of oxygen (saturation) was assumed to follow a first order reaction[3-5]. The reaction rate can be expressed as

$$-d[H_2A] / dt = k[H_2A] \ldots \text{ (proportional to } A_{450})$$

where $[H_2A]$ is the concentration of remaining ascorbic acid during the course of autoxidation, t is the time and k is the first-order rate constant which can be calculated from a plot of $\log[H_2A]$ *vs.* time. It gives a straight line with a slope $-k / 2.303$. If k_d is defined as the difference between the first-order rate constants in the presence and absence of copper ions, as defined by Khan & Martell[3], then the specific rate constant (K) independent of the metal ion concentration is expressed with the equation

$$K = k_d / [Cu(II)]$$

II. *Kinetic Findings and Rate Constants*

In order to investigate uncatalyzed and cupric amino acid chelate–catalyzed oxidation of AA in acetate-buffered solution, the Log (absorbance) *vs.* time data were analyzed with a linear regression approach. Such a curve is given for the Cu(II)-alanine-ascorbic acid system in Figure 1, using 3.14×10^{-7} M Cu(II) and varying concentrations of the amino acid (1.0, 5.0, 10.0 and 20.0×10^{-4} M). The rate of change of log (absorbance) *vs*. time (i.e. slope of linear curves in Figure 1) decreased as the amino acid concentration was increased, showing catalytic inhibition of AA autoxidation with Cu-binding. The specific rate constants (K) independent of Cu(II) concentration were found from the slopes of k_d *vs* [Cu(II)] curves such as the Cu(II)-aspartic acid-AA system (Figure 2). The specific rate constants found with the help of regression analysis are tabulated in Table 1. The K values found for all Cu-chelate systems studied were lower than that of Cu(II) alone. Generally for all amino acids, K values significantly decreased as the amino acid concentrations were increased, showing inhibition of Cu-catalyzed autoxidation with increased Cu-binding by amino acids. Among the tested amino acids, the most and least inhibitive Cu-binding ligands were histidine and glutamic acid,

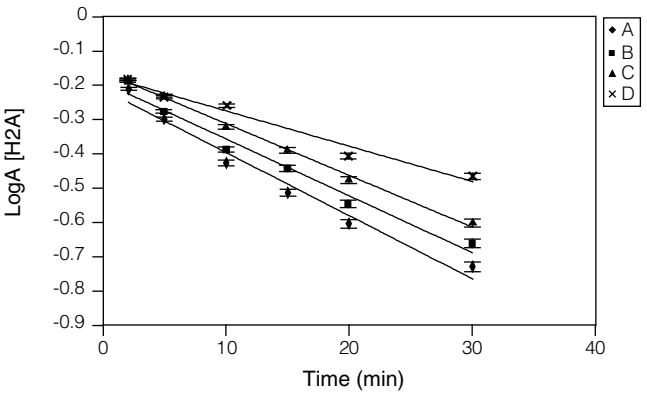

Figure 1. Kinetic curves for copper-catalyzed autoxidation of AA Cu(II) at 3.14×10^{-7} M; alanine at (A: 1, B: 5, C: 10, D: 20) $\times 10^{-4}$ M (Log A/ $[H_2A]$ indicates log value of CUPRAC absorbance due to ascorbic acid; error bars indicate standard deviations for n = 3 data points).

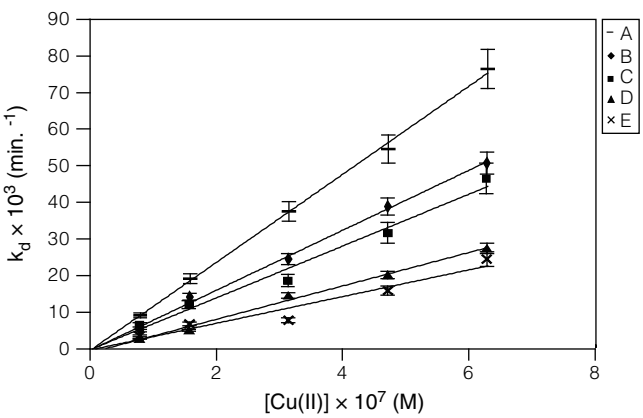

Figure 2. k_d as a function of Cu(II) concentration in aspartic acid-containing solutions buffered to pH 4.5 with acetate; aspartic acid concentrations: [(A : 0, B : 1, C : 5, D : 10, E : 20) $\times 10^{-4}$ M];
k_d = difference between first-order rate constants in the presence and absence of Cu(II); slopes of lines yield K: specific rate constants (error bars indicate standard deviations for n = 6 data points).

