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Review

Comparing the superoxide-generating activities of plant peroxidase and the action of prion-derived metallopeptides: Towards the development of artificial redox enzymes

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ABSTRACT

It is widely accepted that plant peroxidases (EC 1.11.1.7) can catalyze the generation of superoxide anion upon oxidation of substrates in the presence of hydrogen peroxide. Similarly, recent studies have shown that peptides derived from human prion protein (PrP) catalyses the generation of superoxide coupled to oxidation of neurotransmitters and their analogues. As human PrP possesses four putative copper-binding regions, the binding to copper confers the catalytic activities to PrP and derived peptides. Recent demonstrations suggested that PrP-derived copper-binding peptides catalyze the generation of superoxide in peroxidative manner involving hydrogen peroxide as electron acceptor and aromatic compounds or phenolics as electron donors. The least components required for the reaction were shown to be (i) short peptides with copper-binding capability, (ii) copper ions, (iii) hydrogen peroxide, and (iv) amines or phenolics. Notably, tyrosine residue(s) on PrP itself can be a good phenolic substrate, thus the superoxide-generating reaction could be completed within copper-bound PrP supplied with hydrogen peroxide. According to earlier studies, at least single histidine (His or H) residue is required for binding of copper, and the catalytically active copper-binding motif within PrP-derived peptides was determined to be X-X-H (where X can be any amino acids followed by His). This review covers the latest results performing and explaining the mechanism of catalytic activities found in copper-bound short peptides derived from PrPs, by comparing the mechanism for the reactions catalyzed by natural plant peroxidase. Furthermore, chemical and biological approaches for designing the novel small-sized artificial enzymes mimicking the natural peroxidase are described.

KEYWORDS: artificial metalloenzyme, peptide engineering, plant enzyme, PrP

INTRODUCTION

Catalysts can be defined as the set of different types of materials as follows:

$$\{Cs\} = \{\{OCs\}, \{ICs\}, \{BCs\}\} \quad (1)$$

where *C*s are catalysts, *OC*s are organocatalysts, *IC*s are inorganic catalysts, and *BC*s are biocatalysts. While *OC*s represented by organic molecules such as proline-type [1] or guanidine-type catalysts [2] and *IC*s represented by inorganic molecules or complex such as metal-based catalysts [3] can be clearly defined based on their chemical natures, the term *BC*s simply suggests the origins but not the natures of these catalysts. In fact, within the set of *BC*s, there are two different subsets as follows:

$$\{BCs\} = \{\{Es\}, \{Ns\}\} \quad (2)$$

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where *E*s are enzymes and *N*s are nucleozymes.

Here, the nature of *BC*s components, both *E*s and *N*s can be confirmed as follows:

$$E\text{s} \in \{\{C\text{s}\} \cap \{\text{proteins} \cup \text{peptides}\}\} \quad (3)$$

$$N\text{s} \in \{\{C\text{s}\} \cap \{\text{RNA} \cup \text{DNA}\}\} \quad (4)$$

Furthermore, within the set of *E*s, there are two different subsets, as follows:

$$\{E\text{s}\} = \{\{E_n\text{s}\}, \{E_a\text{s}\}\} \quad (5)$$

where E_ns are natural enzymes presented in or produced by biological organisms, and E_as are artificial enzymes newly designed or engineered in the laboratory.

Among the enzymes defined above, a great number of both natural and artificial enzymes directly bind metals or possess prosthetic groups such as iron-centered hemes functioning as the center of catalytic reactions [4]. Therefore, catalytic activities of such enzymes can be attributed to the chemistry of metals. Thus, following proposition can be supported.

$$P(E\text{s}) = \exists\, E\text{s} \in \{IC\text{s}\} \quad (6)$$

This type of enzymes are so called metalloenzymes. Recent review article on metalloenzymes stated that an artificial metalloenzyme would be ideally designed *de novo* from the 20 natural amino acids and the *de novo* design of a metalloprotein is based on the construction of polypeptide sequence which is not directly correlated to any natural protein and that can fold in well-defined three dimentional structure capable of binding metal ions [4]. However, it is far easier to learn the best motifs of catalytic peptides from the naturally existing active enzymes or proteins, or to modify the motifs using the natural peptide sequences as the platforms of engineering.

One of recent successes in metalloenzyme engineering is the modification of a hemoprotein, myoglobin (Mb). Yeung *et al.* have re-designed the natural Mb into a functional nitric oxide reductase, by creating a novel non-heme iron binding site in the distal pocket of Mb [5]. By this way, leaning from and modification of natural proteins are of great importance in designing a novel class of artificial enzymes.

