The Effect of Neighboring Amino Acid Residues and Solution Environment on the Oxidative Stability of Tyrosine in Small Peptides

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ABSTRACT

The effects of neighboring residues and formulation variables on tyrosine oxidation were investigated in model dipeptides (glysyl tyrosine, N-acetyl tyrosine, glutamyl tyrosine, and tyrosyl arginine) and tripeptide (lysyl tyrosyl lysine). The tyrosyl peptides were oxidized by light under alkaline conditions by a zero-order reaction. The rate of the photoreaction was dependent on tyrosyl pK_a, which was perturbed by the presence of neighboring charged amino acid residues. The strength of light exposure, oxygen headspace, and the presence of cationic surfactant, cetyltrimethylammonia chloride had a significant effect on the kinetics of tyrosyl photooxidation. Tyrosine and model tyrosyl peptides were also oxidized by hydrogen peroxide/metal ions at neutral pH. Metal-catalyzed oxidation followed first-order kinetics. Adjacent negatively charged amino acids accelerated tyrosine oxidation owing to affinity of the negative charges to metal-ions, whereas positively charged amino acid residues disfavored the reaction. The oxidation of tyrosine in peptides was greatly affected by the presence of adjacent charged residues, and the extent of the effect depended on the solution environment.

KEYWORDS: Tyrosine, oxidation, kinetics, charge-effect, ionic strength, antioxidants.

INTRODUCTION

Oxidation of amino acid residues is an important chemical pathway by which proteins can degrade.[1,2] Oxidized proteins can have altered biological activity, biological half-life, and immunogenicity.[3,4] According to the biochemical literature, methionine, cysteine, histidine, tryptophan, and tyrosine residues are most susceptible to oxidation.[2,5,6] However, most of the pharmaceutical research has focused on the oxidation of methionine and cysteine, and the oxidation of other amino acids in protein formulations has not been investigated thoroughly. A recent review highlights the importance of tyrosine and tryptophan oxidation in the stability of therapeutic proteins.[7]

Tyrosine is 1 of the 20 naturally occurring amino acids present in many peptides and proteins. The biochemical literature has documented that tyrosine readily oxidizes in biological systems.[3,8-10] These experiments were aimed at studying the relation of tyrosine oxidation to certain physiological and pathological processes. The predominant oxidation product was dihydroxyphenylalanine (or dopa), which further oxidized to a complex polymeric pigment, melanin,[9,10] whose molecular weight can range from 500 to 30 000 Da. Some investigators have shown that the formation of dityrosine was also possible.[10,11] Nonetheless, the reaction conditions and by-products are expected to be much more diverse in pharmaceutical situations because of the complex environmental and processing factors. The oxidation of tyrosine is generally believed to follow a free-radical pathway[8,10] that may begin with the formation of a phenoxy radical that propagates a chain reaction. A singlet-molecular-oxygen-mediated mechanism has also been proposed.[9,12] The mechanism of oxidation is complicated, where many reactive oxygen species are involved and the rate limiting step of the chain reaction may change during the course of the reaction. Hence, a kinetic model fully describing the oxidation reaction is difficult to propose and, very often, the overall observed rate of reaction is useful. Oxidative degradations can follow zero-order kinetics,[13] first-order kinetics, or second-order kinetics.[14] Self-accelerated kinetics is also possible in photolytic reactions.[15]

Packaging and formulation factors can often play an important role in the stability of protein/peptide products. For example, oxygen present in the headspace of parenteral packaging can dissolve in aqueous solutions and induce oxidative reactions.[2,16] Lower environmental temperature would not necessarily decrease the rate of oxidation because the aqueous solubility of oxygen is inversely proportional to temperature.[5,14] Therefore, one of the strategies for minimizing oxidation in a liquid formulation is to remove oxygen. Exposure to light can further accelerate the oxidation reaction.[14,16]

Solution pH has a significant effect on tyrosine oxidation because the pK_a of the tyrosyl group is 10.1,[17] and the ionized tyrosine is more susceptible to oxidation than the

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un-ionized form.[12,18] Many adjuvants are added to parenteral formulations to stabilize proteins. For example, salts are used to maintain isotonicity and balance ionic interactions between protein molecules.[2] Increase in ionic strength of the salted solution has been shown to affect the rate of degradation reactions in proteins.[19,20] Surfactants are also common additives to many protein formulations as solubilizers and stabilizers.[2] Nonionic surfactants such as polysorbates may contain peroxides that are introduced in the bleaching step of manufacturing.[21] These peroxide species can catalyze oxidation of protein formulations.[22] A process of removing peroxides from polyethylene glycol solutions was discussed recently.[23] Ionic surfactants often exhibit strong interaction with charged groups on proteins,[2,24] which could potentially influence the reaction of these groups. In addition, formulations are often contaminated with transition metal ions such as Cu^{2+} and Fe^{3+} that originate from chemical reagents or from the containers.[5] It was documented that even trace amount of metal ions can catalyze oxidative reactions.[10,25,26]

