Role of neighbouring amino acid residue in horseradish peroxidase-catalyzed *o,o'*-dityrosine crosslink in Tyrosyl dipeptides

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Dityrosine is a fluorescent biomolecule and it is a specific biomarker for oxidative stress. Though dityrosine cross link provides structural rigidity to proteins, increased level of dityrosine crosslink is associated with numerous diseased states. It is important to find out whether the local environment of Tyr has any role in determining the rate of formation of dityrosine. A detailed study has been done with substrates as C-terminal Tyr-containing dipeptides of all possible combinations. In the HRP-catalyzed oxidation of L-Tyr-containing dipeptides with H_2O_2, the rate of dimerization is higher for all dipeptides compared to that of free L-Tyr. When the neighbouring amino acid residue is a positively charged amino acid residue like Lys or Arg, the enhancement in rate of formation of corresponding dityrosine has been observed. With the negatively charged amino acid residues like Glu or Asp as neighbouring amino acid residues, the decrease in rate has been observed. However, no dityrosine formation has been observed with Cys-Tyr. Instead, the formation of disulfide (Tyr-Cys-Cys-Tyr) has been observed. The enhancement in rate has been observed with externally added L-Arg. The oxidation trend is likely to follow the pK_a of neighbouring amino acid residue. As the pK_a of L-Arg and L-Lys are higher, the rate of dityrosine formation is higher for dipeptides Arg-Tyr and Lys-Tyr. The decrease in rate of dityrosine formation of peptides Asp-Tyr and Glu-Tyr are attributed to the lower pK_a of Asp and Glu, respectively. However, the appreciably higher rate of dityrosine formation of Phe-Tyr is attributed to the presence of strong hydrophobic environment around the active site of HRP.

Introduction

o,o'-Dityrosine is one of the oxidative products of L-tyrosine. A couple of tyr-derivatives formed under oxidative/nitrative conditions are shown in Figure 1. *o,o'*-Dityrosine is a fluorescent molecule and is formed as a result of normal post-translational process. Unlike other oxidized products, dityrosine is resistant to proteolysis which makes it as a specific biomarker for oxidative stress.[1] In dityrosine, the *ortho*-position of two tyrosine units is linked by the formation of a stable covalent C-C linkage. It shows characteristic UV absorbance at 320 nm in physiological pH and at 285 nm in acidic pH.[2] When excited at 320 nm, it shows characteristic fluorescence emission at 410 nm.

Dityrosine cross-links are commonly observed in structural and insoluble proteins. It provides structural rigidity and elasticity to proteins and hence it is found in the cell wall of insects. The natural existence of dityrosine has been widely observed from skin protein of arthropods to prothyroid hormone.[3] In addition to natural existence, the formation of dityrosine has been induced in several structural and non-structural proteins. Peroxidases like HRP, LPO, TPO, CIP and MPO are known to catalyze the oxidation of tyrosine to dityrosine with H_2O_2.[4] The reactive oxygen species (ROS),[5] reactive nitrogen species (RNS),[6] ozone treatment[7] and radiation exposure[4f,8] are known to induce dityrosine formation. The oxidizing agents like potassium hexacyanoferrate(III), vanadium(V)-oxyfluoride and phenyliodine(III)-bis(trifluoroacetate) are also known to induce dityrosine formation.[9] To detect dityrosine in proteins, antibodies like G6 are employed. Dityrosine cross link based biomaterials are also widely reported.[10]

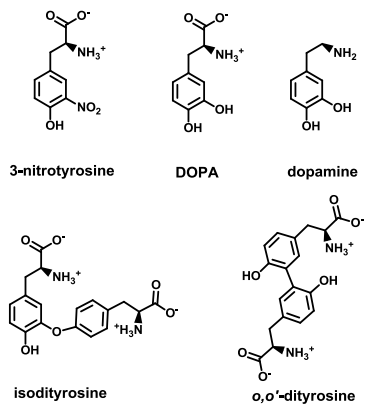


Figure 1 Tyrosine derivatives formed under oxidative conditions.

Although dityrosine is formed as a result of normal post-translational process, increase in levels of dityrosine is associated with the number of diseased states like eye-cataracts, atherosclerosis and neurodegenerative diseases like Alzheimer's and Parkinson's.[8g,11] The aggregations of a few Tyr-containing peptides/proteins in a couple of diseased states are listed in Table 1.

There are a couple of reports in the literature dealing with the role of neighbouring amino acid residues in the iodination/ phosphorylation/ nitration and oxidation of tyrosine in tyrosyl peptides. The LPO-catalyzed iodination of different tyrosine-containing dipeptides have been studied.[12] It has been observed that

in all cases, the rate of iodination of tyrosine in dipeptides was less compared to that of free L-Tyr. Among the dipeptides, the rate of iodination is much faster in the dipeptides where the tyrosine residue is in C-terminal compared to that of N-terminal. However it is reverse in the case of dipeptides of glutamic acid. The rate of iodination is as follows at neutral pH : Tyr > Tyr-Gly = Tyr-Ala ≥ Tyr-Val = Tyr-Leu = Tyr-Phe = Phe-Tyr > Leu-Tyr = Val-Tyr = Ala-Tyr ≥ Gly-Tyr. Dipeptides of lysine also follow the same trend: Tyr > Tyr-Lys > Lys-Tyr. The trend is reverse in the case of glutamic acid containing dipeptides as Tyr > Glu-Tyr ≥ Tyr-Glu. Unlike LPO-catalyzed iodination, in the case of chemical modification, Gly-Tyr is readily iodinated compared to that of free L-Tyr.[13] Moreover, HRP does not catalyze the iodination of any of the tyrosine containing peptides or tyrosine under the experimental conditions employed. Hence it is clear that the rate of iodination not only depends on the nature of the neighbouring amino acid residue, but also on the iodinating species and the enzyme catalyzing these reactions.

