Running title: Ozone-induced peptides as plant prions

Superoxide generation catalyzed by the ozone-inducible plant peptides analogous to prion octarepeat motif

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Abstract

Ozone-inducible (OI) peptides found in plants contains repeated sequence consisted of a hexa-repeat unit (YGHGGG) repeated for 8-10 times in tandem and each unit tightly binds copper. To date, the biochemical roles for OI peptides are not fully understood. Here, we demonstrated that the hexa-repeat unit from OI peptides behaves as metal-binding motif catalytically active in the $O_2^{\bullet-}$-generation. Lastly, possible mechanism of the reaction and biological consequence of the reactions are discussed by analogy to the action of human prion octarepeat peptides.

Key words

Reactive oxygen species, Ozone-inducible peptide, Stress response, Copper ion, Prion

Ozone is a major secondary air pollutant, as one of important environmental factors threatening the living plants, often reaching high concentrations in the urban areas under strong daylight. Studies are now suggesting that a steep increase in global background concentrations of ozone is in progress and thus the impact of atmospheric ozone to living plants including valuable crops might be severer in the future world.[1] Despite of great efforts to identify the physiological and biochemical elements of ozone tolerance and/or hyper-sensitivity in plants, the pictures obtained to date are not clear enough.[2] As part of such efforts, our recent studies revealed a signaling pathway within plant cells, in which ozone-exposed plant cells determine their fate to survive or to initiate localized apoptotic cell death, involving the members of reactive oxygen species (ROS), calcium signaling events, and anion channel regulations on plasma membrane.[3, 4] Several lines of studies also suggested that generation and/or removal of ROS may be the key signaling events determining the plant behaviors at cellular level under the ozone stresses.[5, 6]

As one of plant responses to ozone, ozone-inducible (OI) genes have been isolated from saltbush (*Atriplex canescens*) and their expression was shown to be responsive not only to ozone, but also to other environmental stresses such as SO_2 and water deficit.[7] To date, two isotypes (OI2-2, 158 amino acids and OI14-3, 119 amino acids) of OI peptides have been reported and they possess a common characteristic repeat unit consisting of hexa amino acids, YGHGGG; repeating it for 8-10 times in tandem. Due to the presence of this repeat unit, OI peptides are considered as putative members of glycine-rich proteins (GRPs).[7] In general, GRPs are known to be inducible by wounding, drought and water-deficient and they were firstly discovered as cell wall-associated proteins and one of their key molecular function was assumed to act as wound-responsive factors contributing to the strengthening of the cell wall.[8] Secondary structures of both the general GPRs and OI peptides were reported to be rich in β-pleated sheets.[7, 8] Furthermore, No et al.[7] have proposed that carboxyl terminal regions in OI peptides may interact with the cell wall components through the tyrosyl residues being exposed from the peptide backbones while the amino termini of the peptides may function as the putative signaling modules. Interestingly, it has been reported that the tyrosyl residues on GRPs behave as phenolic substrates for the cell wall peroxidase and thus capable of forming the covalent bonds with dehydrogenated coniferyl alcohol moieties within the cell wall components, finally leading to enhanced lignification.[9] Indeed, plant apoplast including cell walls is highly rich in

peroxidase activity which favors various phenolics and amines in order to catalyze the generation of radical species often resulting in generation of ROS.[10] By analogy, it has been suggested that there would be roles for tyrosyl residues on OI peptides similarly to those on GPRs, possibly by contributing to some redox reactions involving ROS with respect to the signaling events during cellular responses.[11]

Figure 1A shows partial amino acid sequences in two OI peptides sharing the identical repeat motif (GGGYGH) which is repeated for 9-times and 7 times in OI2-2 and OI14-3, respectively. The each repeat unit in OI peptide was shown to form a complex with transition metals, chiefly copper ion in the physiological pH range as illustrated (Fig. 1C) based on the spectroscopic studies by measuring the cyclic dichroism (CD) and nuclear magnetic resonance (NMR).[11]