Oxidation is often controlled by the incorporation of antioxidants to the formulations.[2] There are 3 classes of antioxidants. The first-class antioxidants are the phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene, propyl gallate, and vitamin E. Reducing agents such as ascorbic acid, sodium metabisulfite, sodium sulfite, and thioglycerol belong to the second class of antioxidants. They act as scavengers in closed systems, where they oxidize preferentially over the protein. The third-class antioxidants are chelating agents such as EDTA, citric acid, and thioglycolic acid. These antioxidants remove trace metal ions that can initiate oxidation reactions by forming a complex with the metal ions.

The objective of this study was to investigate systematically the influence of neighboring amino acid residues and formulation factors on the oxidation of tyrosine in model dipeptides and tripeptides (Figure 1). The results of this study will aid formulation scientists in developing stable formulation for proteins containing labile tyrosine residues.

Materials and Methods

Materials

L-tyrosine, N-acetyl tyrosine (N-Ac-Tyr), glysyl tyrosine (Gly-Tyr), and glutamyl tyrosine (Glu-Tyr) were obtained as free bases and lysyl tyrosyl lysine (Lys-Tyr-Lys) as acetate salt from Sigma Chemical Co (St Louis, MO). Tyrosyl arginine (Tyr-Arg) acetate salt was purchased from ICN Biomedicals Inc (Costa Mesa, CA) and from Bachem Bioscience Inc (King of Prussia, PA). All the model peptides were L-isomers. These materials were used as received without further purification.

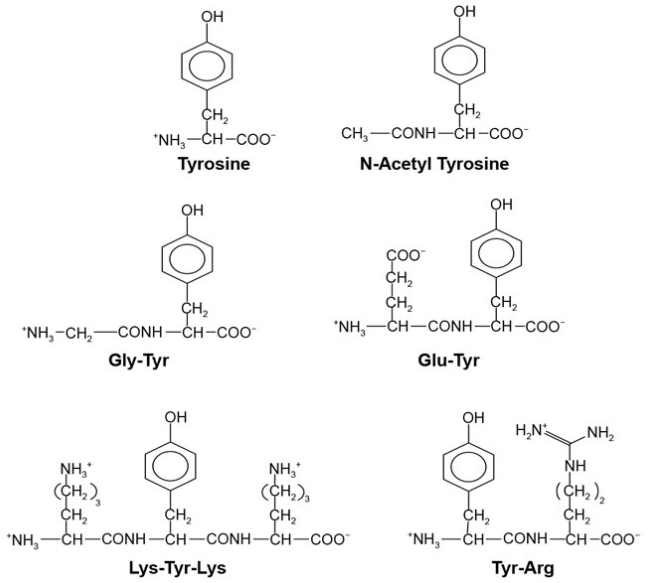

Figure 1. Structures of tyrosine, N-acetyl tyrosine, Gly-Tyr, Glu-Tyr, Tyr-Arg, and Lys-Tyr-Lys.

Hydrogen peroxide (30%), sodium chloride, cetyltrimethylammonia chloride, and ascorbic acid were purchased from Aldrich Chemicals (Milwaukee, WI). Ammonia solution (29%), cupric sulfate, sodium metabisulfite, and butylated hydroxyanisole were purchased from Sigma Chemical Co. High-performance liquid chromatography (HPLC)-grade trifluoroacetic acid (TFA) and EDTA disodium salt dihydrate were obtained from J.T. Baker Chemicals, (Phillipsburg, NJ). HPLC-grade ammonia acetate and ferric chloride were from Fisher Chemicals (Fair Lawn, NJ). Polysorbate 80 was from UniQema (Wilmington, DE), and sodium lauryl sulfate was from Brand-Nu Labs Inc (Meriden, CT).

Methods

Photo-oxidation of Tyrosyl Peptides

The tyrosyl compounds were dissolved in 0.01 M ammonia acetate solution at a concentration of 2 mM. The pH of solutions was adjusted to 6.8, 8.3, 9.3, and 10 with the addition of ammonium hydroxide. The ionic strength of the solutions was adjusted to 0.01, 0.1, and 0.3 M by adding appropriate amounts of NaCl. Some samples were purged with purified nitrogen to remove dissolved oxygen. The solutions were exposed to simulated solar radiation (290-800 nm) in a Heraeus Xenon Lamp Suntester (Heraeus Inc, Phoenix, AZ). The irradiance to which samples were exposed was set at 2 different levels, 380 and 760 W/m^2. The temperature inside the Suntester was maintained at 30°C ± 1°C by an intercooler. To study the effect of surfactants, 0.5% (wt/wt) sodium lauryl sulfate (anionic), cetyltrimethylammonia chloride (cationic), and polysorbate 80 (nonionic) were added to the peptide

solutions. To study the effectiveness of antioxidants to retard oxidation, 0.56 mM of sodium metabisulfite, ascorbic acid, or BHA was incorporated into the peptide solutions. Aliquots of samples were taken from the reacting solutions at 5 different time points within 6 hours and analyzed by HPLC/UV/mass spectrometry (MS). All the studies were conducted in duplicate.