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Table 1. The specific rate constants at pH 4.5 for autoxidation of ascorbic acid catalyzed with amino acid-Cu(II) complexes

	AMINO ACID CONCENTRATION (M)							
	0.1×10^{-3}		0.5×10^{-3}		1.0×10^{-3}		2.0×10^{-3}	
AMINO ACID	$K \pm S_k$ (M^{-1} min^{-1})	r^2	$K \pm S_k$ (M^{-1} min^{-1})	r^2	$K \pm S_k$ (M^{-1} min^{-1})	r^2	$K \pm S_k$ (M^{-1} min^{-1})	r^2
Alanine	$(7.72 \pm 0.21) \times 10^4$	0.995	$(7.09 \pm 0.45) \times 10^4$	0.977	$(6.76 \pm 0.28) \times 10^4$	0.990	$(5.94 \pm 0.36) \times 10^4$	0.978
Aspartic acid	$(8.13 \pm 0.22) \times 10^4$	0.997	$(7.02 \pm 0.41) \times 10^4$	0.986	$(4.49 \pm 0.14) \times 10^4$	0.996	$(3.64 \pm 0.40) \times 10^4$	0.953
Asparagine	$(7.92 \pm 0.39) \times 10^4$	0.990	$(6.77 \pm 0.53) \times 10^4$	0.976	$(4.67 \pm 0.49) \times 10^4$	0.957	$(3.24 \pm 0.33) \times 10^4$	0.961
Phenylalanine	$(7.05 \pm 1.20) \times 10^4$	0.896	$(5.00 \pm 0.24) \times 10^4$	0.991	$(3.67 \pm 0.25) \times 10^4$	0.981	$(3.35 \pm 0.32) \times 10^4$	0.965
Glycine	$(8.12 \pm 0.98) \times 10^4$	0.944	$(7.79 \pm 0.90) \times 10^4$	0.948	$(6.30 \pm 0.57) \times 10^4$	0.969	$(5.82 \pm 0.12) \times 10^4$	0.998
Glutamic acid	$(9.94 \pm 0.52) \times 10^4$	0.989	$(9.27 \pm 0.46) \times 10^4$	0.990	$(8.32 \pm 0.66) \times 10^4$	0.975	$(6.54 \pm 0.48) \times 10^4$	0.979
Glutamine	$(8.68 \pm 0.72) \times 10^4$	0.973	$(8.00 \pm 0.68) \times 10^4$	0.972	$(7.90 \pm 0.23) \times 10^4$	0.997	$(5.64 \pm 0.45) \times 10^4$	0.975
Histidine	$(7.98 \pm 0.48) \times 10^4$	0.986	$(3.67 \pm 0.41) \times 10^4$	0.952				

For Cu(II) alone (without amino acid) $K \pm S_k$ (M^{-1} min^{-1}) = $(11.89 \pm 0.20) \times 10^4$ ($r^2 = 0.999$)

S_k = The standard deviation of K values, r^2 = regression coefficient (squared)

K = Specific rate constant independent of concentration, $K = \frac{k_d}{[Cu(II)]}$

respectively.

In general, the kinetic properties of Cu-catalyzed autoxidation of AA in the presence of amino acids (Table 1) can be summarized as follows:

- The reaction rates increased with increasing Cu(II) concentration.
- For different combinations of Cu(II) and amino acids, the oxidation rate decreased with increasing amino acid concentration, especially at high amino acid levels, e.g. 1.0×10^{-3} - 2.0×10^{-3} M.
- In Cu-catalyzed autoxidation of AA, the specific rate constants (K) decreased with increasing amino acid concentrations.
- The effects of the pairs; glutamic acid and glutamin, aspartic acid and asparagine, or glycine and alanine, were not significantly different.
- The inhibitive effect of phenylalanine was greater than that of alanine.
- Since the inhibitive effect of histidine was predominant at increasing levels of the amino acid, kinetic experiments could not be designed at relatively high concentrations (*i.e.*, 1.0×10^{-3} - 2.0×10^{-3} M).
- The rate of change of inhibition observed with increasing amino acid concentration was greater for aspartic acid than for glutamic acid (as reflected in the variation of the corresponding K values tabulated in Table 1).