Previously, we have been engaged in the biochemistry and bioengineering of redox active peptides derived from human prion protein (PrP).[12-14] The repeated peptidic motifs active in Cu-binding found in human PrP was shown to be active biocatalysts mimicking the action of superoxide anion radical ($O_2^{\bullet-}$)-generating plant peroxidase which utilizes phenolics and amines as e^- donors and H_2O_2 as e^- acceptor.[10, 12, 15] Interestingly, phenolic moiety in tyrosyl residue on PrP or peptides could be the $O_2^{\bullet-}$-generating substrate catalyzed by PrP-derived Cu-binding motifs.[16] Since the His-containing repeated motif found in plant OI peptides has structural similarity required for Cu binding and redox catalysis in PrP-derived sequence,[12] and the repeat motif itself contains the Tyr-residue, it is natural to expect that OI peptides natively possess $O_2^{\bullet-}$-generating activity through self-oxidation in the identical manner reported for PrP-derived peptides. In the present study, based on above working hypothesis, we assessed the $O_2^{\bullet-}$-generating activity of metal-binding motif derived from plant OI peptides.

A series of peptides chemically synthesized and purified on high pressure liquid chromatography, was obtained from the custom peptide service department of Sigma Genosys Japan (Ishikari, Japan). The purities of the four OI hexa-repeat motif-related peptides, namely GGGYGH, YGHGGG, HGGGYG, and GGGFGH, were 95.29%, 99.39%, 99.39% and 98.20%, respectively. To prevent the peptide from forming self-circularization, amino and carboxyl terminal of each peptide was acetylated and amidated, respectively.

To detect the generation of $O_2^{\bullet-}$, an $O_2^{\bullet-}$-specific chemiluminescence agent, *Cypridina* luciferin analog (2-Methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one; Tokyo Chemical

Industry, Tokyo) designated as CLA was used. The other chemicals used in this study were of reagent grade, and were purchased from Sigma (St. Louis, MO). The peptides and other chemicals were dissolved in 50 mM potassium phosphate buffer (pH 7.0). The molar ratios among the components in the reaction mixture (200 μl in total), *viz*., the peptides of interest, Cu^{2+} and H_2O_2 were approximately 1 : 3 : 3 (that is each reaction mixture contained 0.15 mM peptides, 0.5 mM $CuSO_4$, 0.5 mM H_2O_2). In our previous report, the molar ratios between substrate (tyrosine) and Cu-peptide complex showing high catalytic activity were determined by dose-dependent manner.[16]

Generation of $O_2^{\bullet-}$ was monitored by the chemiluminescence of CLA with a luminometer (Luminescensor PSN AB-2200-R, Atto, Tokyo), and the yield of chemiluminescence was expressed as relative luminescence units (rlu), as previously described.[12] CLA is a specific probe for $O_2^{\bullet-}$ (and singlet oxygen to a lesser extent), but not that of H_2O_2 or the hydroxyl radical.[17] According to our previous studies, the signal for singlet oxygen can be minimized by avoiding the use of high concentrations of organic solvents such as ethanol not exceeding 2% (v/v) in the reaction mixture.[18] Thus the induced chemiluminescence recorded here reflects the generation of $O_2^{\bullet-}$ rather than singlet oxygen. Calibration of CLA-chemiluminescence reflecting the changes in $O_2^{\bullet-}$ level was carried out by dropping the solution of potassium superoxide (KO_2) dissolved in DMSO onto the CLA containing media. Approximately, 2000 rlu indicates 5 nmol/ml of superoxide.

According to the previous reports, His residues on PrP-derived peptides are essential to anchor the copper and Tyr residues also on PrP-derived peptides behave as phenolic substrates for Cu-bound motif-catalyzed peroxidative reaction, finally releasing $O_2^{\bullet-}$.[16] Here, by analogy, catalytic activity for the repeat motif derived from OI peptides was examined. Since in the repeated region, the hexapeptide unit is repeated directly in tandem, six different repeat hexamers can be selected, each starting and ending with different amino acids. Here, we randomly selected three sequences, namely GGGYGH, YGHGGG, and HGGGYG. Obviously any of these sequences can be considered as a single repeat unit, but eventually differed in the topological locations of His residue (*i.e.* at both termini or middle) on each single repeat peptide (Fig. 1B).

Figure 2A shows the typical traces of H_2O_2-fueled $O_2^{\bullet-}$ generation catalyzed by OI repeat unit peptides, which has been measured as the transient increases in CLA-chemiluminescence.

