

RESEARCH PAPER

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## Fluorescent monitoring of copper-occupancy in His-ended catalytic oligo-peptides

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### ABSTRACT

Controlled generation of reactive oxygen species (ROS) is widely beneficial to various medical, environmental, and agricultural studies. As inspired by the functional motifs in natural proteins, our group has been engaged in development of catalytically active oligo-peptides as minimum-sized metalloenzymes for generation of superoxide anion, an active member of ROS. In such candidate molecules, catalytically active metal-binding minimal motif was determined to be X-X-H, where X can be most amino acids followed by His. Based on above knowledge, we have designed a series of minimal copper-binding peptides designated as G<sub>n</sub>H series peptides, which are composed of oligo-glycyl chains ended with C-terminal His residue such as GGGGGH sequence (G<sub>5</sub>H). In order to further study the role of copper binding to the peptidic catalysts sharing the X-X-H motif such as G<sub>5</sub>H-conjugated peptides, we should be able to score the occupancy of the peptide population by copper ion in the reaction mixture. Here, model peptides with Cu-binding affinity which show intrinsic fluorescence due to tyrosyl residue (Y) in the UV region (excitation at *ca.* 230 and 280 nm, and emission at *ca.* 320 nm) were synthesized to score the effect of copper occupancy. Synthesized peptides include GFP-derived fluorophore sequence, TFSYGVQ (designated as Gfp), and Gfp sequence fused to C-terminal G<sub>5</sub>H (Gfp-G<sub>5</sub>H). In addition, two Y-containing tri-peptides derived from natural GFP fluorophores, namely, TYG and SYG were fused to the G<sub>5</sub>H (TYG-G<sub>5</sub>H and SYG-G<sub>5</sub>H). Conjugation of metal-binding G<sub>5</sub>H sequence to GFP-fluorophore peptide enhanced the action of Cu<sup>2+</sup> on quenching of intrinsic fluorescence due to Y residue. Two other Y-containing peptides, TYG-G<sub>5</sub>H and SYG-G<sub>5</sub>H, also showed intrinsic fluorescence which is sensitive to addition of Cu<sup>2+</sup>. There was linear relationship between the loading of Cu<sup>2+</sup> and the quenching of fluorescence in these peptide, suggesting that Cu<sup>2+</sup>-dependent quenching of Y-reside-derived fluorescence could be a measure of copper occupancy in the peptides. Lastly, the fate of Y residue in the Cu-loaded peptides under oxidative condition in the presence of H<sub>2</sub>O<sub>2</sub> was discussed based on the Cu/H<sub>2</sub>O<sub>2</sub>-dependent changes in fluorescence spectra.

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## Introduction

Controlled generation of reactive oxygen species (ROS) is widely beneficial to various medical, environmental engineering, and agricultural fields including clinically applied immunological modulations,<sup>2,7</sup> degradation of polluting organic compounds,<sup>23</sup> inactivation of bacterial cells for the hygienic purpose,<sup>19</sup> activated sludge process,<sup>22</sup> and direct and indirect agricultural pest controls targeting pathogens and host plants, respectively.<sup>16,29</sup>

In the last decade, our group has been engaged in development a novel classes of engineered biocatalysts including catalytic oligonucleotides (functional DNA sequences,<sup>10</sup> and peptides,<sup>11</sup> designed to catalyze the production and/or removal superoxide anion radicals

(O<sub>2</sub><sup>•-</sup>) through understanding and modification of natural catalytic proteins of animal and plant origins.

For example, we found that peptides derived from human and mammalian prion proteins (PrPs)<sup>14,24</sup> and plant stress-responsive peptides<sup>25</sup> have catalytic nature although they are not considered as enzymes at present. Actually, the kingdoms of plants and animals are rich in such small peptides with high affinity to metal ions which might aid in catalysis. By mimicking such natural peptide, novel series of minimal-sized oligo-peptidic artificial enzymes catalyzing the generation of O<sub>2</sub><sup>•-</sup> in peroxidase-like manner requiring hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and electron donating substrates such as phenolics or amines were developed.<sup>11,21</sup>

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The first criterion for consisting such minimal peroxidase-like small peptides is the presence of His-containing motif(s) required for binding to metals (chiefly copper), and then, free-form and/or peptide-bound form of substrates fuels the reaction.<sup>27</sup> Our preliminary studies on PrP-derived peptides have pointed that His residues (at least single His) are required for anchoring of Cu onto PrP-derived peptides,<sup>13,14</sup> and eventually, the catalytically active Cu-binding minimal motif was determined to be tri-peptidic sequence X-X-H, where X can be most amino acids followed by His.<sup>12,27</sup> An engineering example of a catalytic peptide sequence sharing XXH motif for development of biosensing and bioengineering materials was reported.<sup>21</sup> Accordingly, one of X-X-H motif derivatives, Gly-Gly-His (G-G-H) sequence was introduced onto the glycidyl methacrylate grafted on the polyethylene platform (porous hollow fiber membrane). Chemiluminescence assay revealed that loading of Cu<sup>2+</sup> on the peptide-conjugated membrane conferred the catalytic activity to it, thus, catalyzing the generation of O<sub>2</sub><sup>•-</sup> upon addition of a pair of substrates (H<sub>2</sub>O<sub>2</sub> and tyramine).