Metal-Catalyzed Oxidation of Tyrosyl Peptides

The tyrosyl peptides were dissolved in 0.01 M ammonium acetate solution at a concentration of 2 mM. The pH of sample solutions was at 6.8. The peptide solutions were incubated with 0.15% hydrogen peroxide and 25 μM $CuSO_4$ or $FeCl_3$ at 25°C ± 0.5°C. To study the effect of metal ion concentration, the level of $CuSO_4$ was adjusted at 0, 25, and 100 μM. To study the effectiveness of antioxidants to retard oxidation, 0.56 mM of sodium metabisulfite, ascorbic acid, BHA, or 100 μM EDTA was incorporated in the peptide solutions. The sample solutions were analyzed periodically during the 72-hour experiment period by HPLC/UV/MS. The studies were performed in duplicate.

Analysis of Model Tyrosyl Peptides

The analytical method employed a Hewlett Packard 1100 liquid chromatography (LC) system (Agilent Technologies, Inc., Santa Clara, CA) coupled with a diode array detector and a single quadruple atmospheric pressure mass spectrometer. The samples of oxidized peptides were eluted on a Phenomenex Prodigy ODS-3 reverse phase column (5 μm, 100Å, 3.2 × 250 mm [Phenomenex, Torrance, CA]). The mobile phase gradients were compound specific. For tyrosine and Gly-Tyr, the mobile phase changed from 0.01% TFA/methanol (95:5) at 0 minute to 0.01% TFA/methanol (70:30) at 15 minutes. For Glu-Tyr, the mobile phase changed from 0.01% TFA/methanol (85:15) at 0 minute to 0.01% TFA/methanol (60:40) at 15 minutes. For N-Ac-Tyr, the mobile phase changed from 0.01% TFA/methanol (80:20) at 0 minute to 0.01% TFA/methanol (50:50) at 20 minutes. For Tyr-Arg and Lys-Tyr-Lys, the mobile phase changed from 0.01 M ammonia acetate/methanol (95:5) at 0 minute to 0.01 M ammonium acetate/methanol (70:30) at 15 minutes. The flow rate was 0.5 mL/min. Atmospheric pressure ionization/electrospray (API-ES) mass spectra of the eluents were acquired in the *m/z* range from 100 to 700. The fragmentation voltage of the mass spectrometer was adjusted between 70 and 160 V to optimize the abundance of the fragment ions. The spray chamber temperature was 350°C, the drying gas flow was 12.0 L/min, and the nebulization pressure was 50 psi. The HPLC traces were also monitored by diode array detection at 220 and 275 nm.

Results and Discussion

Photo-oxidation of Model Tyrosyl Peptides and Effect of pH

The disappearance of the parent peptide molecules as a result of photo-oxidation at pH 6.8, 8.3, 9.3, and 10 was monitored by HPLC/UV/MS. The photo-oxidation reaction followed apparent zero-order kinetics, which was reflected by a linear relationship between peptide concentration and time. The rate constants were calculated from the slope of the time dependence profiles. Such apparent zero-order behavior has been reported for many compounds of pharmaceutical interest[27-29] and can be explained theoretically by the deduction of Mendenhall.[16]

Figure 2 shows that the rate constant of tyrosine photo-oxidation depends both on the pH of the solution and immediately adjacent amino acid residue. The rate constant of photo-oxidation (k_0) of Tyr, Gly-Tyr, Glu-Tyr, N-Ac-Tyr, Tyr-Arg, and Lys-Tyr-Lys at pH 6.8 were 0.031 ± 0.012, 0.017 ± 0.006, 0.022 ± 0.01, 0.003 ± 0.0006, 0.189 ± 0.006, and 0.0152 ± 0.012 mM/h, respectively. The rate constant gradually increased with increase of pH from 6.8 to 10, where the amount of the ionized phenolic species of tyrosine increased. This finding can be attributed to the fact that the ionized form of tyrosine is a stronger absorbing group[17] and possesses higher quantum yield than the unionized form in the oxidation reaction.[18]

Furthermore, higher reaction rate was observed in Tyr-Arg, Lys-Tyr-Lys, and tyrosine than in Gly-Tyr, Glu-Tyr, and N-Ac-Tyr. The difference in reaction rate among the tyrosyl compounds appears related to the variability of their pK_a,