In this review article, recent approaches for leaning and modification of natural plant peroxidase reactions leading to release of superoxide anion radicals ($O_2^{\bullet-}$) are covered. It is widely accepted that plant peroxidases can catalyze the generation of reactive oxygen species (ROS), chiefly $O_2^{\bullet-}$ upon oxidation of substrates such as aromatic monoamines or phenolics in the presence of H_2O_2 [6]. By assuming that peptides (both natural and artificial) with catalytic activity can be considered as enzymes in a wide sense as defined above (eq. 3, 5, 6), both plants [7] and animals [8] are rich in such small peptidic metalloenzymes catalyzing the generation of $O_2^{\bullet-}$. The key features of reported peroxidase-like small peptides include the presence of histidine (His)-rich motifs, binding to copper, and requirement for aromatic monoamines or phenolics as substrates [7].

Similarly, recent studies have shown that peptides derived from human prion protein (PrP) catalyses the generation of $O_2^{\bullet-}$ coupled to oxidation of aromatic monoamines or phenolics which are neurotransmitters and their analogues [9]. As human PrP possesses four different putative copper-binding motifs, the binding to copper confers the catalytic activities to PrP and derived peptides. Recent demonstrations suggested that PrP-derived copper-binding peptides catalyze the generation of $O_2^{\bullet-}$ in peroxidative manner involving H_2O_2 as electron acceptor and aromatic amines or phenols as the electron donors [9, 10].

The following sections cover the latest results performing and explaining the mechanism of catalytic activities found in copper-bound short peptides derived from PrP, by comparing the mechanism for the reactions catalyzed by natural plant peroxidase. Furthermore, chemical biological approaches for designing the novel small-sized artificial metalloenzymes mimicking the peroxidase are described.

Natural plant peroxidases catalyzing the reactions yielding superoxide

Plants are rich sources of enzymes involved in production and removal of ROS [11]. A group of pioneering researchers of peroxidases of plant origins in Switzerland metaphorically described that plant peroxidases possess more functions than

a ‘Swiss army knife’ [12]. Indeed, highly diversified functions of plant peroxidases including regulation of H_2O_2 level, oxidation of various substrates, generation of ROS coupled to oxidation of aromatic monoamines such as phenylethylamine [13, 14] and phonolics such as salicylic acid [15] in living plants have been reported to date. In plants, peroxidases achieve a great deal of oxidation reactions essential for the cells, using H_2O_2 as an electron (e^-) acceptor and a variety of substrates as e^- donors [6]. By synthesizing or inducing certain isoforms of peroxidase at specific timing and localization, thus by making use of a variety of plant peroxidase functions, the living plants can respond to and combat a wide variety of biotic and abiotic factors threatening the plants [16, 17]. As intensively discussed among the plant research community, the oxidation of phenolics is one of the key functions of the ‘knife’ [12]. The formulae shown below originally proposed for describing the mechanism for salicylic acid-dependent generation of $O_2^{\bullet-}$ in plant system [18, 19] suggest that the byproducts of peroxidase-catalyzed oxidation of phenolics are necessarily involved in generation of $O_2^{\bullet-}$.

$$N^{(3)} + H_2O_2 \rightarrow I^{(5)} + H_2O \quad (7)$$

$$I^{(5)} + PhOH \rightarrow II^{(4)} + PhO^{\bullet} \quad (8)$$

$$II^{(4)} + PhOH \rightarrow N^{(3)} + PhO^{\bullet} \quad (9)$$

$$2\ PhO^{\bullet} + 2\ O_2 \rightarrow 2\ PhO^{+} + 2\ O_2^{\bullet-} \quad (10)$$

where N, I, II are native ferric peroxidase and its Compounds I and II, respectively. $PhO^{\bullet}$ and PhO^{+} are free radical species and the two-electron oxidized intermediate product derived from phenolics (PhOH), respectively. Numbers in the small brackets indicate the formal oxidation states of the heme within the enzyme. Here, PhOH behaves as the e^- donor while H_2O_2 acts as the e^- acceptor. Then the released $PhO^{\bullet}$ may react with O_2 to form $O_2^{\bullet-}$. As $O_2^{\bullet-}$ can be readily converted to H_2O_2, one cycle of PhOH-oxidizing peroxidase reaction started with single unit of H_2O_2 results in yield of two units of $O_2^{\bullet-}$ equivalent to two units of H_2O_2, thus ROS members are amplified [6].