Each peptide was mixed up with copper solution allowing the Cu-peptide complex formation, prior to starting the reaction by addition of H_2O_2. Although, known peroxidative $O_2^{\bullet-}$-generating reactions catalyzed by plant peroxiases,[10] methemoglobin,[19] or PrP-derived peptides[12, 13, 16] require both H_2O_2 and phenolics (or other e^- donors), the reaction was initiated by addition of only H_2O_2 to the reaction mixture containing the Cu-loaded repeat unit peptides since each of these peptides possesses one Tyr residue as a putative e^- donor.

Following the addition of H_2O_2, both GGGYGH and YGHGGG peptides showed long-lasting catalytic profiles of $O_2^{\bullet-}$ generation whereas the HGGGYG peptide showed no detectable response. Statistical comparisons of $O_2^{\bullet-}$-generating activities among the peptides were carried out as shown in Fig. 2B. As for a comparison, a hexa-glycyl peptide (GGGGGG) was used in place of OI repeat motif peptides, but no catalytic activity was observed (Fig. 2B). Furthermore, supplementation of phenolics (such as free tyrosine, tyramine, and mono- and di-hydroxybenzoates, known to be good substrates for the PrP-Cu complex-catalyzed superoxide generating reactions) to GGGGGG solution prior to addition of Cu and H_2O_2 failed to confer the catalytic activity to the GGGGGG peptide,[11] suggesting the lack of catalytic activity in GGGGGG is not due to the lack of e^--donating Tyr residue (as a intra-molecular substrate) but most likely due to the lack of His residue. In the previous reports, supplementation of free tyrosine to Y-residue lacking PrP-derived peptides successfully resulted in oxidative burst converting H_2O_2 to $O_2^{\bullet-}$.[16]

Since the peptide with His residue located at the carboxyl terminal (GGGYGH) showed the highest activity and that with amino terminal His residue (HGGGYG) showed no activity, not only the presence but also the location of His residue is one of critical requirement for the reaction, similarly to the previously reported results with model His-containing Cu-binding peptides.[20]

To assess the importance of tyrosine residue located in the repeat region as putative intra-molecular substrate within the catalyst as reported for several redox active enzymes such as cyclooxygenase-2 [21] and ribonucleotide reductases,[22] in which corresponding reactions proceed *via* the formation of a tyrosyl radical; a tyrosine-to-phenylalanine (Y-to-F) substituted mutant peptide (GGGFGH) was synthesized and used for comparison. As predicted, the Cu-GGGFGH complex showed no activity for the generation of $O_2^{\bullet-}$ after the addition of H_2O_2. On the other hand, by adding free tyrosine as a substrate into the reaction mixture containing

GGGFGH and copper, the H_2O_2-dependent $O_2^{\bullet-}$-generation was regained (Fig. 3A, B). This result is consistent with the view that the Tyr moiety on the peptide is essential for conversion of H_2O_2 to $O_2^{\bullet-}$ catalyzed by copper-binding OI peptides.

Requirement for free Tyr or Tyr residue in H_2O_2-dependent $O_2^{\bullet-}$-generating reaction suggest that tyrosyl radical (one form of phenoxy radicals) is transiently formed for one-electron reduction of di-oxygen by analogy to the plant peroxidase reaction H_2O_2-dependently producing $O_2^{\bullet-}$ by coupling to oxidation of salicylic acid to form phenoxy radical.[15]

At present, we do not have enough experimental data to judge if the reaction observed is due to intra-molecular interaction between the Cu-bound catalytic moiety and the Tyr. Figure 4 illustrates three different models proposed for interactions between Tyr residues and Cu-binding motif within the intact native peptides, namely (1) inter-molecular interaction allowing the peptide-to-peptide interaction in which one molecule of activated OI peptide may attack the neighboring homogenous molecules at Tyr residues (Fig. 4A), (2) intra-molecular interaction model allowing the repeat-to-repeat interaction in which activated Cu-centered reaction complex within one repeat unit may attack the neighboring repeat units at Tyr residues (Fig. 4B), and (3) intra-repeat interaction allowing the movement of electron between Tyr residue and Cu within the same repeat unit (Fig. 4C). Each model must be critically examined in the future studies.