Table 1 Aggregation of Tyr-containing proteins/peptides in diseased states

Protein	No. of Tyr residues	Disease associated due to aggregation
Amyloid-β-peptide	1	Alzheimer's disease
α-Synuclein	4	Parkinson's disease
Human lens Crystallin protein		
α-Crystallin-A	6	Eye cataract
α-Crystallin-B	2	

Even for phosphorylation of Tyr, the peptide sequence around the tyrosine residue plays a key role. The presence of acidic amino acid residues like aspartic acid and glutamic acid, adjacent to Tyr provides substrate specificity to tyrosine kinases.[14]

For nitration of L-tyrosine, there is no specific amino acid sequence criteria exist. However, it has been suggested that the presence of negatively charged amino acid residues namely aspartic acid or glutamic acid in close proximity to Tyr residue enhances nitration, whereas the presence of cysteinyl or methionine residues in close proximity decreases Tyr-nitration.[15]

Considering the oxidation of tyrosine, there are a couple of reports dealing with the chemical environment around tyrosine. The products of oxidation range from DOPA, TOPA, dityrosine, isodityrosine, trityrosine, pulcherosine and insoluble polymers. Free Tyr and a couple of Tyr-containing peptides of varying chain lengths have been subjected to oxidation. The oxidation trend largely depends on the nature of the oxidant employed. Different groups have employed different oxidants, different Tyr-containing peptides and obtained different trends for the oxidation of tyrosine. Bayse *et al* have studied the oxidation of free Tyr in both the isomeric forms L- and D catalyzed by peroxidases HRP and LPO in the presence of H_2O_2.[4a] Dityrosine is the major product formed under the initial rate conditions. It has been observed that LPO- preferentially catalyzes the oxidation of L- isomer more readily than D-isomer. However HRP preferentially oxidizes D-isomer.

Eickhoff *et al* have studied the oxidation of simple Tyr-derivatives with different oxidizing agents like potassium hexacyanoferrate(III), vanadium(V)-oxyfluoride, phenyliodine(III)-bis(trifluoroacetate) and HRP/H_2O_2.[9] The formation of both dityrosine (biphenyl linkage, C_o-C_o coupling) and isodityrosine (biphenyl ether linkage, C_o-O coupling) were observed with all oxidants employed except HRP/H_2O_2. HRP/H_2O_2 produces dityrosine almost exclusively. The formation of dityrosine as an exclusive product has also been observed in the Tyr- containing linear and cyclic peptides with HRP/H_2O_2. This regioselectivity is attributed to the complex association of peptides themselves or their association with the peptidic environment of HRP.

Tien has studied MPO-catalyzed oxidation of free L-Tyr and a few Tyr-containing dipeptides and tripeptides in the presence of H_2O_2.[16] The rate of dityrosine formation was found to be inversely proportional to the length of the peptide chain. This observation was attributed to the less accessibility for the substrates of longer chain to the active site of MPO. The enzyme MPO is found to be inhibited by Cys and Glutathione (GSH) but not by Met. Hence the presence of Cys residue may provide a partial protection of proteins from cross-linkage. Steffensen *et al* studied the cross linking of a few tyrosyl peptides with H_2O_2 catalyzed by CIP.[4e] The rate and degree of polymerization have been again found to be dependent on chain length and bulkiness of neighbouring amino acid residue.

Metal-catalyzed oxidation systems like Cu^{2+}/H_2O_2 is also known to induce dityrosine formation. In 2007, Zhang *et al* have studied the effect of charged amino acid residues adjacent to tyrosine in small peptides.[17] The oxidation of these Tyr-containing peptides was carried out by both metal catalyzed system and photo-oxidation. However, the products of the oxidation have not been characterized. In the metal catalyzed systems, negatively charged neighboring residues enhance the oxidation of tyrosine, due to the affinity of negative charges to positive metal ions. In metal-catalyzed systems, positively charged neighbouring residues decrease the rate of Tyr-oxidation. However, in case of photo-oxidation, positively charged amino acid residues enhance the rate of Tyr-oxidation owing to their increased stability for ionized Tyr. In photo-oxidation, negatively charged amino acid residues decrease the rate of Tyr-oxidation.

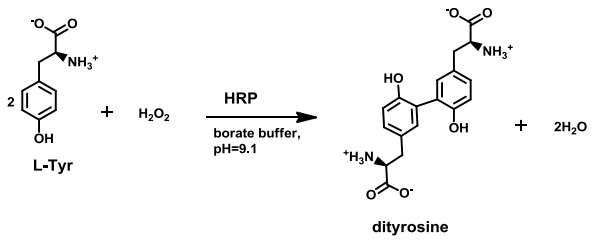
Although these earlier reports provide some basic idea about Tyr-oxidation, there is no complete study with a single oxidizing system. As the trend is found to be dependent on the nature of the oxidizing system, the objective of our study is to find out the role of neighbouring amino acid residue in Tyrosyl peptides with a particular oxidizing system. We have chosen HRP/H_2O_2 system as it almost exclusively produce dityrosine under initial rate conditions. Dipeptides of the type X-Tyr, where X represents all proteinogenic amino acids other than Tyr are used as substrates.

Results and Discussion

Horseradish peroxidase catalyzed dityrosine formation-Fluorometric study

As dityrosine is highly fluorescent, we have followed the formation of dityrosine by fluorometric study as reported in the literature.[18] Free L-Tyr and Tyrosyl peptides have been treated with HRP/H_2O_2 in borate buffer at pH 9.1 (Scheme 1 and 2). Only 0.5 equivalent of H_2O_2 with respect to L-Tyr or tyrosyl peptides have been used in order to avoid further oxidation. The reaction has been carried out in basic pH as the formation of dityrosine and hence the change in fluorescent intensity is maximum at pH ~ 9.[18] When excited at 320 nm, dityrosine shows a characteristic fluorescent emission at 408 nm. As the reaction proceeds the increase in F. I. due to the formation of dityrosine has been observed. Initially, the F. I. corresponding to the formation of dityrosine increases linearly with

time and it saturates over time (Figure 2). The change in F. I. /min under initial rate conditions has been determined.



Scheme 1 Formation of dityrosine from free L-Tyr in the presence of H_2O_2 catalyzed by HRP.

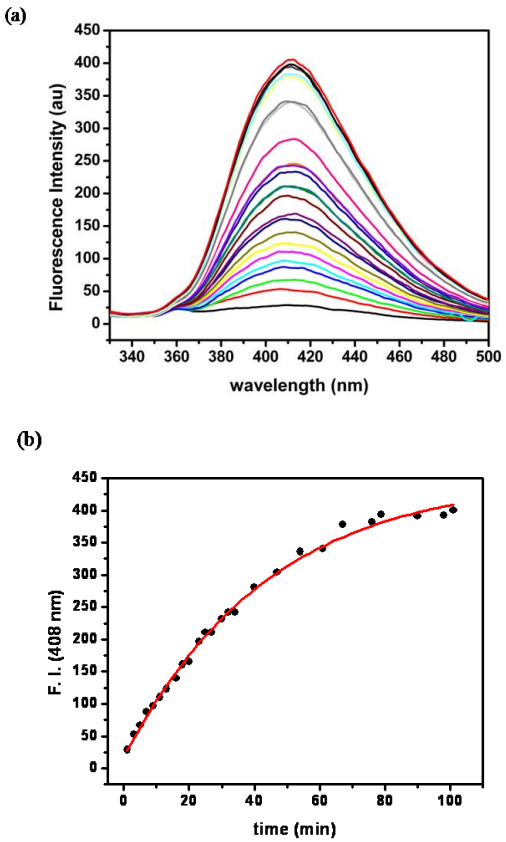
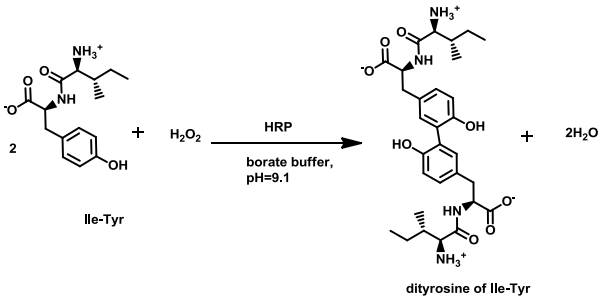