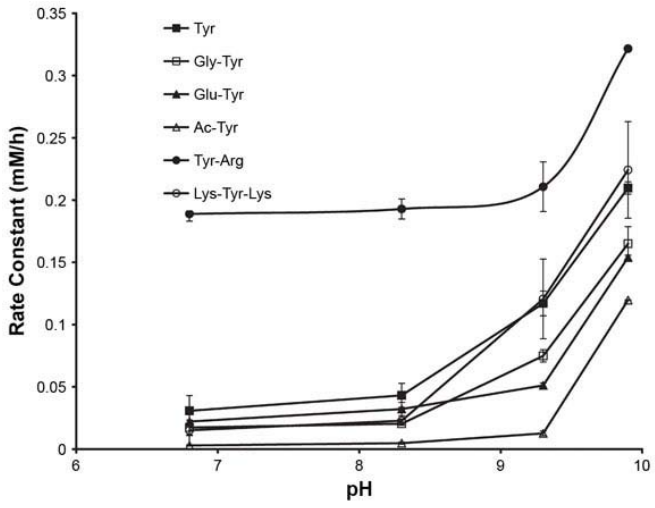

Figure 2. The effect of pH on the rate of photo-oxidation of model tyrosyl compounds by simulated sunlight (irradiance = 760 W/m^2).

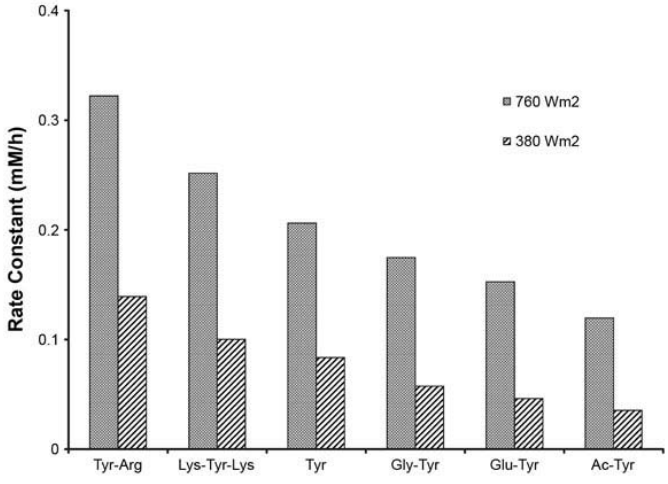

Figure 3. Effect of light intensity on the rate of photo-oxidation of model tyrosyl compounds (2 mM) in 0.01 M NH_4Ac/NH_4OH buffer (pH 10).

which is induced by differently charged neighboring groups. The phenolic side chain is in a more positively charged environment in Tyr-Arg, Lys-Tyr-Lys, and tyrosine than in Gly-Tyr, Glu-Tyr, and N-Ac-Tyr. There are more ionized phenolic species of Tyr-Arg, Lys-Tyr-Lys, and tyrosine present at the same pH than those of Gly-Tyr, Glu-Tyr, and N-Ac-Tyr. Hence, Tyr-Arg, Lys-Tyr-Lys, and tyrosine were more reactive than Gly-Tyr, Glu-Tyr, and N-Ac-Tyr. Tyr-Arg was extremely unstable toward photo-oxidation even at pH 6.8. This was possibly a result of the stabilization of the ionized tyrosyl group by Arg side chain. The stabilization will decrease the pK_a of the tyrosyl OH and result in a much larger fraction of ionized species at pH 6.8, which is susceptible to oxidation. The measured tyrosine pK_a was 0.7 units lower in Tyr-Arg than in Tyr (Zhang J., Radhakrishnan V., and Kalonia D.S., unpublished data, October 1996).

Effect of Light Intensity and Nitrogen Purge on the Kinetics of Photo-oxidation

Feitelson et al[30] studied the effect of flash intensity on the photo-ionization of p-cresol and tyrosine and found that the yield of phenoxyl radicals was directly proportional to the light intensity at pH 11. Shimizu[31] reported that at pH 3.5 with 283 nm excitation, the rate of reaction was directly proportional to light intensity. The experiments in the present study were conducted with steady-state photolytic exposure with a broad spectrum of sunlight. It demonstrated a similar effect of light intensity on oxidation rate as shown in Figure 3. The intensity of light stress had a significant effect on the rate of photo-oxidation. By lowering the light intensity from 760 to 380 W/m^2, the zero-order rate constant was proportionally reduced for all the model tyrosyl compounds. In addition, the elimination of dissolved oxygen by purging the peptide solutions with nitrogen, significantly slowed down photo-oxidation (Figure 4). Under de-aerated conditions, the variation among the tyrosyl compounds diminished. This finding is possibly because the rate-limiting step of the photoreaction is the excitation of oxygen from the ground state to singlet state. It has been shown that the subsequent step, where tyrosine is oxidized by the singlet oxygen is very fast with the reaction rate constant of $0.8 \times 10^7\ M^{-1}s^{-1}$.[32] When the solution was purged with nitrogen, the concentration of dissolved oxygen became small.