Here, we presented the data in support of the roles for OI peptides as natural pro-oxidants in plants inducible in response to oxidative stress under exposure to ozone. In contrast, earlier work proposed that Cu-peptide complex formed between copper ions and OI peptides possibly behaves as superoxide dismutase (SOD) mimics capable of removing $O_2^{\bullet-}$.[11] Therefore, some anti-oxidative roles for OI peptides in plant cells has been proposed as a working hypothesis.[11] Such controversial roles were also proposed for human PrP behaving as anti-oxidant (by mimicking SOD)[23] and as pro-oxidant (by mimicking ROS-generating peroxidase).[12] Assuming that OI peptides possess both the $O_2^{\bullet-}$-forming and $O_2^{\bullet-}$-scavenging activities depending on the conditions such as the concentrations of reaction substrates, products, and co-factors; it is highly likely that these peptides, upon binding to Cu, may drastically alter the equilibrium of ROS, thus affecting the fate or behaviors of the plant cells under the oxidative stress.

Apart from plant systems, a series of peptidic redox catalysts derived from PrPs harboring Cu as one of key co-factors has been reported. It has been reported that human PrP has

His-containing specific motifs for binding certain amount of copper ions.[24] At least one His residue is required for anchoring Cu ion to each motif.[25, 26] Human PrP has four distinct copper-binding motifs. Among such motifs, the octarepeat motif possessing 4-time-repeated amino acid octet (PHGGGWGQ), has been studied in details. Such motifs, when isolated or synthesized as short peptides, shows catalytic activities for conversion of H_2O_2 to $O_2^{\bullet-}$.[12, 16, 20] The key criterion required for Cu-binding motifs in PrP and other synthetic peptides to be redox active catalysts, is the presence of His residue positioned after at least dimer or longer sets of any amino acid sequence except for Met and Cys.[16, 20] As OI peptides are proposed to be localizing to the cell wall, ROS production may contribute to the plant defense mechanism by enhancing the cell wall cross linking and strengthening.

GRPs including OI peptides have been discovered in many of higher plants and known to localize mostly in the cell wall.[8,27] In the glycine-rich region of general GRPs, the amino acid sequence contains the motif of $(Gly\text{-}X)_n$ where X could be frequently glycine but also histidine, tyrosine, alanine, serine, valine, phenylalanine or glutamic acid.[27] In case of the $(Gly\text{-}X)_n$ having occasional His residues as X, the motif can be reconsidered as $(Gly_n\text{-}His)_n$ which is exactly matching with the structural criteria for behaving as Cu-binding and $O_2^{\bullet-}$-generating biocatalysts as shown in PrP-derived peptides,[20] thus suggesting that GRPs may possibly function as catalytic proteins capable of ROS generation in many plant species depending on combination of phenolic substrates including free Tyr and Tyr residues on proteins. The case of the repeat regions in OI peptides needs slight modification of the model. The repeat regions possess the GGGYGH repeat motif, which can be reconsidered as $(Gly_3\text{-}Tyr\text{-}Gly\text{-}His)_n$ or $(X_5\text{-}His)_n$ where X can be both Gly and Tyr. According to the PrP model, elongation of $(X_n\text{-}His)$ with 'n' greater than 3 (up to 10) linearly increases the catalytic activity upon binding to Cu.[20] Therefore, high catalytic activity of GGGYGH is due to the structure matching the "X_n-His (n>3)" structure. In addition, free Tyr and Tyr residues can be good substrates for Cu-loaded X_n-His peptides during conversion of H_2O_2 to $O_2^{\bullet-}$,[16] the Tyr residue within each repeat unit in OI peptides can be a target of self catalysis.

The proposed roles for OI peptides include (1) ROS removal, (2) ROS generation, (3) copper homeostasis, and (4) macromolecule cross-linking, all similarly to PrP, therefore we would like to consider the OI peptides as the plant models for prion-like proteins functionally analogous to the octarepeat in PrP. In this topic, further critical works are required for deepen

our knowledge on the behaviors and roles of small protein and peptides in biological systems.

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Figure legends

Figure 1.