In place of phenolics, aromatic monoamines could be used as another group of active substrates [13, 14]. Pinontoan and his colleagues have shown that aromatic monoamine-dependent oxidative burst can be widely observed not only in plants but also in yeast cells and human hemoglobin [20, 21].

In case of salicylic acid oxidation by plant enzymes, experimental evidence in support of the production of salicylate radical species (one of $PhO^{\bullet}$) has been obtained using electron spin resonance spectroscopy by employing ascorbate as a sensitive spin trapper [19]. After above studies, the likely structures of the radical and derived cationic intermediate were proposed by Gozzo [22]. In addition, the involvement of Compound I and II as the intermediate species required for salicylate-dependent and monoamine-dependent $O_2^{\bullet-}$ generation was spectroscopically confirmed [23, 24].

In plants, the salicylic acid-dependently produced $O_2^{\bullet-}$ acts as a chemical signal required for development of defense mechanism against pathogenic microbes [18] and closure of stomata on leaves [25]. In model plant cells, TPC1 calcium-permeable cation channel is a likely target of the salicylic acid signal transduction pathway mediated with $O_2^{\bullet-}$ [26].

Apart from H_2O_2-requiring reaction, plant peroxidases are also capable of $O_2^{\bullet-}$ generation depending on the substrates. Metabolism of indole-3-acetic acid (IAA), the principal form of auxin in higher plants, is of great interest to plant biologists. Plant peroxidases are considered to be involved in the metabolism of IAA, by oxidizing IAA *via* two different mechanisms: a conventional H_2O_2-dependent pathway and one that requires O_2 but not H_2O_2 [27-29].

The conventional peroxidase cycle for the oxidation of various substrates coupled to the consumption of H_2O_2 proceeds as follows:

$$N^{(3)} + H_2O_2 \rightarrow I^{(5)} + H_2O \quad (7)$$

$$I^{(5)} + S \rightarrow II^{(4)} + P \quad (11)$$

$$II^{(4)} + S + H^{+} \rightarrow N^{(3)} + H_2O + P \quad (12)$$

where S and P are the substrate and product of its one-electron oxidation, respectively [6].

IAA can be oxidized by plant peroxidases, chiefly by horseradish peroxidase (HRP) in model experiments, by this mechanism but there is no strict substrate specificity in this conventional H_2O_2-dependent peroxidase cycle [6]. The plant

peroxidases including HRP oxidize IAA also *via* the H_2O_2-independent pathway requiring molecular oxygen (O_2), since most peroxidases of plant origins (but not animal or microbial origins) are considered to be highly specific IAA oxygenases, which possess the domains structurally similar to a specific motif in auxin-binding proteins [27]. The proposed reaction cycle for IAA oxidation is initiated *via* the formation of a ternary complex [28], enzyme-IAA-dioxygen, yielding IAA cation radicals and $O_2^{\bullet-}$ as by-products as follows [29]:

$$E + IAA \leftrightarrow [E\text{-}IAA] \quad (13)$$

$$[E\text{-}IAA] + O_2 \leftrightarrow [E\text{-}IAA\text{-}O_2] \quad (14)$$

$$[E\text{-}IAA\text{-}O_2] \rightarrow E + IAA^{\bullet+} + O_2^{\bullet-} \quad (15)$$

where E and $IAA^{\bullet+}$ stands for enzyme and IAA cation radicals, respectively. Thus, plant peroxidases are capable of catalyzing the IAA-dependent generation of $O_2^{\bullet-}$ in the absence of H_2O_2. However, depending on the concentrations of H_2O_2 and IAA, plant enzymes are readily inactivated and degraded by forming P-670 pigment which is an irreversibly inactivated form [30]. While the nature of the enzyme during formation of enzyme-substrate complexes such as [E-IAA] and [E-IAA-O_2] has not been identified [6, 29], medical application of horseradish peroxidise-labelled antibodies and IAA has been proposed as a novel $O_2^{\bullet-}$-generating system for cancer cell-targeted and controlled cell death induction [31].

As above, the $O_2^{\bullet-}$-generating reactions catalyzed by plant peroxidise can be dissected into the H_2O_2-dependent peroxidise cycle and and H_2O_2-independent oxygenase cycle as illustrated (Fig. 1). This model is often referred to as the hourglass model due to its shape [6, 15, 32]. In addition to the oxygenase cycle, we have recently observed that the conventional peroxidise cycle represented by involvement of Compound II can be mimicked in the absence of H_2O_2 depending on the conditions given as discussed below.