Figure 2 Fluorometrically monitored time courses showing HRP-catalyzed conversion of Tyr to dityrosine. The reaction mixture contained 2.5 mM L-Tyr, 1.2 mM H_2O_2, 0.25 µM HRP in 0.1 M boric acid-sodium borate buffer, pH=9.1. For fluorescent measurement samples were diluted 500 fold with 0.1 M sodium bicarbonate, pH=9.7. Excitation, 320 nm; and Emission, 408 nm. (a) Wavelength scan (b) Time scan.

We have also extended this study to a couple of Tyr – containing peptides. With respect to time the increase in formation of dityrosine of Ile-Tyr is shown in Figure 3. The formation of dityrosines of different tyrosyl dipeptides are shown in Figure 4. The change in F. I. /min for the formation of dityrosine of different tyrosyl peptides is shown in Table 2. The change in F. I. / min is different for different peptides. Some residues are likely to favor dityrosine formation, whereas some other residues disfavour this reaction. For example the change in F. I./min and hence the formation of dityrosine is less for negatively charged neighbouring residues as compared to that of other residues. An interesting analogy between Leu and Ile has also been observed. Although Leu and Ile are isomers, the change in F. I. /min for the formation of corresponding dityrosine for Leu-Tyr is almost 2-3 times higher than that of Ile-Tyr. This suggests that the residue adjacent to Tyr may have some influence in dityrosine formation.



Scheme 2 Formation of dityrosine of Ile-Tyr from Ile-Tyr in the presence of H_2O_2 catalyzed by HRP.

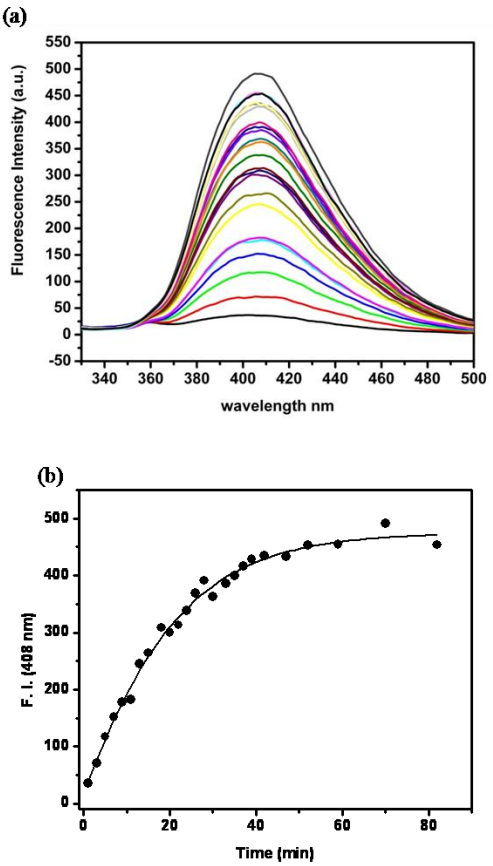


Figure 3 Fluorometrically monitored time courses showing HRP-catalyzed conversion of Ile-Tyr to its corresponding dityrosine. The reaction mixture contained 2.5 mM Ile-Tyr, 1.2 mM H_2O_2, 0.25 µM HRP in 0.1 M boric acid-sodium borate buffer, pH=9.1. For fluorescent measurement samples were diluted 500 fold with 0.1 M sodium bicarbonate, pH=9.7. Excitation, 320 nm; and Emission, 408 nm. (a) Wavelength scan (b) Time scan.

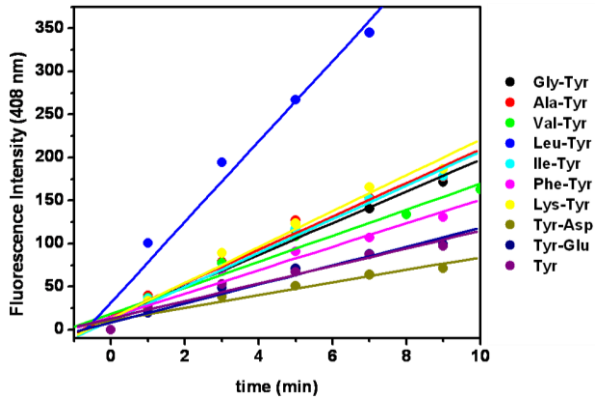


Figure 4 Fluorometrically monitored time courses showing HRP-catalyzed conversion of different tyrosyl peptides to their corresponding dityrosines. The reaction mixture contained 2.5 mM tyrosyl peptide, 1.2 mM H_2O_2, 0.25 µM HRP in 0.1 M boric acid-sodium borate buffer, pH=9.1. For fluorescent measurement samples were diluted 500 fold with 0.1 M sodium bicarbonate, pH=9.7. Excitation, 320 nm; and Emission, 408 nm.

Table 2 The change in fluorescent intensity per min corresponding to the formation of dityrosine of different Tyrosyl peptides is shown. The reaction mixture contained 2.5 mM tyrosyl peptide, 1.2 mM H_2O_2, 0.25 µM HRP in 0.1 M boric acid-sodium borate buffer, pH=9.1. For fluorescent measurement samples were diluted 500 fold with 0.1 M sodium bicarbonate, pH=9.7. Excitation, 320 nm; and Emission, 408 nm.