In human PrP, His96 centered in G-G-G-T-H-S-Q-W-N sequences is considered as one of Cu-binding sites. Effect of His position on the catalytic activity in PrP-derived peptides was examined by comparing the H-S-Q-W-N (His-started pentapeptide) and the G-G-G-T-H (His-ended pentapeptide).<sup>11</sup> While reaction with tyramine (given as a model phenolic substrate) and G-G-G-T-H peptide resulted in robust production of O<sub>2</sub><sup>•-</sup>, the H-S-Q-W-N peptide showed no catalytic activity, suggesting that G-T-H motif within the His-ended pentapeptide is one of X-X-H motif derivatives. As the catalytic activities among G-G-G-T-H and shorter derivatives (G-G-T-H and G-T-H) were compared, the importance of the *N*-terminal glycy-chain elongation for maximal redox activity in *C*-terminal His anchored peptides was implied.<sup>11</sup> Furthermore, the likely common structure formed by Cu/X-X-H motif complex found in Cu-binding motifs in human PrP including octarepeat region<sup>9</sup> helical region,<sup>27</sup> neurotoxic region<sup>12</sup> was proposed to be semi-planar shape resembling the structure of metal-centered heme in which metallic element is coordinated by planarly arranged four nitrogen atoms, by analogy to the structure of Ni/X-X-H metallopeptides.<sup>5,6</sup>

As inspired by the natural PrP-derived G-G-G-T-H sequence, we have designed a series of simplified model peptides designated as G<sub>*n*</sub>H series peptides, which are composed of oligo-glycyl chains (G<sub>*n*</sub>) ended with *C*-terminal His residue.<sup>11</sup> As expected, importance of the elongated *N*-terminal G<sub>*n*</sub> chain with anchoring His was confirmed by comparing the G<sub>*n*</sub>H series peptides (*n* = 2, 3, 4, 5 and 10) and G<sub>*n*</sub> series peptides lacking His.

Notably, G<sub>*n*</sub> series lacking the metal-binding motif showed no catalytic activity even in the presence of free Cu<sup>2+</sup>. In G<sub>*n*</sub>H series, G<sub>3</sub>H tetrapeptide showed a detectable increase in production of O<sub>2</sub><sup>•-</sup>, and peptides with longer chain showed higher activity, confirming the importance of *N*-terminal G<sub>*n*</sub> chain length. Data suggested that the requirement for the *N*-terminal G<sub>*n*</sub> elongation is nearly fulfilled at between G<sub>5</sub> and G<sub>10</sub>,<sup>11</sup> and amazingly, unlikely to conventional enzymes, the catalytic activity in these metal-binding peptides survive the heating treatment such as autoclaving and the repeated freeze and thaw cycles.<sup>28</sup>

Fluorometry often provides strong approaches for studying the molecular interaction.<sup>9</sup> We have previously assessed (1) the quenching of Tb<sup>3+</sup> fluorescence by PrP-derived metal-binding peptides and (2) the Cu<sup>2+</sup>-dependent quenching of intrinsic fluorescence in human PrP octarepeat peptide sequence. Quenching of Tb-fluorescence by interacting peptides implied the important role for His-ended peptidic sequence sharing X-X-H motif (in case of human PrP's octarepeat region, P-Q-H). On the other hand, quenching of intrinsic peptide fluorescence due to the presence of a tryptophan (W) residue by copper ion suggested that classically known H-G-G-G motif in PrP<sup>3</sup> forms an active motif in metal binding. Taken together, in the mammalian PrP octarepeat regions, in which P-H-G-G-G-W-G-Q is repeated for four (human) to six (bovine) times, two distinct metal binding motifs, namely, X-X-H motif (in this case, Q-P-H tripeptide sequence) and H-G-G-G motif, could be overlaid by sharing common His residue (Q-P-H-G-G-G) and thus co-existed and synergically capturing the metals.<sup>9</sup>

In order to further study the role of copper binding to the biocatalysts sharing the X-X-H motif such as G<sub>*n*</sub>H series catalytic peptides, we should be able to score the occupancy of the peptide population by copper ion in the reaction mixture. In the present study, we attempted to monitor the binding of copper to G<sub>*n*</sub>H catalytic peptides by designing the chimeric molecule fusing fluorescent oligo-peptide sequence derived from green fluorescence protein (GFP) and G<sub>5</sub>H sequence.