As an example with modified Mb reaction was given in the introductory section [5], both the leaning from and modification of the properties of natural enzymes are of great importance in designing a novel class of catalysts. Recently, soybean peroxidase (SBP), one of widely used model plant enzymes, was used for designing the

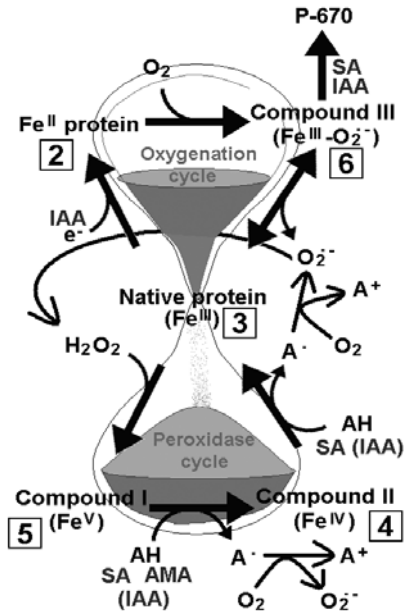

Fig. 1. Hourglass model that summarizes the inter-conversions among active and inactive forms of peroxidase intermediates. The model was modified from earlier works [6, 15, 32]. The boxed numbers indicate the formal oxidation states of the enzyme and its intermediates.

novel catalytic path independent from H_2O_2 [32]. Interestingly, addition of nitric oxide to SBP in the absence of H_2O_2 resulted in formation of an intermediate species of protein which is spectroscopically resembling the Compound II of the enzyme, which is one of two catalytically active forms in peroxidase cycle. The NO-induced intermediate was shown to be catalytically active capable of returning to the native form in response to addition of substrates, therefore, novel gaseous regulation of SBP-mediated reaction cycle without involvement of H_2O_2 was proposed. In this approach, SBP is still used as an oxidative catalyst for peroxidase substrates but from the point of the mechanism of reactions, the mode of action observed should be no longer described as peroxidative.

Plant peptides with superoxide-generating activity

Ozone is a major secondary air pollutant, threatening the living plants as one of important environmental factors, often reaching high concentrations in the urban areas under strong daylight. Several lines of studies 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 [33, 34]. Recent studies revealed the signaling pathways involving the members of ROS within the living plant cells, in which ozone-exposed cells determine their fate to survive or to initiate the localized apoptotic cell death [35, 36]. One of key factors required for plant defense against ozone toxicity was shown to be endogenous peroxidase [35].

In addition to peroxidases, small peptides reportedly participate in the plant responses to ozone [7]. As examples of plant responses to ozone, ozone-inducible (OI) genes have been isolated from several plants such as 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, suggesting the multiple roles for these genes [38]. 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, Y-G-H-G-G-G; 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 designated as GRPs [37].

In general, GRPs are known to be inducible by wounding, drought and water-deficient in plants, as they were firstly discovered as the members of cell wall-associated proteins. Therefore, one of their key molecular functions suggested was to act as wound-responsive factors contributing to the strengthening of the cell wall [38]. The secondary structures of both the general GPRs and OI peptides were reported to be rich in β-pleated sheets [37, 38] as their similarity to prion proteins (PrPs) from animal systems was suggested [7].

Recently, Yokawa *et al.* [7] have demonstrated that the hexa-repeat unit found in OI peptides behaves as a metal-binding motif and the model peptides derived from the hexa-repeat in OI peptides actively catalyzed the generation of $O_2^{\bullet-}$. Interestingly, in the above report, possible mechanism of the reaction and biological consequence of the reactions catalyzed by OI peptides were discussed by analogy to the action of human PrP's octarepeat peptides (Fig. 2).

Cu-binding sites on prion proteins (PrPs)

Importance of metalloproteins in neurobiology has been shown both as oxidant and antioxidant in neurodegenerative processes (Opazo, 2003). Cu is an essential trace element but its redox reactivity leads to the risk of damage to the cells and tissues, especially in neurodegenerative diseases such as Menkes' and Wilson's diseases occurring *via* disorders of Cu metabolism, and Alzheimer's disease and 'prion' diseases, the two major conformational diseases, as documented [39-41]. In the cases of β-amyloid accumulation in Alzheimer's disease and of α-synuclein accumulation in Parkinson's disease, the evidence for involvement of ROS such as H_2O_2 and hydroxyl radicals ($HO^{\bullet}$) in the toxic mechanisms have been documented, suggesting that fundamental molecular mechanisms underlying the pathogenesis of cell death in neurodegenerative diseases could be attributed to the production of ROS that stimulates the formation of abnormal protein aggregates [42, 43].