Peptides	change in F. I. /min
Tyr	7.28±0.10
Gly-Tyr	16.38±0.34
Ala-Tyr	17.33±0.25
Val-Tyr	13.88±0.18
Leu-Tyr	40.83±0.68
Ile-Tyr	16.06±0.04
Phe-Tyr	11.75±0.30
Lys-Tyr	18.09±0.03
Tyr-Asp	5.69±0.09
Tyr-Glu	8.54±0.31

Horseradish peroxidase catalyzed dityrosine formation-HPLC study

Although the change in F. I. /min for different tyrosyl peptides shows some trend, it does not provide the exact rate for dityrosine formation. In order to find out the rate, the reaction has been followed using HPLC. The reaction has been carried out in phosphate buffer at physiological pH of 7.5. The decrease in concentration of L-Tyr or tyrosyl peptides has been followed with respect to time (Figure 5). The fraction corresponding to dityrosine has been isolated and characterized by mass spectrometry and ^{1}H NMR. With some of the tyrosyl peptides the formation of a small amount of trityrosine has also been observed along with the formation of dityrosine. The plot of concentration of Ala-Tyr with time upon formation of corresponding dityrosine is shown in Figure 6. From the slope of this plot, the initial rate has been determined. The initial rate for the formation of dityrosine of different tyrosyl peptides are shown in Table 3. The bar diagram showing the initial rate for the formation of dityrosine of different tyrosyl peptides is shown in Figure 7. Irrespective of the nature of the neighbouring amino acid residue, all tyrosyl peptides show higher initial rate compared to free L-Tyr.

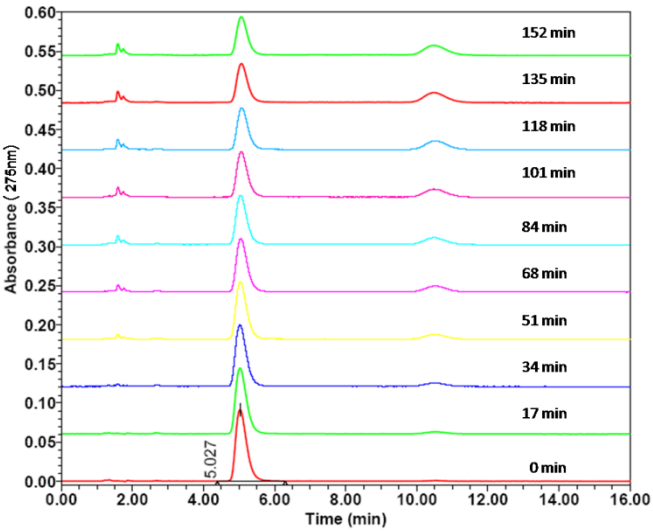


Figure 5 Stack plot of HPLC chromatogram showing the formation of dityrosine from L-Tyr over a period of time. The reaction mixture contained 2.13 mM L-Tyr, 1.22 mM H_2O_2, 0.06 µM of HRP in 0.1 M phosphate buffer, pH=7.5 at 20 °C.

Among the aliphatic amino acid residues no general trend is observed in the formation of dityrosine. However, the analogy between Leu and Ile is again observed as in the case of fluorescent studies. Although Leu and Ile are isomers, the initial rate of dityrosine formation with Leu-Tyr is almost 2-3 times higher than that of Ile-Tyr. The dipeptide Phe-Tyr shows appreciably higher rate for corresponding dityrosine formation. This is due to the presence of a strong hydrophobic environment in the vicinity of active site of HRP (Phe 68, Phe 142, Phe 179 and exposed heme edge with heme methyl C18H_3 and heme meso proton C20H).[19] However, Trp-Tyr shows only less rate for corresponding dityrosine formation as indole ring of Trp do undergo oxidation during the reaction. When the neighbouring amino acid residue is Cys, the formation of corresponding dityrosine has not been observed. Instead, the formation of disulfide corresponding to Tyr-Cys-Cys-Tyr has been observed. This is in accordance with the literature where the MPO/H_2O_2 catalyzed oxidation of L-Tyr to dityrosine is inhibited by externally added Cys.[16] Even nitration of Tyr-Cys has not been observed with MPO/H_2O_2/NO_2^- system. Instead the formation of corresponding disulfide is observed.[15b]

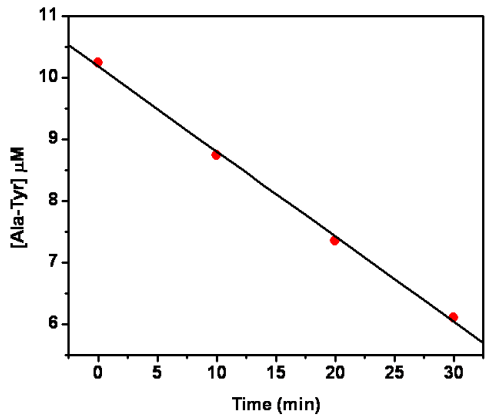


Figure 6 The plot showing the decrease in concentration of tyrosyl peptide, Ala-Tyr versus time upon formation of respective dityrosine. The reaction mixture contained 2.13 mM Ala-Tyr, 1.22 mM H_2O_2, 0.06 µM of HRP in 0.1 M phosphate buffer, pH=7.5 at 20 °C. The reaction was followed by reverse-phase HPLC and the amount of Ala-Tyr oxidized to dityrosine at a given time was calculated from the calibration plot.

Table 3 The initial rate corresponding to the formation of dityrosine of different Tyrosyl peptides is shown. The reaction mixture contained 2.13 mM L-Tyr, 1.22 mM H_2O_2, 0.06 µM of HRP in 0.1 M phosphate buffer, pH=7.5 at 20 °C.

Peptides	initial rate, v_0 (µM/min)[a]	Peptides	initial rate, v_0 (µM/min)[a]
Tyr	7.07±0.43	Cys-Tyr	-[b]
Gly-Tyr	49.7±1.17	Ser-Tyr	34.93±1.54
Ala-Tyr	26.45±1.34	Thr-Tyr	15.45±0.78
Val-Tyr	16.75±0.18	His-Tyr	24.36±1.06
Leu-Tyr	47.56±0.25	Lys-Tyr	43.02±0.62
Ile-Tyr	19.37±0.92	Arg-Tyr	77.67±0.58
Pro-Tyr	23.38±0.52	Asp-Tyr	11.72±0.86
Phe-Tyr	49.34±0.58	Glu-Tyr	12.66±0.86
Trp-Tyr	14.24±0.39	Asn-Tyr	25.44±1.65
Met-Tyr	34.48±0.31	Gln-Tyr	20.54±1.76

[a] Calculated from the initial 5-10% of the reaction by following the decrease in peak area due to tyrosine or tyrosyl peptides. [b] No dityrosine formation has been observed.