DISCUSSION

Kinetic evaluation of experimental data showed that Cu-catalyzed autoxidation of ascorbic acid (AA) in acetate-buffered solution at pH 4.5 conformed to pseudo first-order kinetics. The autoxidation rate constant increased with Cu(II) concentration, and decreased with amino acid concentration for a given Cu(II) level[24].

Since acetate was used as the buffer medium (at pH 4.5), the possibility of acetate complexation of Cu(II) should be considered in kinetic evaluation. The cumulative stability constants ($Log\beta_n$) of the Cu(II)–acetate complexes are 2.16 and 3.2 for mono- and bis- complexes, respectively[25]. Authors of this work have recently established that aspartame, a dipeptide synthesized from phenylalanine and aspartic acid, significantly inhibited Cu-catalyzed oxidation of AA in acetate-buffered media[26]. Free aquated Cu^{2+} ion and the weak Cu(II)-acetate complex were shown to catalyze AA oxidation to a greater extent than the relatively stable Cu(II)-aspartame complex. The stability constants ($Log\beta_n$) of the Cu(II)–aspartame complexes were 6.097 and 10.84 for CuL^+ and CuL_2, respectively[27], rendering cupric ion complexation with aspartame predominant in acetate-buffered solutions.

Khan and Martell[4] postulated the following mechanism for ascorbic acid (H_2A) autoxidation catalyzed by a transition metal (M) chelated with a ligand (L) in the form of a relatively stable complex (ML^{n+}) :

$$H_2A \longleftrightarrow HA^- + H^+$$

$$HA^- + ML^{n+} \longleftrightarrow MHLA^{(n-1)+}$$

$$MHLA^{(n-1)+} \longrightarrow MHLA^{\bullet(n-1)+}$$

$$MHLA^{\bullet(n-1)+} \xrightarrow{rapid} A^{\bullet-} + ML^{(n-1)+}$$

$$A^{\bullet-} + ML^{n+} \xrightarrow{rapid} A + ML^{(n-1)+}$$

In this catalytic process, the catalyst ML^{n+} chelate can be regenerated by oxidation of $ML^{(n-1)+}$ with dissolved oxygen (thus, ascorbic acid, denoted as H_2A, is 2-e oxidized to dehydroascorbic acid, A). In the multi-step process, the slow, rate-limiting step is the formation of the ternary transition complex, $MHLA^{(n-1)+}$ from monohydrogen ascorbate (HA^-) and Cu(II)-chelate (ML^{n+}). If the binary M-L chelate is extremely stable, the ternary transition complex may not form quantitatively. Weak bonding in the transition complex may lead to easy deformation of this complex during electron transfer. In this respect, Khan and Martell[4] postulated that the autoxidation rate of AA in the presence of cupric chelates would decrease with increasing stability of the metal complexes, and found linear relationship in the catalytic activity of chelates as a function of logarithmic stability constant of the corresponding complex. In accordance with this postulate, the extremely stable cupric chelates with phenanthroline, dipyridyl[4], and thiourea[28] were shown to be completely inactive in catalyzing AA autoxidation. The postulated mechanism requires that the copper chelate with the amino acid ligand form a ternary transition complex with the ascorbic acid monoanion (HA^-) which may be converted to the Cu(I)-chelating amino acid complex and the radical product of ascorbic acid ($HA^{\cdot}$) in a slow reaction[8]. The ascorbyl radical responsible for the ultimate reaction with molecular oxygen (ascorbic acid itself being converted to dehydroascorbic acid)[7] was detected in the Cu(II)-histidine-AA reaction system by ESR techniques[9]. In this work, AA autoxidation was expected to be more inhibited in the presence of more stable Cu(II)-L chelates with amino acids due to weaker bonding of the transition complex with hydrogen ascorbate (HA^-).

The specific rate constants (K) for AA autoxidation listed in Table 1 reveal that the inhibitive potency of Cu(II)-ligand chelates for the tested amino acids followed the order: histidine > phenylalanine ≥ asparagine ≥ aspartic acid ≥ alanine ≥ glycine > glutamine > glutamic acid.