Effect of Surfactants on Photo-oxidation

The experiment with surfactants demonstrates that, 0.5% cationic cetyltrimethylammonium chloride significantly increased the rate of photo-oxidation of tyrosine, Gly-Tyr, Glu-Tyr, and N-Ac-Tyr. The reaction rate of Tyr-Arg and Lys-Tyr-Lys was not influenced by the surfactant. This is indicative of the electrostatic interaction between the cationic surfactant and tyrosine, Gly-Tyr, Glu-Tyr, and N-Ac-Tyr. The interaction may result in lower tyrosyl pK_a and, in turn, a higher rate of photo-oxidation. The interaction of cetyltrimethylammonium chloride with the positively charged Tyr-Arg and Lys-Tyr-Lys is weak due to electrostatic repulsion, therefore no effect of the surfactant on reaction rates was observed for these 2 peptides. In addition, the variation in reaction rates among the model peptides decreased in the presence of cetyltrimethylammonium chloride, which suggests that the intermolecular interaction between the cationic surfactant and the model peptides dominates the intramolecular charge effect between tyrosyl and neighboring groups. The anionic sodium lauryl sulfate and non-ionic polysorbate

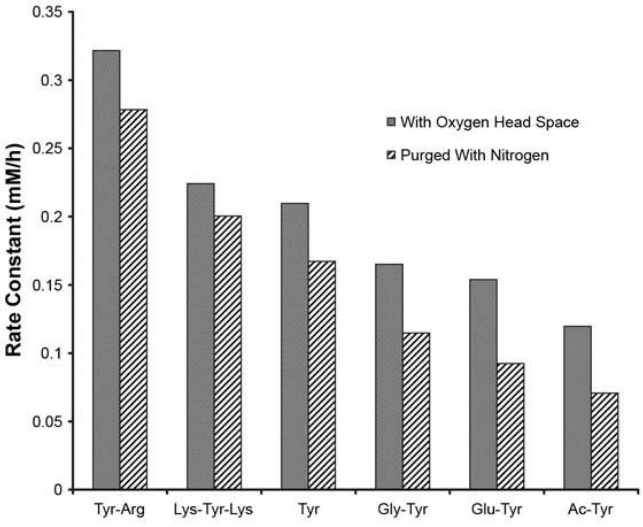

Figure 4. Effect of nitrogen purging on the rate of photo-oxidation of model tyrosyl compounds (2 mM) in 0.01 M NH_4Ac/NH_4OH buffer (pH 10, irradiance = 760 W/m^2).

80 did not significantly affect the rate of oxidation, although the oxidation of tyrosine and Lys-Tyr-Lys appeared slightly sensitized by polysorbate 80, possibly because of the presence of peroxide species in the surfactant.

Metal-Catalyzed Oxidation of Model Tyrosyl Peptides

The metal-catalyzed oxidation of amino acids is thought to involve the formation of HO· radical by means of the Fenten reaction:

$$H_2O_2 + Fe^{2+}(Cu^{+}) \rightarrow Fe^{3+}(Cu^{2+}) + OH^{-} + HO\cdot \quad (1)$$

The kinetics of tyrosine oxidation by the hydrogen peroxide (0.15%)/Cu^{2+} (25 μM) system differed from the one observed in photo-oxidation. Metal-catalyzed oxidation at pH 6.8 was faster and followed apparent first-order kinetics, where the logarithmic concentration vs time profile is linear. This observation suggests that the initiation step of the chain reaction (ie, creation of the phenolic radical by metal ions) may be rate limiting. A complete kinetics model is difficult to propose because the reaction can involve many radical species and propagate via many steps. Nonetheless, the overall reaction rate constants were calculated from the slope of the logarithmic concentration vs time profile.

As shown in Figure 5, the rate of metal-catalyzed oxidation varies among the peptides depending on which amino acid residues are located adjacent to tyrosine. However, the effect of neighboring groups on this reaction was almost opposite to what was observed in photo-oxidation. The negatively charged Glu-Tyr and N-Ac-Tyr underwent faster degradation than the other tyrosyl compounds: the positively charged Tyr-Arg and Lys-Tyr-Lys were the least degraded. A possible explanation is that the negative carboxyl groups on Glu-Tyr and N-Ac-Tyr were attractive to the metal ion, which facilitated the Fenton chemistry to occur in close proximity of the molecules. The positive side-chains of Tyr-Arg and Lys-Tyr-Lys were repulsive to the metal ion and the oxidation was disfavored.