## Materials and methods

### Peptides and chemicals

Model peptides with Cu-binding affinity which show intrinsic fluorescence in the UV region were synthesized to score the effect of copper occupancy. The peptides were obtained from the custom peptide service department of Sigma Genosys Japan, Ishikari, Hokkaido, Japan. The amino acid sequences of the peptides chemically synthesized were purified on high pressure liquid

chromatography prior to the experimental use. Other chemicals such as  $\text{CuSO}_4$  and salts for buffer used in this study were of reagent grade purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Synthesized peptides include GFP-derived fluorophore sequence, TFSYGVQ (designated as Gfp), and Gfp sequence fused to GGGGGH, thus, designated as Gfp-G<sub>5</sub>H. Note that the synthesized peptide sequences corresponding to GFP fluorophore do not show green fluorescence without post-translational process for developing the molecular rigidity in living cells. Instead, intrinsic fluorescence due to presence of the tyrosyl residue (Y) can be expected. Therefore, two Y-containing tri-peptide sequences found in natural GFP fluorophores, namely, TYG and SYG were fused to the Cu-binding GGGGGH sequence (thus, designated as TYG-G<sub>5</sub>H and SYG-G<sub>5</sub>H, respectively).

### Fluorometric analysis

Intrinsic fluorescence from the 30  $\mu\text{M}$  peptides with and without loading of copper ions were detected in potassium phosphate buffer (50 mM, pH 7.0) using a fluorescence spectrophotometer (F-4500 Hitachi High-Technol. Co., Tokyo). The three-dimensional (3D) spectral measurement of fluorescence was carried out at the excitation wavelength between 200 and 700 nm with 5 nm intervals and emission wavelength between 200 and 700 nm with 5 nm intervals.

To perform full occupancy of peptide with metals, the concentrations of metals of interest tend to be higher than the peptide concentration as previously observed in fluorescence measurements with prion-derived oligopeptides.<sup>9,13</sup> High concentration of metals in phosphate-based reaction mixture often cause a slight turbidity which sensitively interferes with fluorescence measurement by developing an intensive diagonal band of scattered light over the fluorescence contour plot. To avoid such situation, peptides needs to be maintained at around 30  $\mu\text{M}$  (so that metal concentration attaining full occupancy can be lowered) as described in our previous reports.<sup>9,13</sup>

## Results and discussion

### Enhanced $\text{Cu}^{2+}$ -dependent changes in intrinsic fluorescence spectra of a GFP-fluorophore peptide conjugated with metal binding sequence

GFP fluorophore-derived peptides tested here showed non-green intrinsic fluorescence at UV region (Fig. 1). Upon excitation at 230 nm (peak a) and 280 nm (peak b), both Gfp-G<sub>5</sub>H (TFSYGVQ-GGGGGH) and Gfp

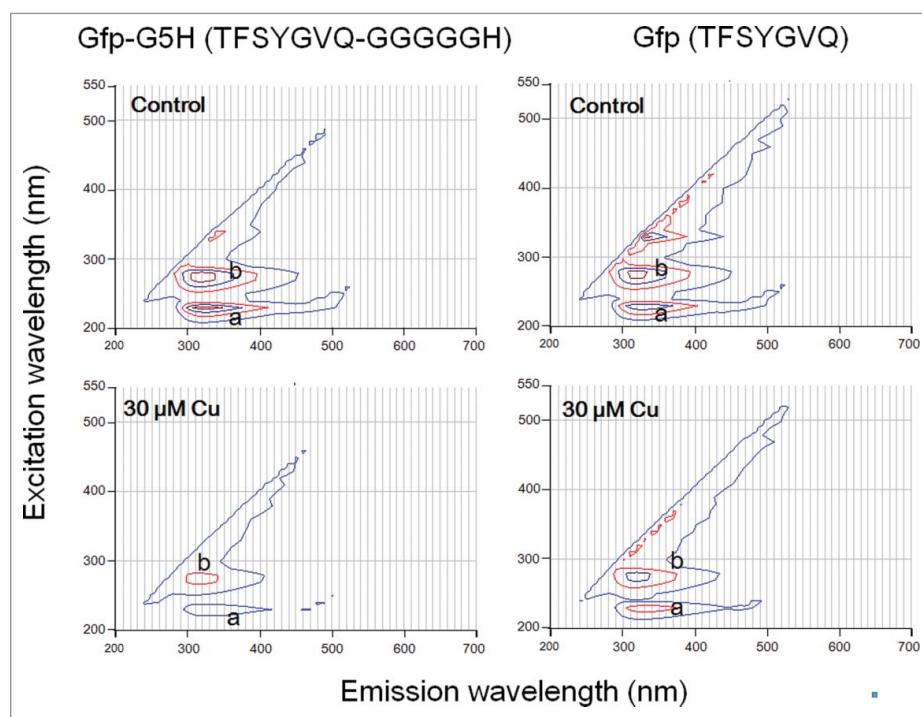
(TFSYGVQ) showed fluorescence emission at around 320 nm. Fluorescence signals at the peak a (230 nm excitation/320 nm emission) by 30  $\mu\text{M}$  Gfp-G<sub>5</sub>H and Gfp showed tendency to be quenched in the presence of 30  $\mu\text{M}$   $\text{Cu}^{2+}$  by 61.6 % and 32.0 %, respectively. Under the same conditions, the fluorescence signals at the peak b (280 nm excitation/320 nm emission) by Gfp-G<sub>5</sub>H and Gfp showed  $\text{Cu}^{2+}$ -dependent quench by 48.5 % and 22.7 %, respectively. These data suggested that presence of Cu may partially quench the fluorescence of Gfp-heptapeptide lacking the Cu-binding motif due to non-specific interaction. Addition of C-terminal G<sub>5</sub>H sequence doubled the extent of fluorescence quenching induced by  $\text{CuSO}_4$ , thus suggesting the impact of selective binding of copper onto the G<sub>5</sub>H sequence conjugated to the sequence with fluorophore. Therefore, we can expect that Cu-dependent quenching of UVC-excited UVA fluorescence by Gfp-G<sub>5</sub>H can be used as a measure of Cu-occupancy in G<sub>5</sub>H domain.