Among the amino acid residues having polar side chains as adjacent to Tyr, negatively charged amino acid residues like Asp and Glu decrease the formation of dityrosine whereas positively charged neighbouring residues like Arg and Lys enhances the formation of dityrosine. This scenario is likely to be correlated with the pK_a of side chains of amino acids. The pK_a of Arg side chain is 12.48, which is the maximum among the entire amino acids shows maximum rate for the formation of dityrosine. The amino acids Asp and Glu having lower pK_a values respectively as 3.65 and 4.25 show lower rate for dityrosine formation. This observation is in line with the study by Zhang *et al*.[17] In the photo-oxidation of Tyrosyl peptides, the positively charged residues enhanced the rate of oxidation whereas negatively charged residues decreased the rate of oxidation. However the products of the oxidation have not been characterized.[17] The pK_a of the phenolic –OH of tyrosine is 10.1. Tyr residues adjacent to positively charged residues are found to be more ionized than that in neutral and negatively charged residues. The positively charged residues provide stabilization to ionized tyrosine and ionized tyrosines are more susceptible to oxidation. The pK_a of Tyr side chain is found to be reduced by 0.7 units in Tyr-Arg than in free Tyr. Hence the initial rate of dityrosine formation of polar tyrosyl peptides is likely to follow the trend of pK_a of side chain of amino acids.

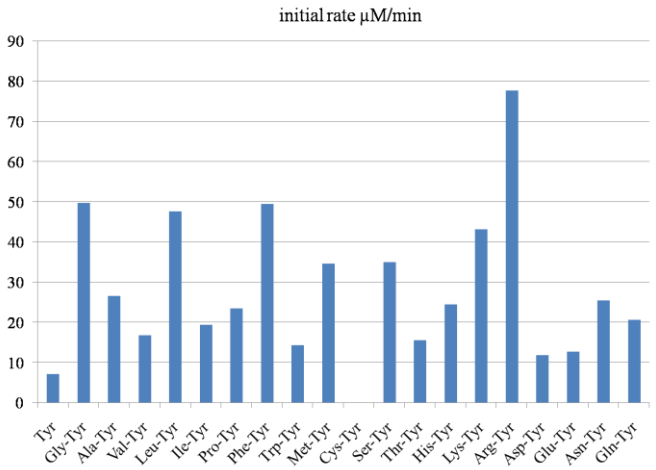


Figure 7 The bar diagram showing the initial rates for the formation of dityrosine of different Tyrosyl peptides.

Determination of kinetic parameters for dityrosine formation

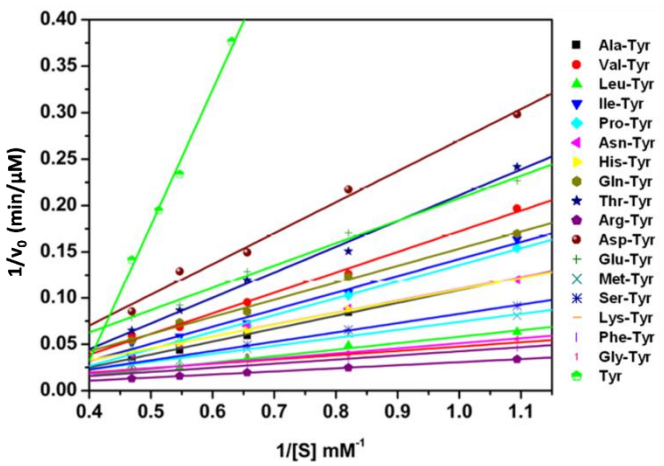


Figure 8 Lineweaver-Burk plots: The effect of substrate (Tyr or tyrosyl peptides) concentration in HRP (0.06 µM)-catalyzed oxidation of tyrosine or tyrosyl peptides in the presence of H_2O_2 (1.22 mM) in 0.1 M phosphate buffer, pH=7.5 at 20 °C.

In order to determine the kinetic parameters the reaction has been carried out with different concentrations of the substrates at pH 7.5. By keeping the concentration of HRP and H_2O_2 as constants, the initial rate (v_0) has been determined for each concentrations of the substrate (tyrosine or tyrosyl peptide). The kinetic parameters like maximum velocity (V_{max}), Michaelis constant (K_M), turn over number (k_{cat}) and catalytic efficiency (η) for the oxidation of Tyr or tyrosyl peptides has been determined (Table 4). Double-reciprocal plots (Lineweaver-Burk plots) of initial rate (v_0) vs concentration of

substrate give families of linear lines for all the substrates (Figure 8). The linear lines correspond to different substrates and indicate that the rates increase linearly with the concentration of the substrate. From the slope and intercept of each linear line, the kinetic parameters have been determined (Table 4).

Table 4 The kinetic parameters for the HRP (0.06 µM) -catalyzed oxidation of Tyr / tyrosyl peptides to dityrosine with H_2O_2 (1.22 mM) in 0.1 M phosphate buffer, pH=7.5 at 20 °C.

Substrate	V_{max} (µM/min)	K_M (µM)	k_{cat} (min^{-1})	η ($M^{-1} min^{-1}$) (k_{cat} / K_M)
Tyr	1.81	2.64	29.7	11.25×10^6
Gly-Tyr	314.47	17.03	5155.25	30.27×10^7
Ala-Tyr	32.62	4.54	534.69	11.78×10^7
Val-Tyr	19.89	4.43	326.11	73.61×10^6
Leu-Tyr	85.47	5.97	1401.15	23.47×10^7
Ile-Tyr	24.09	4.43	394.93	89.15×10^6
Pro-Tyr	21.67	3.95	355.22	89.92×10^6
Phe-Tyr	492.61	21.88	8075.57	36.91×10^7
Met-Tyr	84.03	7.23	1377.5	19.05×10^7
Ser-Tyr	58.11	5.81	952.6	16.4×10^7
Thr-Tyr	15.05	4.18	246.72	59.02×10^6
His-Tyr	55.56	7.07	910.75	12.88×10^7
Lys-Tyr	1000	46.0	16393.4	35.64×10^7
Arg-Tyr	396.83	13.18	6505.41	49.36×10^7
Asp-Tyr	8.51	3.63	139.47	38.46×10^6
Glu-Tyr	29.17	7.065	478.22	67.69×10^6
Asn-Tyr	53.59	6.88	878.53	12.77×10^7
Gln-Tyr	31.67	5.86	519.11	88.59×10^6

V_{max} represents the maximum rate attained by the system at saturating substrate concentrations. The substrates Lys-Tyr, Arg-Tyr and Phe-Tyr show higher V_{max} values whereas Asp-Tyr and Glu-Tyr exhibits lower V_{max} values for their oxidation to corresponding dityrosine. As observed in the case of initial rates for dityrosine formation, the V_{max} value is appreciably more for Ile-Tyr compared to that of Leu-Tyr. K_M is the Michaelis constant and represents the affinity of substrate towards the enzyme. The value of K_M is inversely proportional to the affinity of substrate towards the enzyme. K_M value is also dependent on conditions such as temperature and pH. Free L-Tyr shows the least K_M value whereas the substrates Lys-Tyr and Phe-Tyr show higher K_M values under the experimental conditions employed. k_{cat} represents the turn over number for the enzyme and represents the maximum number of substrate molecules converted to product by per enzyme per minute. For the substrate Lys-Tyr maximum k_{cat} value is observed. Among the polar neighbouring residues, negatively charged residues (Asp and Glu) show lower k_{cat} value compared to positively charged residues (Lys and Arg). For Phe-Tyr appreciably higher k_{cat} value is observed. The value, k_{cat}/ K_M is a measure of how efficiently an enzyme converts a substrate in to product. The highest k_{cat}/ K_M value is observed for Arg-Tyr whereas lower values are observed for Asp-Tyr and free L-Tyr. For the substrate Trp-Tyr the kinetic parameters could not be determined as the indole ring of tryptophan also undergoes oxidation during the reaction. The products of oxidation other than dityrosine have not been characterized.