Deposition of abnormal protein fibrils is a prominent pathological feature of many different "protein conformational" diseases, including prion dementias, Alzheimer's disease, Parkinson's disease and motor neuron disease [42]. PrPs are causative agents for transmissible spongiform encephalopathies in mammalian brains [44]. Recent studies have shown that PrPs can form a novel group of Cu-binding proteins possibly involved in redox reactions [45, 46].

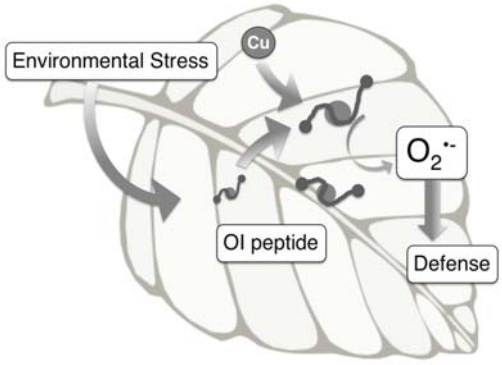

Fig. 2. The hypothetical role for copper-binging plant OI-peptide acting against environmental stress. Generation of $O_2^{\bullet-}$ catalyzed by OI-peptide in oxidatively stressed plants may result in cell wall strengthening and protection of plants from pathogenic microbials, by analogy to the actions of macromolecule plant peroxidases.

Human PrP has four Cu-binding sites in the "octarepeats" region (PrP 60-91) in which amino acid sequence P-H-G-G-G-W-G-Q appears four times in tandem and each repeat possibly binds single Cu^{2+} at physiological neutral and basic range of pH [47]. In chicken, the Cu-binding sites analogous to the octarepeats are known as hexa-repeats with each repeat consisting of the amino acid sequence H-N-P-G-Y-P and here again His residues play a key role in anchoring of Cu [48].

Earlier, *in vitro* studies have shown that the actual least motif in the octarepeats necessarily required for binding of Cu consists of 5 amino acids H-G-G-G-W [49] or 4 amino acids H-G-G-G [47]. In general, the most relevant copper binding mode for human PrP would involve the formation of a 4:1 octarepeat:Cu complex through coordination of one single Cu molecule by four His residues on the 4 octarepeat segments of a single PrP molecule [50]. Morante *et al.* [51] showed that partial occupancy of Cu on bovine PrP is manifested by binding of Cu to PrP in the intermolecular or inter-octarepeat orientations while total occupancy of Cu is manifested by intra-repeat binding of Cu to the octarepeat region.

There are additional Cu-binding sites on PrP such as amino acid regions 92-96 (G-G-G-T-H) [46], 124-126 (K-H-M) [52] and 180-193 (V-N-I-T-I-K-Q-H-T-V-T-T-T-T) [53], and all studies suggested that His residue in each region (or each repeat unit) plays a key role in anchoring of Cu (Fig. 3).

Interestingly, the metal binding properties of these PrP-derived peptides can be directly assessed by loading of fluorescent metal ions such as Tb^{3+} [54]. Currently, our group is screening a series of active metal-binding peptides based on the real-time fluorescence monitoring using the Tb^{3+} binding model (unpublished results).

As suggested above, many of metalloproteins possibly behave both as oxidants and as antioxidants in biological systems. As expected, the PrP-derived Cu-binding small peptides were shown to be active in both protection of biological components from the oxidative stress due to metal actions and enhancement of oxidative process, depending on the conditions given.

Binding of copper and protection of cells and DNA

Oxidative damage to DNA is reportedly promoted in the presence of ROS such as $HO^{\bullet}$, which can be generated through the Fenton-type or Harbor-Weiss-type reactions in the presence of the ions of transition metals (chiefly copper and iron), ascorbic acid and/or H_2O_2 [55]. Tb^{3+} and other rare earth element ions shown to be removed by PrP-derived peptides also catalyzes the degradation of DNA possibly due to similar mechanism [56].

Among the members of ROS, $HO^{\bullet}$ is the most highly reactive species oxidizing any neighboring molecules. Therefore, generation of $HO^{\bullet}$ in the biological systems results in immediate damages to DNA molecules and the subsequent DNA degradation may further leads to apoptotic reaction and carcinogenesis in the living cells. Such oxidative stress-mediated DNA fragmentation and chromosomal dysfunction play key roles in mammalian cell death mechanisms [57]. Through the DNA-degrading reactions in $Cu(II)/H_2O_2$ or Cu(II)/ascorbate systems, the production of a large amount of $HO^{\bullet}$ at physiological pH condition *via* Harbor-Weiss-like reaction has been recorded, by monitoring the level of 8-hydroxyguanosine which is a reliable biomarker for $HO^{\bullet}$-dependent oxidative damage to guanosine residues on DNA [55].