Amino acid sequence of ozone-inducible proteins including repeat region and list of peptides tested. (A) Two sequence of isotype of ozone-inducible protein included 9-times or 7times repeats of GGGYGH. (B) Peptides tested in this work. Underline and asterisk indicates position of tyrosine and the His residue putatively anchoring copper, respectively. GGGFGH is synthesized as a Y-F substituted peptide. Position of F is highlighted. (C) Likely structure of GGGYGH-Cu complex.

Figure 2.

OI-catalyzed H_2O_2-dependent superoxide generation. (A) Typical curves of OI-dependent superoxide generation measured as an increase in CLA-chemiluminescence. An arrow indicates the timing of addition of hydrogen peroxide into reaction mixture. (B) Comparison of superoxide-generating activity depending on the position of tyrosine and histidine residues. Histidine residue located in amino terminal shows higher activity. No production of superoxide was observed in HGGGYG and GGGGGG. Vertical bars on the graph indicate S. E. (n=3).

Figure 3.

Role of tyrosine in the OI-catalyzed reaction. (A) Typical trace of GGGFGH and effect of free tyrosine to GGGFGH. The arrows indicate a timing of addition of hydrogen peroxide, tyrosine, respectively. (B) Assessment of requirement of tyrosine residue on OI-peptide for generation of superoxide. Although no activity was observed by using Y-F substituted mutant peptide, superoxide-generating activity was recovered after addition of free tyrosine as a substrate into the mixture of GGGFGH, copper and H_2O_2. Vertical bars on the graph indicate S. E. (n=3).

Figure 4.

Proposed model of interaction between copper-binding OI-peptide and tyrosine residues. (A) inter-molecular reaction model. (B) intra-molecular reaction model. (C) intra-regional reaction model.

Fig. 1ABC

ozone-inducible protein, isotype

A

OI2-2

-68EESVEDSKHGYYGH [GGGYGH]×9 GGYGGHYPPKETETTQEQKGN

OI14-3

-81EEESVEDSKHGYYGH [GGGYGH]×7 GGHGGHYPPKETKTTQEQNGN

B

Peptides synthesized

GGGYGH*

YGH*GGG

H*GGGYG

GGGFGH*

C

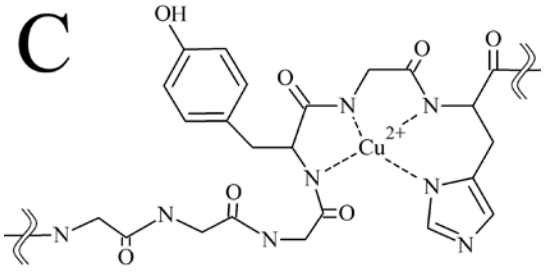
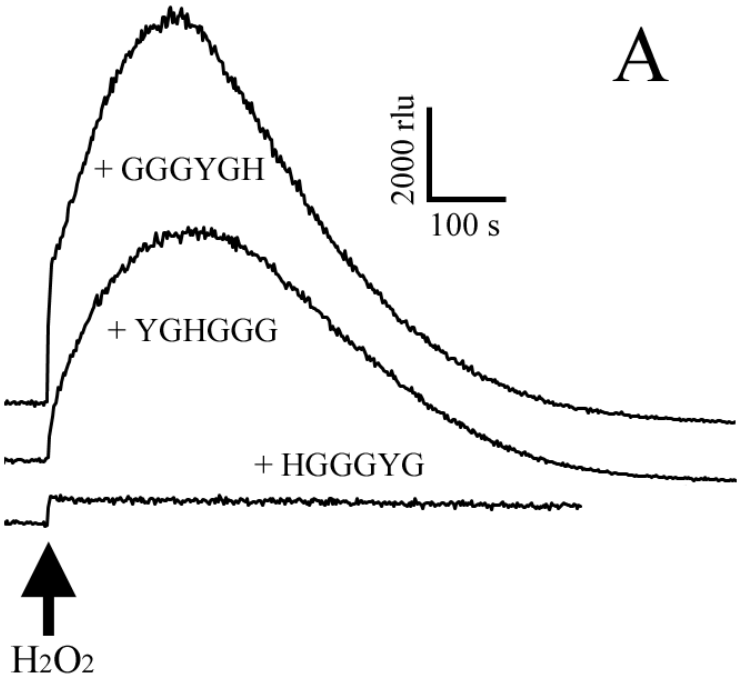