Effect of L-Arginine in dityrosine formation

As the positively charged substrate Arg-Tyr show higher initial rate and k_{cat}/ K_M value, the effect of externally added L-Arg in the oxidation of free L-Tyr to dityrosine have been determined. As the concentration of L-Arg is increased the rate of dityrosine formation is also found to be increased gradually. However, with the further increase in concentration of Arg, the rate decreases (Figure 9). This trend can be explained as follows: The positively charged residue stabilizes the ionized tyrosine. However, with the excess of positively charged environment, the extent of ionization of Tyr itself could be hampered.

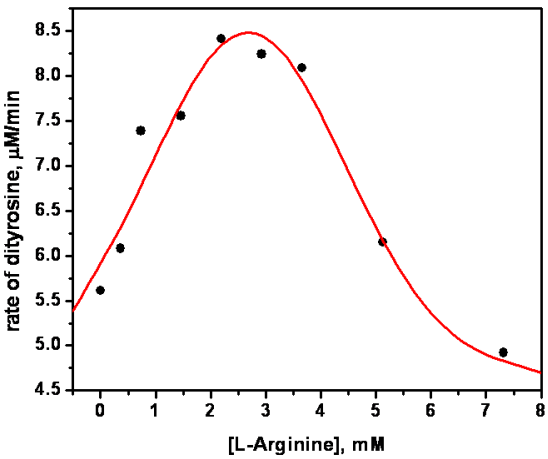
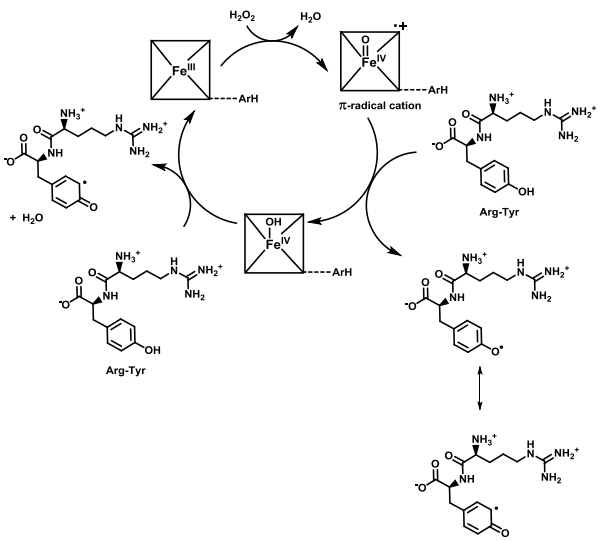


Figure 9 The rate of dityrosine formation of L-Tyr (2.13 mM) in the presence of HRP (0.06 µM) and H_2O_2 (1.22 mM) with varying concentration of L-Arg in phosphate buffer, pH 7.5 at 20 °C.



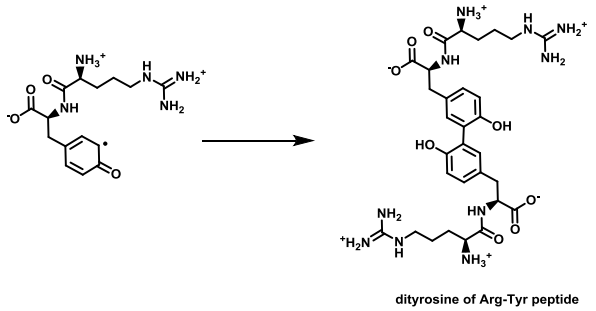


Figure 10 Mechanism of dityrosine formation of Arg-Tyr dipeptide.

Conclusions

o,o'-Dityrosine is a specific marker for oxidative/nitrasative stress. The increase in concentration of dityrosine is associated with several diseased states. A detailed study has been carried out in order to find out the effect of neighbouring amino acid residues in the rate of formation of dityrosine of several tyrosyl dipeptides. Except Cys-Tyr, all other tyrosyl dipeptides, form corresponding dityrosine with HRP/H_2O_2. With Cys-Tyr the formation of corresponding disulfide is observed. The appreciably higher rate of dityrosine formation of Phe-Tyr is attributed to the presence of strong hydrophobic environment around the active site of HRP. Among the polar tyrosyl peptides, the positively charged peptides (Arg-Tyr, Lys-Tyr) undergo dityrosine formation at much faster rate compared to that of negatively charged dipeptides (Asp-Tyr, Glu-Tyr). This trend is in accordance with the pK_a of neighbouring amino acid residues. The positively charged neighbouring residues with higher pK_a stabilizes ionized tyrosine, hence the rate of dityrosine formation is higher for them. As positively charged neighbouring residue, enhances the rate of dityrosine formation, the effect of externally added L-Arg has been studied.

Experimental Section

General Procedure

Horseradish peroxidase was purchased from CALBIOCHEM. L-tyrosine, 3-nitro-L-tyrosine, 3-iodo-L-tyrosine and deuterated solvent D_2O were obtained from Aldrich. The dipeptides Pro-Tyr, Arg-Tyr, Asp-Tyr, Glu-Tyr, Cys-Tyr, Ser-Tyr, His-Tyr, Thr-Tyr, Met-Tyr, Asn-Tyr and Gln-Tyr were commercially purchased from GL Biochem (Shangai) Ltd. Other tyrosyl containing dipeptides were synthesized by following the classical solution phase procedure. HPLC solvents were obtained from Merck. All other chemicals were of highest purity available. ^{1}H (400MHz) and ^{13}C (100.56MHz) NMR spectra were obtained on a Bruker 400 MHz NMR spectrometer. Chemical shifts are cited with respect to SiMe4 as internal standard (^{1}H and ^{13}C). Mass spectral studies were carried out on a Bruker ESI-MS ion-trap. The fluorescence measurements were carried out on Perkin Elmer Luminescence Spectrometer (LS 50B). The HPLC measurements were carried out on a Waters Alliance System (Milford, MA) with a 2695 separation module, 2996 PDA detector and a fraction collector. The HPLC system was controlled by EMPOWER software (Waters Corporation, Milford MA). The assays were carried out in a 1.8 ml sample vials and an in-built auto-sampler was used for sample injection.