Stability constants (Log β_1) at different ionic strengths of the studied amino acids with Cu(II) were as follows: histidine (10.2-10.6) , phenylalanine (7.8-8.0), asparagine (7.9), aspartic acid (8.4), alanine (8.3), glycine (8.2), glutamine (7.75), glutamic acid (7.85)[29]. In order to precisely correlate the stabilities of the amino acid complexes to their inhibition potencies, it is better to calculate the conditional stability constants of these complexes at the working pH of 4.5. Moreover, since amino acids act as bidentate ligands for copper at pH values where they are sufficiently ionized, 1:2 metal (M)-ligand (L) complexes (ML_2) may form simultaneously with 1:1 (ML) complexes, and therefore, it would be more precise to consider the second cumulative stability constans (Log β_2) in the correlations. A theoretical analysis comprising these considerations is given below:

$$M + 2\,L \leftrightarrow ML_2 \quad \ldots \quad \beta_2 = [ML_2] / ([M]\,[L]^2)$$

$[L] = C_{H2L}\,\alpha_L$ (where C_{H2L}: total amino acid concn. ; α_L: relative abundance of L)

For diprotic amino acids (H_2L), $\alpha_L = K_{a1}K_{a2} / ([H^+]^2 + K_{a1}\,[H^+] + K_{a1}K_{a2})$, and for triprotic amino acids (H_3L), $\alpha_L = K_{a1}K_{a2}K_{a3} / ([H^+]^3 + K_{a1}\,[H^+]^2 + K_{a1}K_{a2}\,[H^+] + K_{a1}K_{a2}K_{a3})$. Thus,

$$\beta_2 = [ML_2] / ([M]\,C^2_{H2L}\,\alpha^2_L) \text{ and } \beta'_2 = \beta_2\,\alpha^2_L$$

where β'_2 is the conditional second cumulative stability constant of the ML_2 complex. For an informative evaluation, this constant has to be correlated to inhibition of copper-catalyzed autoxidation (Table 2). The percentage inhibition, I (%), can be defined as:

$$I\,(\%) = 100\,(K_{Cu(alone)} - K_{complex}) / K_{Cu(alone)}$$

For example, for 1.0×10^{-3} M alanine, I (%) = 43.1 by taking $K_{Cu(alone)} = 11.89 \times 10^4$ (Table 1).

The specific inhibition (I_s) is then defined as the mean percentage inhibition per unit concentration of amino acid, i.e. $I_s = I\,(\%) / C_{H2L}$. Statistical analysis (t-test at 95%

Table 2. Specific inhibition (I_s) of copper-catalyzed autotoxidation of ascorbic acid by various amino acids (I_s is correlated to conditional second cumulative stability constant, β'_2)

Amino acid	pK_{a1}	pK_{a2}	pK_{a3}	Log β_1	Log β_2	α_L	β'_2	Log β'_2	I_s
histidine	1.8	6.1	9.2	10.6	18.6	4.88×10^{-7}	9.48×10^5	5.98	2.34×10^5
phenylalanine	2.2	9.1	-	7.9	14.7	2.50×10^{-5}	3.13×10^5	5.50	1.57×10^5
asparagine	2.1	8.8	-	7.9	14.45	5.0×10^{-5}	7.02×10^5	5.85	1.29×10^5
aspartic acid	2.1	3.7	9.6	8.4	15.2	6.85×10^{-6}	7.44×10^4	4.87	1.24×10^5
alanine	2.4	9.7	-	8.3	15.1	6.26×10^{-6}	4.93×10^4	4.69	1.25×10^5
glycine	2.3	9.7	-	8.2	14.9	6.27×10^{-6}	3.12×10^4	4.49	1.15×10^5
glutamine	2.2	9.0	-	7.75	14.25	3.15×10^{-5}	1.76×10^5	5.24	9.88×10^4
glutamic acid.	2.3	4.3	9.7	7.85	14.4	3.86×10^{-6}	3.74×10^3	3.57	6.51×10^4

confidence level)[30] confirmed that a significant correlation did exist between β'_2 and I_s. The valid correlation equations for the data in Table 2 are:

$$\beta'_2 = 5.71\ I_s - 4.61 \times 10^5 ;\ r = 0.7990 \text{ and}$$

$$\text{Log } \beta'_2 = 4.14 \text{ Log } I_s - 16.05 ;\ r = 0.8275.$$

It is clear from these data that as the conditional stability of the copper-amino acid complex increased at the working pH of 4.5, the specific inhibition of the copper-catalyzed ascorbic acid autoxidation increased, in accordance with the hypothesis of Khan and Martell[4]. Since the studied amino acids are the building blocks of proteins, they can have a protective role for the prevention of transition metal–catalyzed ascorbic acid oxidation that may give rise to the generation of free radicals *via* reactions:

2 Cu(II) + ascorbic acid → 2 Cu(I) + dehydroascorbic acid

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

and thereby cause tissue damage. It has been well established in literature that such uncontrolled Fenton reactions produce the most powerful oxidant species, the hydroxyl radical, that attack biological macromolecules such as lipid, protein, and DNA, causing oxidative degradation in these macromolecules and damage in cellular tissues[31,32]. Under certain conditions of transition metal catalysis, ascorbic acid may act as a pro-oxidant instead of its usual function as antioxidant, and the presence of such amino acids as copper sequesterants may be the most efficient means of preventing such degradation reactions. It should be indicated that the concentration of amino acid was optimally taken in excess of either ascorbic acid or Cu(II) in this study to observe the mentioned protective effect. Otherwise, an excess of uncomplexed Cu(II) might easily induce the prooxidant effect of AA, which led some researchers in the past to conclude that the copper-ligating substance increased the rate of Cu-catalyzed autoxidation of AA[33].

The basic amino acid residues existing on proteins that are responsible for copper binding with concomitant formation of bioinorganic complexes exhibiting various oxidant and antioxidant activities have been identified as histidine, cysteine, and tryptophan moieties[34]. There is important evidence showing that those proteins and polypeptides exhibiting pro-oxidant behaviour (i.e. inducing oxidative stress, either through creating reactive oxygen species or inhibiting antioxidant systems) by copper binding may lead to neurodegenerative diseases[35,36]. The specific rate constants show that histidine is the strongest inhibitor of Cu-catalyzed AA autoxidation among the tested amino acids (Table 1). This may be related to the specific health-beneficial role of histidine in being primarily responsible for copper binding and transport in human and mammal serum[37]. The findings of this study may also be useful in understanding the possible synergistic combinations of antioxidants, e.g. a chain-breaking antioxidant (ascorbic acid) and a metal-sequestering antioxidant (amino acid).

CONCLUSIONS

Free Cu^{2+} ion was shown to catalyze AA oxidation to a greater extent than the relatively stable Cu(II)-amino acid complexes. This finding supports the conclusion of Khan and Martell that as the stability of a Cu(II) complex increases, its catalytic activity as a mediator of electron-transfer in AA oxidation decreases[3,4]. Since amino acids are physiologically compatible ligating agents in the food and biochemical era, they may help to protect AA values by retarding transition metal-catalyzed oxidation. Amino acids could also be useful in stabilizing mixture solutions of AA with a transition metal ion, which would otherwise yield Fenton-like reactions[14] causing hydroxyl radical-induced degradations, thereby aiding the conservation of foods and beverages. This is important, as it has been established that antioxidants in food such as ascorbic acid may act as pro-oxidants by reducing transition metal ions like Cu(II) to the lower oxidation state and make the strongly oxidizing hydroxyl radical generation catalytic[38]. The AA-protective effect of amino acids in pharmaceutical products by retarding metal-catalyzed oxidation of AA can be exploited in formulations containing vitamin C with relatively high water activity. The experimental work adopted for following ascorbic acid degradation is also important, as the remaining AA in solution is spectrophotometrically monitored with a copper(II)-neocuproine (CUPRAC) assay[20,21] that is not influenced by amino acids and many other organic compounds that normally interfere with conventional UV-spectrometric assays. Among the studied amino acids, histidine showed the strongest inhibitive effect on copper-catalyzed AA autoxidation. This may be related to the specific function of histidine as copper transport amino acid in the serum of humans and mammals[37]. The findings of this study may also be useful in understanding the possible synergistic combinations of antioxidants, such as ascorbic acid and amino acid combinations.