Fig. 2AB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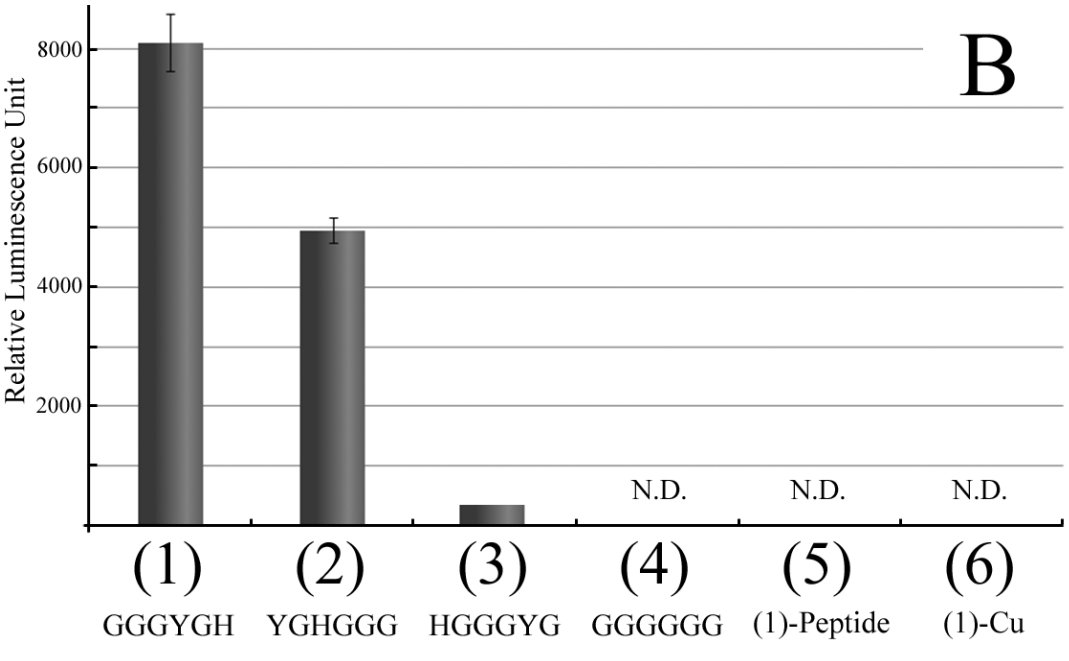
+ YGHGGG
+ HGGGYG
H2O2


B
Relative Luminescence Unit
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6000
4000
2000
N.D.
N.D.
N.D.
(1)
(2)
(3)
(4)
(5)
(6)
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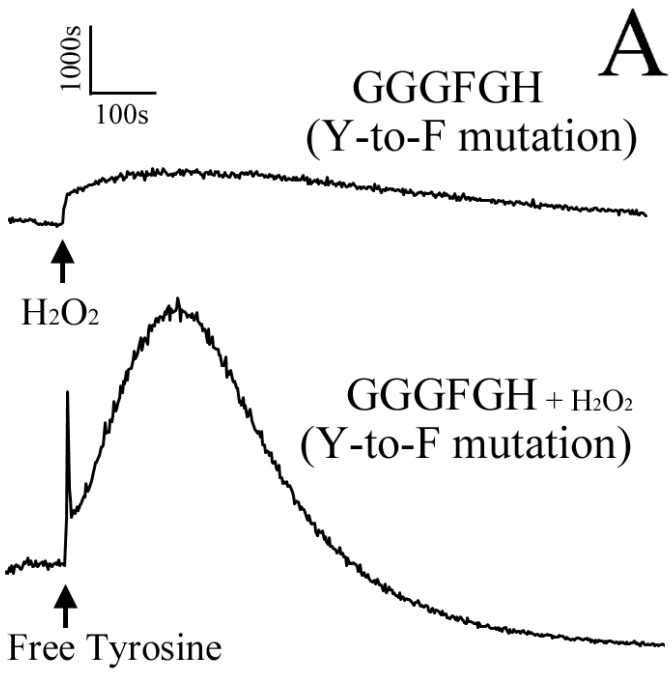
HGGGYG
GGGGGG
(1)-Peptide
(1)-Cu