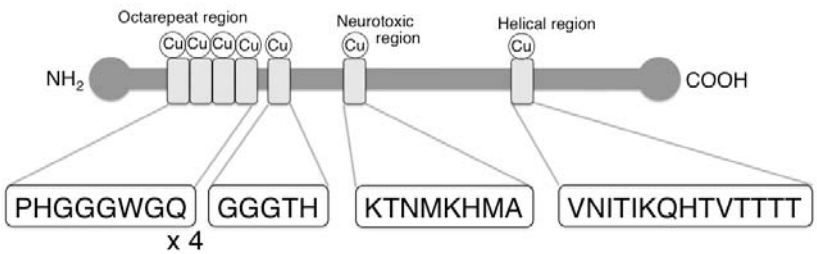

Fig. 3. Amino acid sequences corresponding to the copper-binding motifs in human PrP.

Due to hyper-reactivity of $HO^{\bullet}$ even against water molecules, $HO^{\bullet}$ hardly migrates even a short distance in aqueous phase, thus the generation of $HO^{\bullet}$ at the site vicinal to DNA likely results in enhanced degradation of or damages to DNA. According to earlier works, Cu^{2+} was shown to bind strongly to the guanosine and cytidine bases at physiological pH, eventually perturbing the A-T base pairs and disrupting the double-helical structure of DNA [58]. In addition, Z-DNA structure-like micro-domains, especially at the base guanine, show much higher affinity to binding of Cu^{2+} [59]. Accordingly, the complex between Cu and Z-DNA domains readily results in damaging to DNA molecules (Geierstanger *et al*. 1991).

Taken together, it is tempting to discuss that Cu-mediated Fenton-type reaction also occurs on site within DNA-Cu complex by effectively allowing the reactions between $HO^{\bullet}$ and DNA.

From a gerontological point of view, it is important to seek for the methods to prevent the Cu-mediated DNA damages. By chelating the Fenton catalysts such as Fe and Cu ions, the H_2O_2-dependent formation of $HO^{\bullet}$ can be effectively prevented. Yokawa *et al.* [60] have demonstrated that the tripeptides with X-X-H motif found within PrP-derived catalytic peptides, effectively bind Cu and prevent the DNA degradation caused by Cu-mediated reactions in the presence of H_2O_2 or ascorbic acid (Fig. 4A).

In addition to prevention of oxidative metal toxicity, Kagenishi *et al.* [61] have demonstrated by using a model plant cell culture (*Nicotiana tabacum* L., cell line BY-2) that application of K-T-N-M-K-H-M-A octapeptide derived from human PrP effectively protects the plant cells from the toxicity of copper which initiates the apoptotic signaling in the living plant cells. Copper actually induces a series of biological and chemical reactions in plant cells including the oxidative burst reflecting the production of ROS, such as $HO^{\bullet}$, and stimulation of calcium channel opening, allowing a transient increase in cytosolic Ca^{2+} concentrations. Following these early events completed within 10 min, the development of copper-induced plant cell death can be observed during additional 1 h in a manner depending on the dose of copper (in a μM-to-sub-mM range). As expected, addition of synthetic K-T-N-M-K-H-M-A octapeptide to the plant cells prior to the addition of copper, effectively blocked both the calcium signaling and the cell death (Fig. 4B).

Peroxidase-like catalytic activity yielding ROS

Among PrP-derived Cu-binding short peptides tested for H_2O_2-dependent $O_2^{\bullet-}$-generating activity, the helical sequence was shown to be most active [9], especially, an analog peptide sequence (V-N-I-T-K-Q-H-T-V-T-T-T-T) prepared by deleting a single Ile residue from native helical sequence showed much higher catalytic activity compared to the native sequence [62]. Although the octarepeat (P-H-G-G-G-W-G-Q-)-dependent oxidative burst was only about one fourth of the helical peptide-dependent one, this amino acid octet is repeated