Synthetic Procedure

Synthesis of dityrosine

To a solution of 15 mM L-tyrosine in phosphate buffer of pH 7.5, 2.5 µM HRP and 7.5 mM H_2O_2 in water were added. The reaction mixture was incubated for 20 min. The color of the solution changed to yellow. This solution is injected in to semi-preparative HPLC. Dityrosine was separated in a reverse phase column by using a gradient elution with methanol in 0.1% TFA and water in 0.1% TFA. Dityrosine was characterized by ^{1}H NMR and mass spectroscopic measurements. Yield: 20% ^{1}H NMR (D_2O, ppm), δ 2.96-3.20 (m, 2H), 3.88 (m, 1H), 6.86 (d, 1H), 7.0 (s, 1H), 7.09 (d, 1H); ESI-MS calc. for $C_{18}H_{21}N_2O_6$ $[M + H]^+$: 361.1395; found 361.0907.

Fluorometric study on dityrosine formation with different tyrosine containing peptides

The fluorometric monitoring of dityrosine has been carried out with the slight modification of reported procedure.[17] To 2.5 mM solution of L-Tyr or tyrosyl peptide in borate buffer of pH 9.1, 0.25 µM HRP was added. The reaction was initiated with the addition of 1.2 mM H_2O_2. With respect to time the aliquots of reaction mixture were diluted 500 times with carbonate buffer, pH 9.7 for fluorescent measurements. The excition and emission wavelengths for dityrosine were 320 nm and 408 nm, respectively. With respect to time, the increase in fluorescent intensity at 408 nm due to dityrosine formation has been monitored. The change in F.I. / min for dityrosine formation has been determined for all tyrosyl peptides.

HPLC study on dityrosine formation with different tyrosine containing peptides

The formation of dityrosine of different tyrosyl peptides has been followed by HPLC analysis. The formation of dityrosine has been followed by decrease in concentration of tyrosine/tyrosyl peptides. The decrease in the concentration of tyrosine/tyrosyl peptides was followed by measuring the peak area of tyrosine/tyrosyl peptides at 275 nm and the amount of tyrosine/tyrosyl peptides present in the solution at a given time is determined from the calibration plot obtained by injecting known concentrations of tyrosine/tyrosyl peptides. The initial rate v_0 for the formation of dityrosine of tyrosine/tyrosyl peptides has been determined. In a typical experiment, the incubation mixtures for the HPLC analysis contained tyrosine/tyrosyl peptide (2.13 mM), HRP (0.06 µM) and H_2O_2 (1.22 mM) in phosphate buffer, pH 7.5. The mixture was incubated at room temperature and aliquots (20 µl) injected onto the HPLC column and eluted with gradient solvent system (0.1% TFA in water-MeCN) using a C18 reverse-phase column. The tyrosine (retention time, 5.02 min) and dityrosine (retention time, 10.25 min) are separable. The decrease in the amount of tyrosine/tyrosyl peptides (µg) was calculated from the calibration plot. The chromatograms were extracted at 275 nm.