Fig. 3AB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(Y-to-F mutation)
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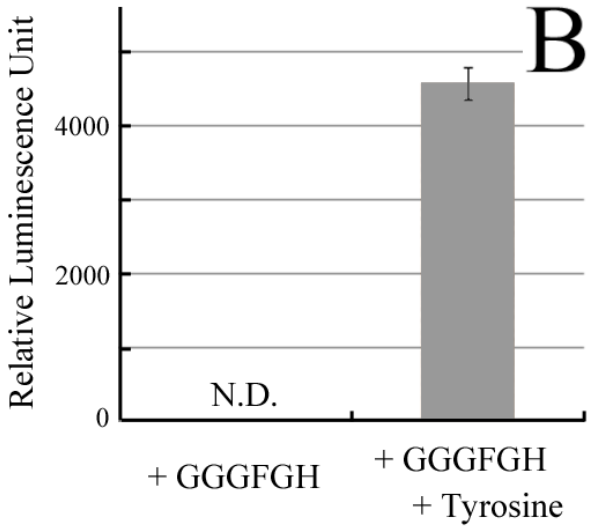
GGGFGH + H2O2
(Y-to-F mutation)
Free Tyrosine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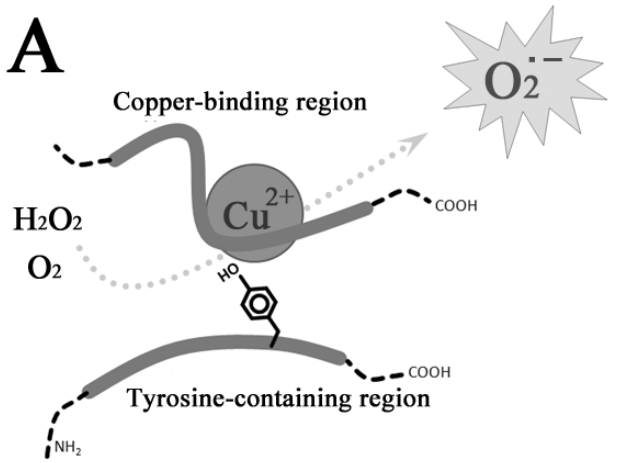
N.D.
+ GGGFGH
+ GGGFGH
+ Tyrosine

Fig. 4ABC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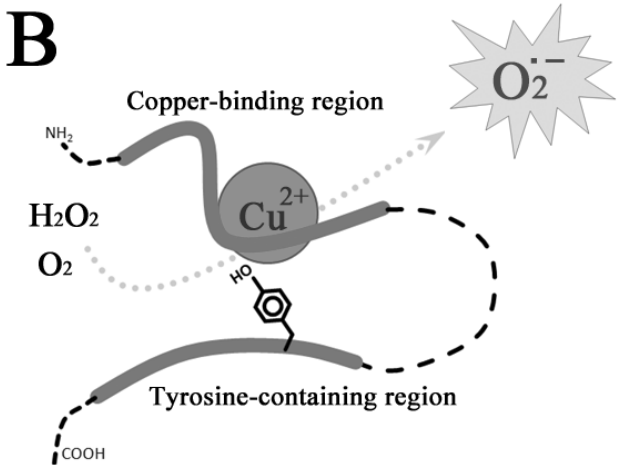
COOH
Tyrosine-containing region
NH_2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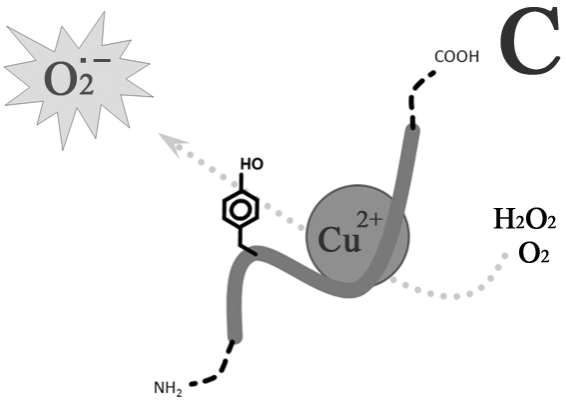
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HO
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