The experiments also revealed a general trend that the rate of metal-catalyzed oxidation increased with pH. However, the correlation of phenolic ionization with reaction rate was not as good as in photo-oxidationH H . The deviation may be attributed to several factors: (1) precipitation of metal ions at alkaline pH, (2) the neighboring side-chains had opposite effect on metal binding to phenolic ionization, and (3) changes in redox potential with pH in metal-catalyzed oxidation. In a different experiment, where the solutions of Tyrosyl peptides were irradiated by simulated sunlight at 760 W/m^2 in the presence of 25 μM Fe^{3+} or Cu^{2+}, no acceleration in the reaction rate was observed. This indicates that Fenton chemistry does not occur in photo-oxidation, and tyrosine is oxidized mainly by photo-ionization and singlet oxygen.

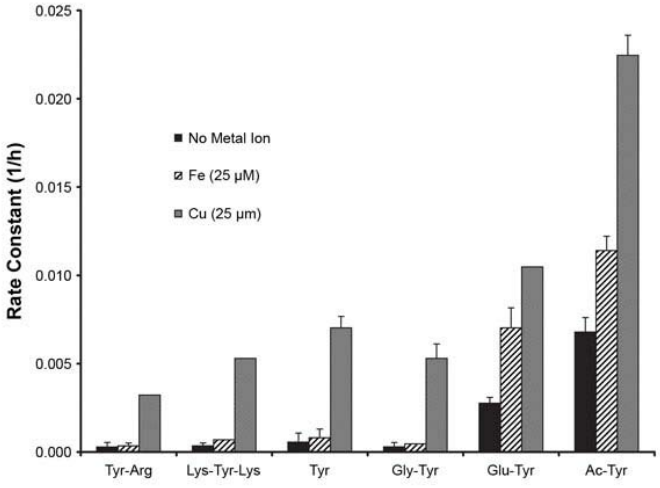

Figure 5. Effect of Fe^{3+} and Cu^{2+} (25 μM) on the oxidation rate of model tyrosyl compounds (2.0 mM) in 0.01 M NH_4Ac buffer (pH 6.8) by 0.15% H_2O_2.

Effect of Metal Ion Concentration and Type on the Kinetics of Metal-Catalyzed Oxidation of Model Tyrosyl Peptides

Tyrosyl residue in model peptides was quite stable in the presence of 0.15% hydrogen peroxide alone at pH 6.8. No significant degradation was observed within 24 hours except for Glu-Tyr and N-Ac-Tyr, which oxidized to a small extent. The rate of oxidation was significantly enhanced by the presence of 25 μM Cu^{2+}. Glu-Tyr and N-Ac-Tyr were the most liable tyrosyl compounds under this reaction condition because of the electrostatic attraction between these 2 peptides and Cu^{2+}. Tyr-Arg and Lys-Tyr-Lys were positively charged and repulsive to Cu^{2+}, hence they were oxidized at a slower rate. When the concentration of Cu^{2+} ions increased from 25 μM to 100 μM, the reaction rate further increased 2- to 4-fold depending on the peptides. In the presence of 100 μM Cu^{2+}, even the positively charged Tyr-Arg and Lys-Tyr-Lys were extensively oxidized with their reaction rate constants increased to almost the same level as the one of Glu-Tyr. This finding suggests that 100 μM Cu^{2+} dramatically promoted the Fenton reaction and produced so many hydroxyl radicals that the interaction between the carboxyl groups and Cu^{2+} ion no longer exerted a dominant effect on the reaction.

A comparison of catalytic efficiency between Cu^{2+} and Fe^{3+} is illustrated in Figure 5. It was shown that both Cu^{2+} and Fe^{3+} were able to catalyze Fenton reaction and increase the rate of tyrosine oxidation. At the same concentration of 25μM, Cu^{2+} ion was a better catalyst than Fe^{3+} ion as higher reaction rates was observed with Cu^{2+} than with Fe^{3+}.

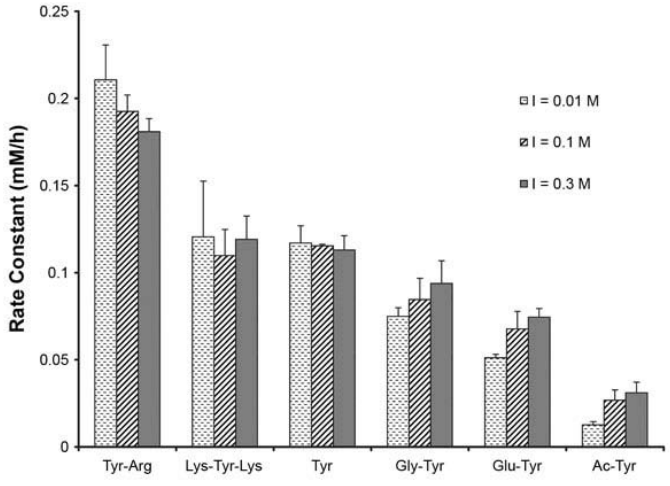

Figure 6. Effect of ionic strength on the rate of photo-oxidation of model tyrosyl compounds (2mM) in 0.01 M NH_4Ac/NH_4OH buffer (pH 9.3, irradiance = 760 W/m^2).