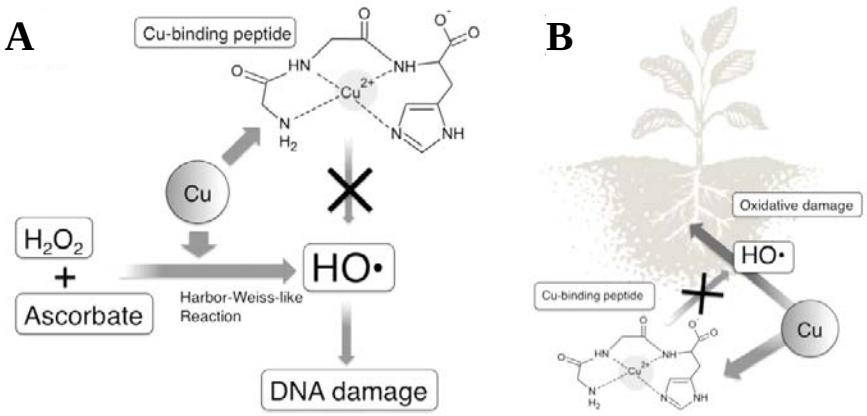

Fig. 4. Possible role of copper-binding peptide for protection of (A) DNA from the metal-dependent degradation and (B) plant roots from the metal toxicity.

4 times in human PrP and 6 times in bovine PrP, therefore, its contribution to oxidative burst in whole protein could be greater than that observed for the single repeat peptide.

Minimal sized peptides with peroxidase-like activity

To date, key involvement of metals (especially Cu) in prion disease has been well documented by a number of works [63-65]. However, two opposing roles for Cu-bound PrPs have been proposed, namely as anti-oxidants and contrary as pro-oxidants enhancing the neurodegenerative process [66]. In both cases, the likely factors associated with generation [9] or removal of ROS [64] within PrPs are Cu-binding sequences highly preserved in PrPs.

A series of works conducted by our group in Kitakyushu, Japan suggested that four distinct peptide sequences corresponding to the putative copper-binding sites containing metal anchoring His residues (His61, His69, His77, His85, His96, His111, and His187) in human PrP function as putative biocatalysts active for generation of $O_2^{\bullet-}$ in the presence of aromatic monoamines [9], free phenolics and tyrosine [8, 62], and tyrosyl residues on proteins [10]. The phenol-dependent $O_2^{\bullet-}$ generation catalyzed by several PrP-derived copper-binding peptides was recently assessed using various phenolics as substrates [8]. Since copper and H_2O_2 are required as cofactors for oxidation of amines or phenols, the reactions were considered to be peroxidase-like reactions catalyzed by the copper-centered peptides [9] (Fig. 5).

Among Cu-binding peptides derived from human PrP, the octarepeat unit (P-H-G-G-G-W-G-Q) performed the monoamine-specific $O_2^{\bullet-}$-generating activity using phenylethylamine as an active substrate [9], by mimicking the $O_2^{\bullet-}$-generating plant enzymes which are sensitive to monoamine oxidase inhibitors [13]. On the other hand, both G-G-G-T-H pentapeptide [8, 9] and a helical motif V-N-I-T-K-Q-H-T-V-T-T-T-T tridecapeptide [9, 10] derived from human PrP showed negligible monoamine-dependent activity while showing higher phenol oxidizing and $O_2^{\bullet-}$ generating activities. Therefore, by choosing the peptide sequences, substrate specifity of novel metalloenzymes can be properly designed.

Based on the results with PrP-derived peptides, our group has designed a series of novel peroxidative biocatalysts as discussed below.

Effects of peptide chain length and position of His residue

In addition to the helical Cu-binding peptide sequence, short region following the octa-repeat sequence in PrP was shown to be a good catalyst for tyramine, tyrosine, and other phenolics [8, 9]. Among the Cu-anchoring His residues in human PrP, we focused on the role of His96, corresponding to the His residue in the catalytically active G-G-G-T-H pentapeptide, and thus the effect of neighboring amino acid chains around the His96 was studied [8]. In human PrP, His96 is located between G-G-G-T sequence and S-Q-W-N sequence. Therefore to see the positional effect of His on the catalytic activity in the derived peptidic catalysts, the H-S-Q-W-N pentapeptide was used for comparison with the G-G-G-T-H pentapeptide. While reaction with tyramine and G-G-G-T-H peptide resulted in robust production of $O_2^{\bullet-}$, the H-S-Q-W-N peptide showed no catalytic activity [8]. This suggests that the position of His residue is highly important for designing novel peroxidase-like artificial metalloenzymes.