### Intrinsic fluorescence in tyrosine-containing peptides showed sensitivity to Cu

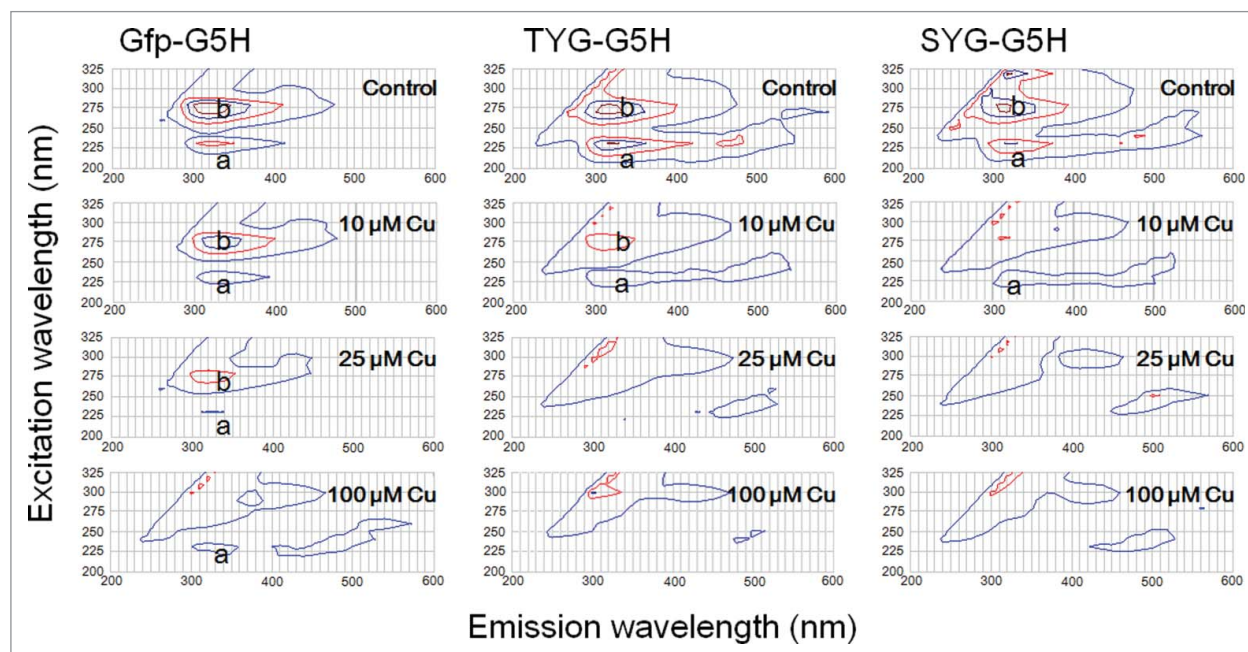
The intrinsic fluorescence signals (the peaks a and b) from the three G<sub>5</sub>H-conjugated GFP-derived peptides (30  $\mu\text{M}$  of Gfp-G<sub>5</sub>H, TYG-G<sub>5</sub>H, and SYG-G<sub>5</sub>H) plotted on 3-dimensional contour graphs were compared. Data showed that the intrinsic fluorescence by all peptides can be quenched in the presence of  $\text{CuSO}_4$  (10, 25 and 100  $\mu\text{M}$ ; Fig. 2). Among three peptides examined, SYG-G<sub>5</sub>H was most sensitive to lower range of  $\text{CuSO}_4$  concentration (10  $\mu\text{M}$ , molar ratio to peptide: *ca.* 0.17–0.33). The fluorescent signals from TYG-G<sub>5</sub>H also showed higher sensitivity to Cu as compared to the signals from Gfp-G<sub>5</sub>H.