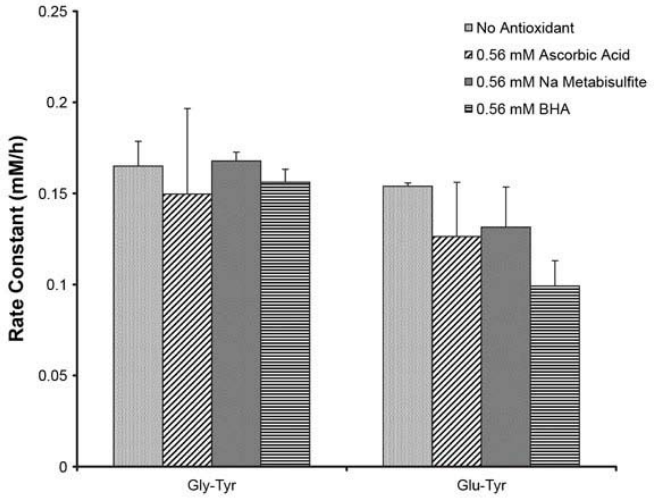

Figure 8. Effect of antioxidants (0.56 mM) on the rate of photo-oxidation of Gly-Tyr and Glu-Tyr (2 mM) in 0.01 M NH_4Ac/NH_4OH buffer (pH 10, irradiance = 760 W/m^2). BHA indicates butylated hydroxyanisole.

Effect of Ionic Strength

Figure 6 shows the effect of ionic strength on the rate of photo-oxidation. The rate constant of Tyr-Arg decreased slightly, whereas the rate constants of Gly-Tyr, Glu-Tyr, and N-Ac-Tyr increased with increase in ionic strength from 0.01 M to 0.3 M. In other words, the difference in the reaction rate among the tyrosyl compounds was reduced as the ionic strength of the solution increased. This result can be attributed to the shielding effect of high salt concentration, which reduced the electrostatic interaction between the tyrosyl phenolic and the adjacent charged groups. This interaction is postulated as the main cause of variation in tyrosyl pK_a and the rate of photo-oxidation. Nevertheless, the effect of ionic strength on the rate of photo-oxidation was small.

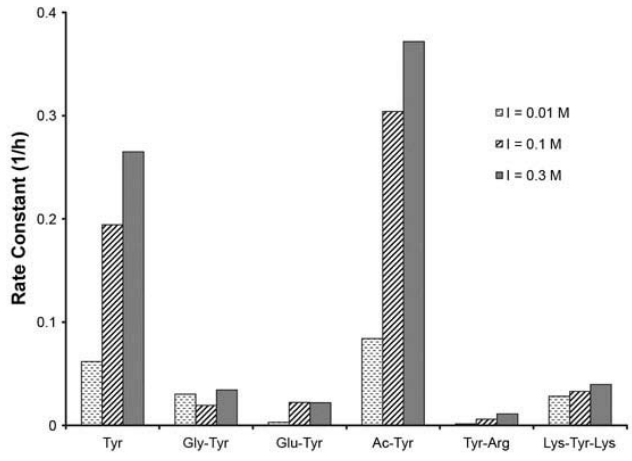

Figure 7. Effect of ionic strength on the rate of Cu^{2+} (100μM) catalyzed oxidation of model tyrosyl compounds (1.2 mM) in 0.01 M NH_4Ac buffer (pH 6.8) with 0.15% H_2O_2.

Unlike in photo-oxidation, ionic strength had a more significant effect on the kinetics of metal-catalyzed oxidation of the tyrosyl compounds. The reaction was accelerated as demonstrated by the 1- to 4-fold increase in the first-order rate constants when ionic strength increased from 0.01 M to 0.3 M (Figure 7). The increase in reaction rate was especially dramatic for tyrosine and N-Ac-Tyr, possibly because they are smaller molecules than the other model peptides and the phenolic side-chains are more accessible to the oxidizing species in the solution.

Effect of Antioxidants

Figure 8 compares the rate constants of photo-oxidation of Gly-Tyr and Glu-Tyr in the presence and absence of ascorbic acid, sodium metabisulfite, and BHA as antioxidants. The data indicated that the antioxidants are essentially ineffective in protecting Gly-Tyr and Glu-Tyr from photo-oxidation.