ABBREVIATIONS

AA:	Ascorbic acid
EDTA:	Ethylenediamine tetraacetic acid
CDTA:	Cyclohexanediamine tetraacetic acid
NTA:	Nitrilotriacetic acid
ESR:	Electron spin resonance
HA^-:	Monoanionic form of ascorbic acid
$HA^{\cdot}$:	Ascorbic acid radical
H_2A:	Undissociated form of ascorbic acid
Nc :	Neocuproine: 2,9-dimethyl-1,10-phenanthroline

CUPRAC: Cupric ion reducing antioxidant capacity
TAC : Total Antioxidant Capacity
UV-Vis : Ultraviolet-Visible
K_a : Acidity constant
β_n : Cumulative complex formation constant
k : First-order rate constant of copper-catalyzed AA autoxidation
k_d : Difference between first-order rate constants in the absence and presence of amino acids
K : Specific rate constant independent of copper concentration
S_K : Standard deviation of K values

REFERENCES

1. Packer, L. and Fuchs, J. 1997. Vitamin C in Health and Disease. p.25 Mercel Dekker. Inc. New York, U. S. A.
2. Martell, A. E. 1982. Chelates of ascorbic acid. Chapter 7. In "Ascorbic Acid : Its Chemistry and Metabolism and Uses". Advances in Chemistry Series 200. p. 153. Seib, P. A. and Tolbert, B. M. eds. American Chemical Society Publishing. Washington, DC. U. S. A.
3. Khan, M. M. T. and Martell, A. E. 1967a. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation. J. Am. Chem. Soc. 89: 4176-4185.
4. Khan, M. M. T. and Martell, A. E. 1967b. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric chelate catalyzed oxidation. J. Am. Chem. Soc. 89: 7104-7111.
5. Ogata, Y., Kosugi, Y. and Morimoto, T. 1968. Kinetics of the cupric salt-catalyzed autoxidation of L-ascorbic acid in aqueous solutions. Tetrahedron 24: 4057-4066.
6. Davies, M. B. 1992. Reactions of L-ascorbic acid with transition metal complexes. Polyhedron 11: 285-321.
7. Sahbaz, F. and Somer, G. 1993. The effect of citrate anions on the kinetics of cupric ion-catalysed oxidation of ascorbic acid. Food Chem. 47: 345-349.
8. Imer, F., Sonmezoglu, I. C. and Kozcaz, M. 2003. The role of buffers on the kinetics of L- ascorbic acid oxidation catalyzed by copper (II). Ital. J. Food Sci. 15: 521-529.
9. Scarpa, M., Vianello, F., Signor, L., Zennaro, L. and Rigo, A. 1996. Ascorbate oxidation catalyzed by bis (histidine) copper(II). Inorg. Chem. 35: 5201-5206.
10. Ohta, Y., Shiraishi, N., Nishikawa, T. and Nishikimi, M. 2000. Copper-catalyzed autoxidations of GSH and L-ascorbic acid: mutual inhibition of the respective oxidations by their coexistence. Biochim. Biophys. Acta 1474: 378-382.
11. Tekman, S. and Oner, N. 1994. Biochemistry in Turkish. In"Genel Biyokimya Dersleri". p.200 Yayinları, I. U. ed. Fakulteler Matbaasi. Istanbul, Turkey.
12. Telefoncu, A. 1992. Medical Biochemistry in Turkish. In"Tip ve Fen Bilimciler icin Biyokimya". p.22 Arkadas Tip Kitaplari. Istanbul, Turkey.
13. Farrell, J. J. 1977. Physical and Chemical Properties of the Copper-Alanine System. An Advanced Laboratory System. J. Chem. Educ. 54: 445-446.
14. Fenton, H. J. H. 1894. Oxidation of tartaric acid in presence of iron. J. Chem. Soc. Trans. 65: 899-911.
15. Tribble, D. L., Thiel, P. M., Van den Berg, J. J. M. and Krauss, R. M. 1995. Differing α-tocopherol oxidative lability and ascorbic acid sparing effects in buoyant and dense LDL. Arterioscler. Thromb. Vasc. Biol. 15: 2025-2031.
16. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130: 432-437.
17. McDonald, J. W. and Johnston, M. V. 1990. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res. Brain Res. Rev. 15: 41-70.
18. Ballantyne, J. S. and Chamberlin, M. E. 1993. Regulation of cellular amino acid levels. Chapter 6. In "Cellular and Molecular Physiology of Cell Volume Regulation". pp.112-129. Strange, K. ed. CRC Press. Boston, U. S. A.
19. Guçlu, K., Sozgen, K., Tutem, E., Ozyurek, M. and Apak, R. 2005. Spectrophotometric determination of ascorbic acid using copper(II)-neocuproine reagent in beverages and pharmaceuticals. Talanta 65: 1226-1232.
20. Apak, R., Guçlu, K., Ozyurek, M. and Karademir, S. E. 2004. A novel total antioxidant capacity index for dietary polyphenols, vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. J. Agric. Food Chem. 52: 7970-7981.
21. Apak, R., Guçlu, K., Ozyurek, M., Karademir, S. E. and Altun, M. 2005. Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: The CUPRAC method. Free Radic. Res. 39: 949-961.
22. Hsieh, Y.-H. P. and Haris, N. D. 1993. Effect of sucrose on oxygen uptake of ascorbic acid in a closed aqueous system. J. Agric. Food Chem. 41: 259-262.
23. Weast, R. C. 1982. CRC Handbook of Chemistry and Physics. 62nd ed. p.D-129 Chemical Rubber Co. Florida, U. S. A.
24. Aldemir, E. 2007. Spectrophotometric investigation of the catalytic effects of certain copper(II) chelates on the oxidation of ascorbic acid. M. Sc. Thesis. p.107 Institute of Science and Technology, Yildiz Technical University, Istanbul, Turkey.
25. Lurie, J. 1978. Handbook Analytical Chemistry. English Translation. 2nd ed. p. 294. Mir Publication. Moscow, USSR.
26. Imer, F., Sonmezoglu, I., Yildogan, B., Kilic, H. and Apak, R. 2008. Kinetic study of copper (II)- and iron (III)- catalyzed oxidation of L-ascorbic acid in