### Linear relationship between loading of $\text{Cu}^{2+}$ and quenching of fluorescence

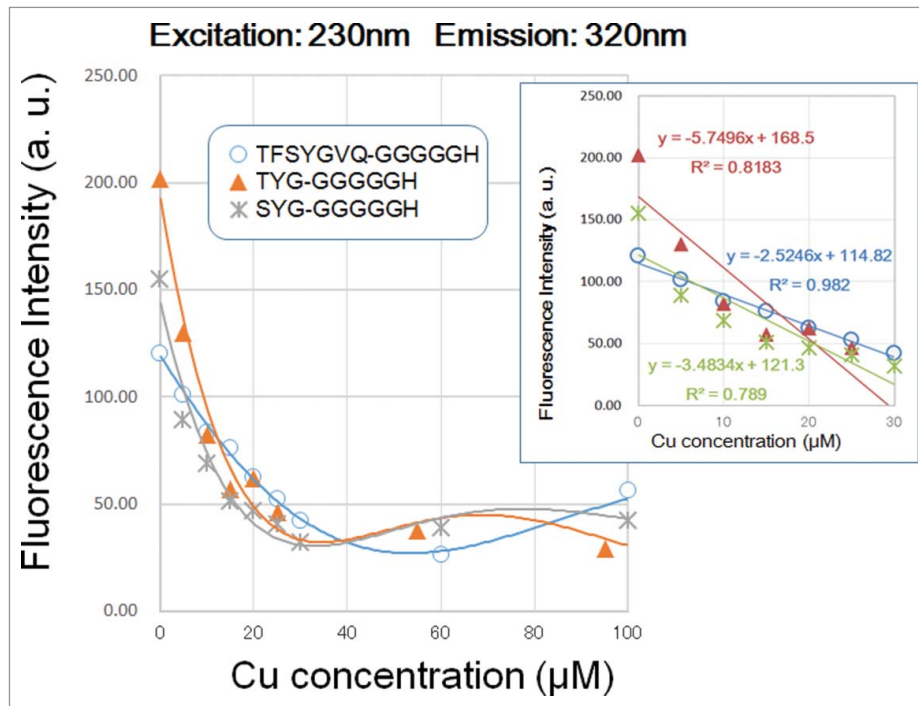
Among three peptides examined, only Gfp-G<sub>5</sub>H showed linear decrease in fluorescent signal along with occupancy with copper in the range between 0.17 and 1.33 of molar ratios of  $\text{Cu}^{2+}$  over peptide (Figs. 3 and 4). Note that within this range of copper concentration, the squared correlation coefficients ( $r^2$ ) for Cu-dependent quenching of fluorescence signals at the peaks a and b in Gfp-G<sub>5</sub>H were 0.982 and 0.989, respectively (Figs. 3 and 4, insets). On the other hand,  $r^2$  for regressions corresponding to Cu-dependent quenching of fluorescence signals by TYG-G<sub>5</sub>H and SYG-G<sub>5</sub>H ranged at relatively low scores between 0.700 and 0.906 (Figs. 3 and 4, insets), suggesting that linear relationship between the quenching of TYG- and SYG-conjugated molecules are