Figure 9 reveals the effect of the antioxidants on the Cu^{2+}-catalyzed oxidation of Gly-Tyr and Glu-Tyr. Ascorbic acid and sodium metabisulfite were not only ineffective, but also accelerated the degradation of Gly-Tyr and Glu-Tyr. This pro-oxidation effect by the reducing agents is consistent with the previous observation in the oxidation of other amino acids.[26,33,34] Ascorbic acid and sodium metabisulfite are electron donors that can accelerate Fenton reaction by reducing the valance of the metal ion, which subsequently generates reactive oxygen radicals by providing an electron and returns to its original valance.[5,26] On the contrary, BHA functioned by inhibiting or interfering with the chain reaction. The reactive free radical species were consumed and terminated by BHA, which was converted to a stable radical

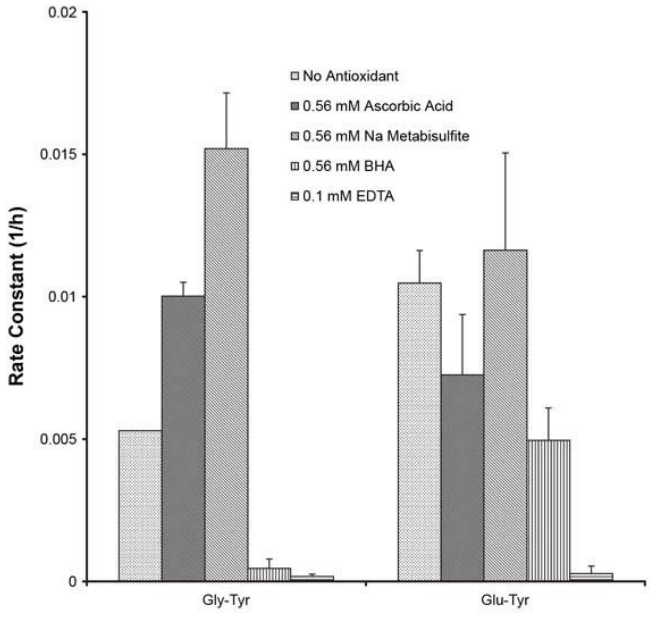

Figure 9. Effect of antioxidants (0.56 mM) on the rate of Cu^{2+} (25μM) catalyzed oxidation of Gly-Tyr and Glu-Tyr (2.0 mM) in 0.01 M NH_4Ac buffer (pH 6.8) with 0.15% H_2O_2. BHA indicates butylated hydroxyanisole.

that could not further propagate because of the steric hindrance of the bulky butyl group. EDTA sequestered the metal ions in the bulk solution and prohibited the catalysis to occur. The study showed that 100 μM EDTA completely inhibited the oxidation reaction catalyzed by 25 μM Cu^{2+}. This result confirmed previous findings that metal-catalyzed oxidation could be prohibited by EDTA at greater than the amount of metal ion present.[10] No catalytic effect of EDTA on the reaction was observed.

By-Products of Tyrosine Oxidation

Photo-oxidation of tyrosine in model dipeptides and tripeptides was nonspecific, a variety of degradation products formed depending on the characteristics of the peptide sequence. Monohydroxyl, dihydroxyl, trihydroxyl, and tetrahydroxyl by-products were observed in the photo-oxidation of tyrosine, Tyr-Arg, and Lys-Tyr-Lys. If tyrosine was connected to glycine residues and the negatively charged glutamic acid or had its amino terminus acetylated, hydroxylation was retarded. Negligible level of degradants was produced in photoreaction as a result of peptide bond breakage. In metal catalyzed oxidation, the reaction yielded mainly monohydroxyl by-products. Several monohydroxyl were detected with Gly-Tyr, Glu-Tyr, and Tyr-Arg. Oxidation was confirmed occurring on the tyrosine residue by mass spectrometry. The monohydroxyl differed possibly in the location of hydroxylation, either the para- or meta- positions of the phenolic ring or the aliphatic carbon of the tyrosine side-chain could potentially be targeted by the hydroxyl radicals. However, the single quadruple mass spectrometer used in this study could not distinguish these structural differences. Two ketone by-products were detected from N-Ac-Tyr. The carbonyl bond was formed when a hydrogen atom was abstracted from the monohydroxyl by-products.

CONCLUSIONS

Tyrosine and tyrosyl peptides (Gly-Tyr, Glu-Tyr, Tyr-Arg, Lys-Tyr-Lys, and N-Ac-Tyr) are susceptible to photo-oxidation and metal-catalyzed oxidation in aqueous solutions. The positive charges on the neighboring residue increased the rate of tyrosine photo-oxidation, while the rate of oxidation decreased if tyrosine was connected to neutral and the negatively charged residues. Unlike photo-oxidation, adjacent negative amino acids accelerated the metal-catalyzed oxidation of tyrosine; positive amino acid residues disfavored the reaction. The oxidation kinetics of tyrosine also depends on the solution conditions such as pH, ionic strength, and oxygen headspace, and the type and concentration of metal ions and other adjuvants present.

ACKNOWLEDGMENTS

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