aspartame-containing aerated solution: protection of ascorbic acid. Ital. J. Food Sci. 20: 5-21.
27. Kholeif, S. and Anderegg, G. 1997. Equilibrium studies of aspartame and some of its degradation products with hydrogen (I) and copper(II) under physiological conditions using potentiometric pH measurements. Inorganica Chim. Acta 257: 225-230.
28. Zhu, B.-Z., Antholine, W. E. and Frei, B. 2002. Thiourea protects against copper-induced oxidative damage by formation of a redox-inactive thiourea-copper complex. Free Radic. Biol. Med. 32: 1333-1338.
29. Berthon, G. 1995. The Stability Constants of Metal Complexes of Amino Acids with Polar Side Chains. Pure and Applied Chemistry (IUPAC). 67: 1117-1240.
30. Miller, J. C. and Miller, J. N. 1993. Statistics for Analytical Chemistry. 3rd ed. p.54 Ellis Horwood, Prentice Hall. London, U. K.
31. Halliwell, B. and Gutteridge, J. M. C. 1989. Free Radicals in Biology and Medicine. p.262 Oxford University Press. Oxford, U. K.
32. Halliwell, B. and Auroma, O. I. 1991. DNA Damage by Oxygen Derived Species: Its Mechanism and Measurements in Mammalian Systems. FEBS Lett. 281: 9-19.
33. Hsieh, Y. P. and Haris, N. D. 1991. Destructive effect of aspartame on ascorbic acid in Cu-catalyzed solutions. J. Agric. Food Chem. 56: 14-16.
34. Opazo, C., Barria, M. I., Ruiz, F. H. and Inestrosa, N. C. 2003. Copper Reduction by Copper Binding Proteins and Its Relation to Neurodegenerative Diseases. Biometals 16: 91-98.
35. Kontusha, A. 2001. Amyloid-β: an antioxidant that becomes a pro-oxidant and critically contributes to Alzheimer's disease. Free Radic. Biol. Med. 31: 1120-1131.
36. Rossi, L., Lombardo, M. F., Ciriolo, M. R. and Rotilio, G. 2004. Mitochondrial dysfunction in neurodegenerative diseases associated with copper imbalance. Neurochem. Res. 29: 493-504.
37. Lee, J., Pena, M. M. O., Nose, Y. and Thiele, D. J. 2002. Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem. 277: 4380-4387.
38. Huang, D., Ou, B. and Prior, R. L. 2005. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53: 1841-1856.