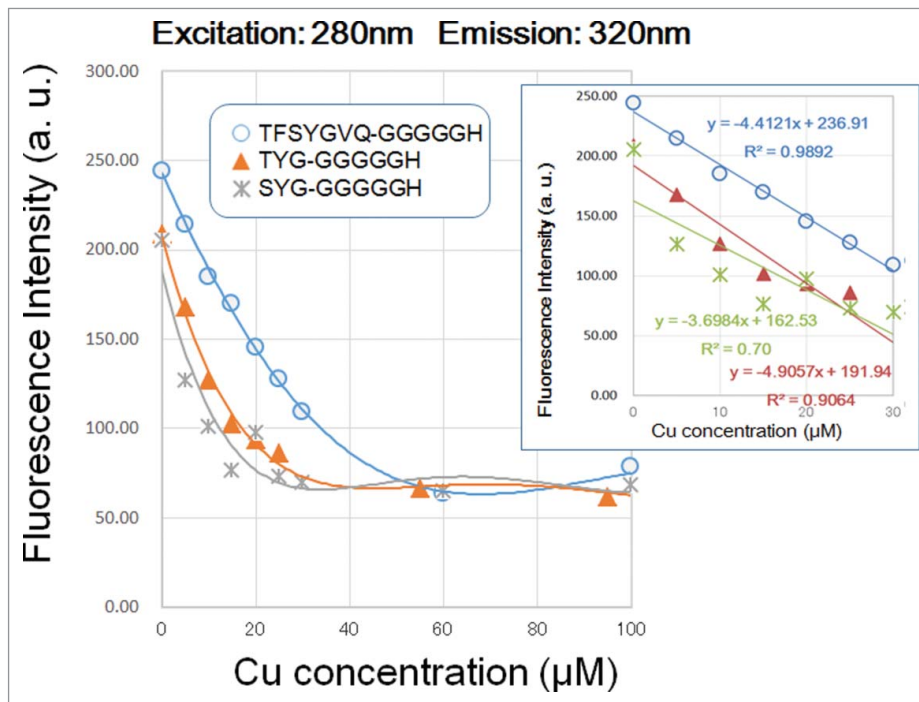
**Figure 1.** Different quenching action of copper ion against intrinsic tyrosine fluorescence in GFP-derived fluorophore sequence with and without fusing to copper binding sequence hexapeptide. Peptides ( $30 \mu\text{M}$ ) used were Gfp-G<sub>5</sub>H (TFSYGVQ-GGGGGH) and Gfp (TFSYGVQ). Peaks of tyrosine fluorescence (emission at ca. 320 nm) were observed with excitation at 230 nm (a) and 280 nm (b). Quenching of fluorescence in the presence of  $30 \mu\text{M}$  CuSO<sub>4</sub> was assessed.



**Figure 2.** Effect of copper ion on quenching of intrinsic fluorescence signals by three GFP fluorophore-derived oligo-peptides conjugated with copper-binding hexapeptide motif. Peptides ( $30 \mu\text{M}$ ) used were Gfp-G<sub>5</sub>H (TFSYGVQ-GGGGGH), TYG-G<sub>5</sub>H (TYG-GGGGGH), and SYG-G<sub>5</sub>H (SYG-GGGGGH). In the absence of CuSO<sub>4</sub>, two typical peaks of tyrosine fluorescence (emission at ca. 320 nm) were observed with excitation at 230 nm (a) and 280 nm (b). Quenching of fluorescence in the presence of 10, 25 and  $100 \mu\text{M}$  CuSO<sub>4</sub> was assessed.



**Figure 3.** Effect of copper concentration on quenching of 230 nm excitation/320 nm emission signals by three G<sub>5</sub>H-conjugated GFP fluorophore-derived oligo-peptides. Peptides (30  $\mu\text{M}$ ) used were as in Figure 2. Quenching of fluorescence was performed with 5–100 and 100  $\mu\text{M}$  CuSO<sub>4</sub> was assessed. Three different symbols represent the data points obtained. Curves were merely approximation of the response (note that they are not regression curves). In the inset, linear relationships between the remitted range of Cu concentration (up to 40  $\mu\text{M}$ ) and the decrease in peptidic fluorescent signals are shown.



**Figure 4.** Effect of copper concentration on quenching of 280 nm excitation/320 nm emission signals by three G<sub>5</sub>H-conjugated GFP fluorophore-derived oligo-peptides. Peptides (30  $\mu\text{M}$ ) used were as in Figure 2. Quenching of fluorescence was performed as in Figure 3. In the inset, linear relationships between the remitted range of Cu concentration (up to 40  $\mu\text{M}$ ) and the decrease in peptidic fluorescent signals are shown.

less significant compared to the Gfp-conjugated molecule. Therefore, we can conclude that the fluorometric kinetics reported by Gfp-conjugated G<sub>5</sub>H peptide is most proportional to the copper occupancy in G<sub>5</sub>H sequence.

Previously, catalytic nature of G<sub>5</sub>H hexapeptide was assessed.<sup>11</sup> It has been shown that catalytic activity in G<sub>5</sub>H peptide requires the binding of copper to it. Furthermore, Michaelis constant ( $K_m$ ) for O<sub>2</sub><sup>•-</sup> production using tyramine as a model substrate for Cu/G<sub>5</sub>H complex (0.15 mM) was determined to be 0.24 mM. Then,  $V_{max}$  at molar basis and weight basis were determined to be 52.91 mmol (O<sub>2</sub><sup>•-</sup>) mmol (peptide)<sup>-1</sup> min<sup>-1</sup> and 0.12 mmol (O<sub>2</sub><sup>•-</sup>) mg (peptide)<sup>-1</sup> min<sup>-1</sup>, respectively.<sup>11</sup> With molar-basis comparison, the catalytic activity looks weak, however, due to its low molecular weight characteristics, weight-basis comparison of the catalytic activity reaches applicable range which is almost 1/6 of purified horseradish peroxidase.<sup>11</sup> It is obvious that this type of approach for creating heat-stable biocatalysts require further innovation. Therefore, we expect that the use of Gfp-fused G<sub>5</sub>H peptide for quantification of Cu-binding to G<sub>5</sub>H motif may contribute to further engineering of the G<sub>n</sub>H-based catalytic peptides.