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References

1. T. DiMarco and C. Giulivi, *Mass Spec. Rev.*, 2007, **26**, 108 and references therein.
2. (a) A. J. Gross and I. W. Sizer, *J. Biol. Chem.*, 1959, **234**, 1611. (b)C. Giulivi and K. J. A. Davies, *Methods Enzymol.*, 1994, **233**, 363.
3. (a) S. O. Anderson, *Biochim. Biophys. Acta* 1964, **93**, 213. (b) F. La Bella, P. Waykole and G. Queen, *Biochem. Biophys. Res. Commun*. 1967, **26**, 748. (c) F. La Bella, P. Waykole and G. Queen, *Biochem. Biophys. Res. Commun.* 1968, **30**, 333. (d) F. W. Keeley, F. La Belle and G. Queen, *Biochem. Biophys. Res. Commun*. 1969, **34**, 156. (e) F. W. Keeley and F. La Belle, *Biochim. Biophys. Acta* 1972, **263**, 52. (f) J. W. Downie, F. La Bella and M. West, *Biochim. Biophys. Acta* 1972, **263**, 604. (g) G. Krishnan and M. H. Ravindranath, *Acta Histochem.* 1972, **44**, 348. (h) K. Ramalingam, *Parasitology*, 1973, **66**, 1. (i) P. Waykole and E. Heidemann, *Connect. Tissue Res.* 1976, **4**, 219. (j) R. H. Fetterer and M. L. Rhoads, *Vet. Parasitol.* 1993, **46**, 103. (k) R. H. Fetterer, M. L. Rhoads and J. F. Jr. Urban, *J. Parasitol.* 1993, **79**, 160. (l) V. Herzog, U. Berndorfer and Y. Saber, *J. Cell. Biol*. 1992, **118**, 1071. (m) E. H. Smail, P. Briza, A. Panagos and L. Berenfeld, *Infect. Immun.* 1995, **63**, 4078. (n) J. Li, B. A. Hodgeman and B. M. Christensen, *Insect Biochem. Mol. Biol*. 1996, **26**, 309. (o) C. A. Foerder and B. M. Shaipiro, *Proc. Natl. Acad. Sci. USA* 1977, **74**, 4214. (p) A. Takahashi, H. Totsune-Nakano, M. Nakano, S. Mashiko, N. Suzuki, C. Ohma and H. Inabe, *FEBS Lett.* 1989, **246**, 117. (q) P. Briza, G. Winkler, H. Kalchhauser and M. Breitenbach, *J. Biol. Chem.* 1986, **261**, 4288. (r) D. P. De Vore and R. J. Gruebel, *Biochem. Biophys. Res. Commun.* 1978, **80**, 993. (s) D. J. Raven, C. Earland and M. Little, *Biochim. Biophys. Acta* 1971, **251**, 96. (t) S. Garcia-Castineiras, J. Dillon and A. Spector, *Science*, 1978, **199**, 879. (u) M. K. McNamara and R. C. Augusteyn, *Exp. Eye Res.* 1980, **30**, 319. (v) J. M. Booij and J. J. Ten-Bosch, *Arch. Oral Biol.* 1982, **27**, 417. (w) F. Hanft and P. Koehler, *J. Agri. Food Chem.* 2005, **53**, 2418.
4. (a) G. S. Bayse, A. W. Michaels and M. Morrison, *Biochim. Biophys. Acta* 1972, **284**, 34. (b) L. A. Marquez, H. B. Dunford, *J. Biol. Chem.* 1995, **270**, 30434. (c) R. Aeschbach, R. Amado and H. Neukom, *Biochim. Biophys. Acta* 1976, **439**, 292. (d) S. Ohtaki, H. Nakagawa, M. Nakamura and I. Yamazaki, *J. Biol. Chem.* 1982, **257**, 761. (e) C. L. Steffensen, M. L. Mattinen, H. J. Andersen, K. Kruus, J. Buchert and J. H. Nielsen, *Eur. Food Res. Technol.* 2008, **227**, 57. (f) D. A. Malencik and S. R. Anderson, *Biochemistry* 1996, **35**, 4375 and references therein. (g) S. O. Andersen, *Acta Physiol. Scand.* 1966, **66**, 1. (h) W. H. Heijnis, H. L. Dekker, L. J. de Koning, P. A. Wierenga, A. H. Westphal, C. G. de Koster, H. Gruppen and W. J. H. van Berkel, *J. Agri. Food Chem.* 2011, **59**, 444.
5. (a) K. J. Davies and M. E. Delsignore, *J. Biol. Chem.* 1987, **262**, 9908. (b) K. J. Davies, M. E. Delsignore and S. W. Lin, *J. Biol. Chem.* 1987, **262**, 9902.
6. (a) J. P. Eiserich, C. E. Cross, A. D. Jones, B. Halliwell and A. van der Vliet, *J. Biol. Chem.* 1996, **271**, 19199. (b) K. Kikugawa, T. Kato and Y. Okamoto, *Free Radic. Biol. Med.* 1994, **16**, 373-382. (c) L. A. MacMillan-Crow, J. P. Crow and J. A. Thompson, *Biochemistry*, 1998, **37**, 1613. (d) S. V. Lymar, Q. Jiang and J. K. Hurst, *Biochemistry*, 1996, **35**, 7855. (e) A. van der Vilet, C. A. O'Neill, B. Halliwell, C. E. Cross and H. Kaur, *FEBS Lett.* 1994, **339**, 89. (f) A. van der Vilet, J. P. Eiserich, C. A. O'Neill, B. Halliwell, C. E. Cross, *Arch. Biochem. Biophys.* 1995, **319**, 341. (g) H. Zhang, J. Joseph, J. Feix, N. Hogg and B. Kalyanaraman, *Biochemistry* 2001, **40**, 7675.
7. H. Verweij, K. Christianse and J. Van Steveninck, *Biochim. Biophys. Acta* 1982, **701**, 180.
8. (a) D. A. Malencik and S. R. Anderson, *Biochemistry* 1994, **33**, 13363. (b) D. A. Malencik and S. R. Anderson, *Biochemistry* 1987, **26**, 695. (c) H. I. Joschek and S. I. Miller, *J. Am. Chem. Soc.* 1966, **88**, 3273. (d) S. S. Lehrer and G. D. Fasman, *Biochemistry* 1967, **6**, 757. (e) T. P. Holler and P. B. Hopkins, *Anal. Biochem.* 1989, **180**, 326. (f) W. A. Prutz, J. Butler and E. L. Laud, *Int. J. Radiat. Biol.* 1983, **44**, 183. (g) P. Guptasarma and D. Balasubramanian, *Curr. Eye Res.* 1992, **11**, 1121. (h) T. G. Huggins, M. C. Wells-Knecht, N. A. Detorie, J. W. Baynes and S. R. Thorpe, *J. Biol. Chem.* 1993, **268**, 12341. (i) T. K. Dalsgaard, J. H. Nielsen, B. E. Brown, N. Stadler and M. J. Davies, *J. Agri. Food Chem.* 2011, **59**, 7939. (j) M. Correia, M. T. Neves-Petersen, P. B. Jeppesen, S. Gregersen and S. B. Petersen, *PLOS ONE* 2012, **7**, e50733.
9. H. Eickhoff, G. Jung and A. Rieker, *Tetrahedron* 2001, **57**, 353.
10. B. P. Partlow, M. B. Applegate, F. G. Omenetto and D. L. Kalpan *ACS Biomater. Sci. Eng.* 2016, **2**, 2108.
11. (a) S. Bhattacharjee, S. Pennathur, J. Byun, J. Crowley, D. Mueller, J. Gischler, R. S. Hotchkiss and J. W. Heinecke *Arch. Biochem. Biophys.* 2001, **395**, 69. (b) Y. Kato, X. Wu, M. Naito, H. Nomura, N. Kitamoto and T. Osawa, *Biochem. Biophys. Res. Commun.* 2000, **275**, 11. (c) M. C. Wells-Knecht, T. G. Huggins, D. G. Dyer, S. R. Thorpe and J. W. Baynes, *J. Biol. Chem.* 1993, **268**, 12348. (d) J. M. Souza, B. I. Giasson, Q. Chen, V. M.-Y. Lee and H. Ischiropoulos, *J. Biol. Chem.* 2000, **275**, 18344. (e) K. Hensley, M. L. Maidt, Z. Yu, H. Sang, W. R. Markesbery and R. A. Floyd, *J. Neurosci.* 1998, **18**, 8126.
12. G. S. Bayse, A. W. Michaels and M. Morrison, *Biochim. Biophys. Acta* 1972, **284**, 30.
13. (a) W. E. Mayberry, J. E. Rall and D. Bertoli, *J. Am. Chem. Soc.* 1964, **86**, 5302. (b) W. E. Mayberry, J. E. Rall and D. Bertoli, *Biochemistry* 1965, **4**, 2606.
14. B. E. Kemp and R. B. Pearson, *Trends Biochem. Sci.* 1990, **15**, 342.
15. (a) J. M. Souza, G. Peluffo and R. Radi, *Free Radic. Biol. Med.* 2008, **45**, 357. (b) H. Zhang, J. Zielonka, A. Sikora, J. Joseph, Y. Xu and B. Kalyanaraman, *Arch. Biochem. Biophys.* 2009, **484**, 134. (c) A. S. Bayden, V. A. Yakovlev, P. R. Graves, R. B. Mikkelsen and G. E. Kellogg, *Free Radic. Biol. Med.* 2011, **50**, 749.
16. M. Tien, Arch. Biochem. Biophys. 1999, **367**, 61.
17. J. Zhang and S. Kalonia, *AAPS PharmSciTech* 2007, **8**, E1.
18. D. A. Malencik, J. F. Sprouse, C. A. Swanson and S. R. Anderson, *Anal. Biochem.* 1996, **35**, 4375.
19. N. C. Veitch, *Phytochemistry* 2004, **65**, 249.