### Fate of tyrosine residue after oxidative reaction

Up to here we mostly discussed the role of His-ended metal binding motif in novel class of catalytic peptides with aid by fluorescent signal which could be attributed to the presence of Tyr residue. We view that Tyr residue has an additional important role in designing the peptidic O<sub>2</sub><sup>•-</sup>-generating catalysts.<sup>15</sup>

Reportedly, supplementation of structurally similar free catecholamine-related chemicals (tyramine or phenylethylamine), a free amino acid Tyr (Y), or Tyr-rich oligopeptides (such as tyrosyl-tyrosyl-arginine, YYR) as model substrates (instead of typical peroxidase substrates such as phenolics or amines) to the reaction mixture containing Cu-bound peptides sharing X-X-H motif such as Cu/VNITKQHTVTTTT (helical Cu-binding motif in mammalian PrP;<sup>24</sup> Cu/G;GGTH (short Cu-binding motif in human PrP;<sup>14</sup> Cu/G;GGGGH (artificial catalyst;<sup>11</sup> Cu/GGGFGH,<sup>25</sup> or Cu/NPGFPH<sup>26</sup> resulted in H<sub>2</sub>O<sub>2</sub>-dependent O<sub>2</sub><sup>•-</sup>-generation. Notably, Y residue-containing peptides with X-X-H motif including Cu/GGGYGH (plant ozone-inducible peptide sequence;<sup>25</sup> Cu/NPGYPH (chicken PrP hexa-repeat sequence;<sup>26</sup> and Cu/FLTEYVA-GGGGGH (Erk1/Erk2 MAP kinase substrate sequence fused with metal binding sequence designated as ErkG<sub>5</sub>H;<sup>15</sup> showed catalytic activity for H<sub>2</sub>O<sub>2</sub>-dependent O<sub>2</sub><sup>•-</sup> generation without supplementation of any free phenolic substrates. These knowledges suggest that Y-residue mimics the role for free phenolics in H<sub>2</sub>O<sub>2</sub>-dependent O<sub>2</sub><sup>•-</sup> generating reactions.

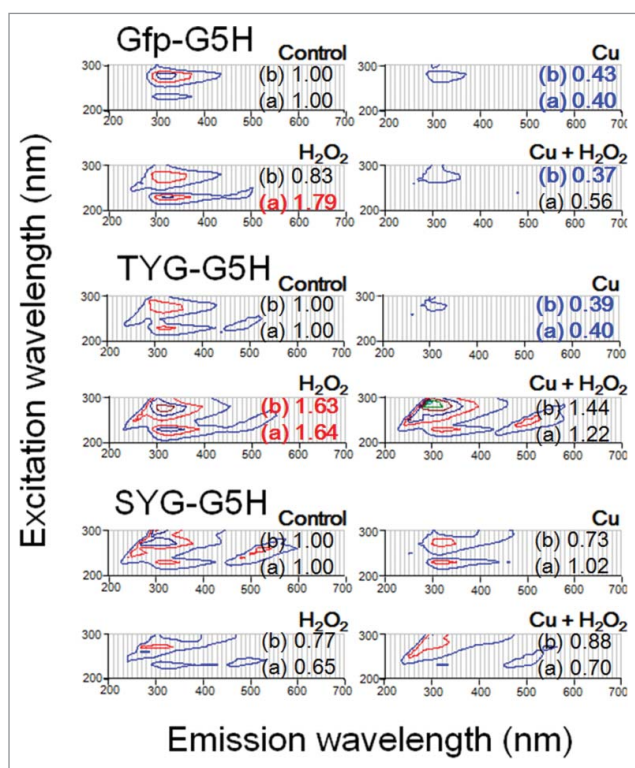
The above view was supported by Y-to-F mutation in chicken PrP sequence<sup>24</sup> and plant ozone-inducible peptide sequence<sup>25</sup> by which Tyr residues on catalytic peptides were replaced with Phe residues. The difference was merely the presence and absence of the OH group on the aromatic ring. Moreover, masking of Y-residue (at OH group) in ErkG<sub>5</sub>H through tyrosyl phosphorylation in Erk1/Erk2 MAP kinase substrate moiety of ErkG<sub>5</sub>H peptide was performed, and catalytic activity for H<sub>2</sub>O<sub>2</sub>-dependent O<sub>2</sub><sup>•-</sup> generation was largely lost in the resultant Y-phosphorylated peptide.<sup>15</sup> To date, ErkG<sub>5</sub>H is the only artificial catalyst which can be attenuated by phosphorylation event.

Involvement of free Y or Y residue in the Cu/peptide complex-catalyzed H<sub>2</sub>O<sub>2</sub>-dependent generation of O<sub>2</sub><sup>•-</sup> suggests that a phenoxy radical derived from Y (tyrosyl radical, Y<sup>•</sup>) can be formed in aid of single electron reduction of molecular oxygen, by analogy to plant enzymes H<sub>2</sub>O<sub>2</sub>-dependently generating O<sub>2</sub><sup>•-</sup> by coupling to oxidation of phenolics (such as salicylic acid) to form phenoxy radicals.<sup>17,18</sup>

There would be two distinct models for the fate of Y residue in model Cu/peptide complex. One likely model is that Y-residue is simply consumed and oxidized by the Cu-bound catalytic center in the presence of H<sub>2</sub>O<sub>2</sub> as observed for various free phenolics.<sup>11</sup> Another likely model is that Y residue lasts longer by repeatedly participating the reaction as shuttle for transferring electron, by analogy to the putative intra-molecular substrate-like roles for Y residues within ribonucleotide reductases,<sup>1</sup> and cyclooxygenase-2,<sup>20</sup> in which corresponding reactions proceed *via* transient formation of Y<sup>•</sup> and recycling of Y.

To obtain a clue to this view, we examined the fate of Y-dependent fluorescence in Gfp-G<sub>5</sub>H, TYG-G<sub>5</sub>H, and SYG-G<sub>5</sub>H peptides after addition of Cu and H<sub>2</sub>O<sub>2</sub> (Fig. 5). Ratio of H<sub>2</sub>O<sub>2</sub> concentration (1 mM) over Cu/peptide concentration (30 μM) was set at excess level since higher range of H<sub>2</sub>O<sub>2</sub> concentration has been employed in the previous studies using G<sub>5</sub>H-based catalysts.

Addition of copper to three peptides largely lowered the fluorescence signals as described earlier in this report. Addition of H<sub>2</sub>O<sub>2</sub> to SYG-G<sub>5</sub>H lowered the fluorescence signals. Contrary, addition of H<sub>2</sub>O<sub>2</sub> enhanced the fluorescence at both peaks a and b in TYG-G<sub>5</sub>H and the peak a in Gfp-G<sub>5</sub>H. The reason why two peaks of Y fluorescence in different peptides showed different sensitivity to H<sub>2</sub>O<sub>2</sub> should be attributed to the fact that even a monomer of phenolic compound often possesses multiple fluorophores within the molecule despite its simple structure as in the case of ferulic acid.<sup>4,8</sup>



**Figure 5.** Changes in UV-excited fluorescence contour spectra in three peptides after addition of copper and/or excess hydrogen peroxide. Peptides used were as in Figure 2, namely Gfp-G<sub>5</sub>H, TYG-G<sub>5</sub>H and SYG-G<sub>5</sub>H. Each peptide (30  $\mu$ M) was treated with none, either or both of CuSO<sub>4</sub> (30  $\mu$ M) or/and H<sub>2</sub>O<sub>2</sub> (1 mM). Numbers after (a) and (b) shown with each spectrum represent the relative changes in fluorescence intensities at peaks a (230 nm excitation/320 nm emission) and b (280 nm excitation/320 nm emission), respectively.

To the combination of Cu and H<sub>2</sub>O<sub>2</sub>, three peptides responded differently. Response to the Cu/H<sub>2</sub>O<sub>2</sub> co-treatment in Gfp-G<sub>5</sub>H was almost identical to the response to Cu alone. Changes in SYG-G<sub>5</sub>H were less obvious. The fluorescence intensities at 230 nm excitation/320 nm emission and 280 nm excitation/320 nm emission corresponding to the peaks a and b in Cu/H<sub>2</sub>O<sub>2</sub> co-treated TYG-G<sub>5</sub>H were seemed to be maintained at higher level compared to control. Note that the peak excitation wavelength at peak b fluorescence slightly shifted from 280 to 290 nm, therefore the product of peptide-catalyzed redox reaction challenging Y-residue under Cu/H<sub>2</sub>O<sub>2</sub> co-treatment must be no-longer intact Y residue. The case in TYG-G<sub>5</sub>H suggests that after possible formation of Y<sup>•</sup> via Cu/peptide-catalyzed H<sub>2</sub>O<sub>2</sub>-dependent reaction, recycling of Y did not sufficiently occur thus a spectral change (shift in the excitation peak) was observed.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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