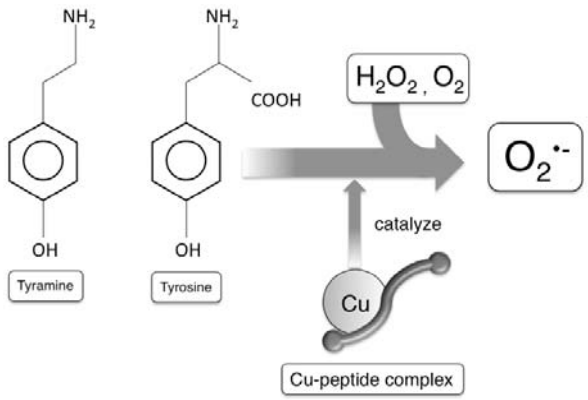

Fig. 5. Superoxide-generating activity catalyzed by prion-derived copper-bound peptide in the presence of phenolic compounds as a substrate. Similarly to plant peroxidase reactions, the $O_2^{\bullet-}$-generating reactions catalyzed by PrP-derived peptides require the involvement of two sets of substrates, namely, e^- acceptors chiefly H_2O_2 and e^- donors such as phenolics and aromatic monoamines, respectively. Here, O_2 can be considered as the final acceptor of e^-, thus, releasing $O_2^{\bullet-}$.

Furthermore, comparison of the catalytic activities among G-G-G-T-H pentapeptide and shorter derivatives with C-terminal His residues (G-G-T-H and G-T-H) suggested the importance of the N-terminal glycyl-chain elongation for manifesting the maximal redox activity.

Designing novel biocatalysts

By analogy to G-G-G-T-H sequence, we have designed a series of simplified model peptides composed of oligoglycyl chains and C-terminal His (G_nH series peptides) as putative biocatalysts. Since above data indicated the importance of the elongation of N-terminal glycyl chain, the peptides designed were differed in N-terminal glycyl chain length.

To test the importance of the C-terminal His, an additional series of peptides were also prepared for comparison. Among the newly designed peptides, the catalytic activity was found only within the G_nH series while none of oligoglycyl peptides lacking His (G_n series) showed catalytic activity. Within the G_nH series (n = 2, 3, 4, 5, and 10), the common minimal motif was G_2H tripeptide which is known as minimal but active element for binding of copper ions (Yang *et al.* 2003). However, catalytic activity of the minimal Cu-binging motif (G_2H tripeptide) was hardly detected but G_3H tetrapeptide showed a detectable increase in production of $O_2^{\bullet-}$, suggesting that N-terminal elongation of the Cu-binding peptide contribute to catalytic activity.

By single amino acid elongation (addition of Gly residue at N-terminal), the catalytic performance was *ca*. 3-fold enhanced (from G_3H to G_4H, and G_4H to G_5H). Furthermore, by elongating G_5H hexapeptide to $G_{10}H$ undecapeptide, catalytic activity was enhanced by *ca.* 2-fold. These data suggest that the presence of the C-terminal His is the primary requirement for catalytic performance, and elongation of N-terminal contribute to the enhancement of the catalytic activity (Fig. 6).

Substrate specificity in G_nH series metalloenzymes

Since earlier studies suggested that tyramine in neuronal tissues [9], free tyrosine, and tyrosine residues on PrP protein [10] can be natural substrates for PrP-derived metallopeptides, the $O_2^{\bullet-}$ generating activities in G_nH peptides were recently assessed with tyramine and tyrosine as substrates [8]. In the presence of two cofactors, Cu^{2+} and H_2O_2; additions of tyramine or tyrosine as a substrate resulted in immediate generation of $O_2^{\bullet-}$ monitored with chemiluminescence probe specific to $O_2^{\bullet-}$.

Although involvement of copper and generation of $O_2^{\bullet-}$ are analogous to tyrosinase which oxidizes tyrosine and polyphenols with concomitant release of $O_2^{\bullet-}$ [67], the roles played by H_2O_2 are largely different in the G_nH series metalloenzymes. While the reactions studied with the G_nH series metalloenzymes require the presence of H_2O_2 as a co-factor (or co-substrate), H_2O_2 is often regarded as an inhibitor of the tyrosinase reaction [68]. On the other hand, plant peroxidases such as HRP are shown to be active in generation of $O_2^{\bullet-}$ upon oxidation of various phenolics and monoamines in the presence of H_2O_2 [6, 19]. Therefore, we can conclude that the modes of reactions catalyzed by PrP-derived peptides and artificial G_nH series peptides are analogous to that of the plant peroxidise reactions.

Fig. 6. Effects of various peptides differed in size as the catalysts for tyramine-dependent $O_2^{\bullet-}$ generating reaction. The position of His residue is highlighted as bold and underlined H. Relative luminescence unit (rlu) represents the relative amount of $O_2^{\bullet-}$ generated in the reaction mixture (modified from Kagenishi *et al.* [9]).

Hydroxylbenzoates and phenols as $O_2^{\bullet-}$-generating substrates for G_nH series metalloenzymes

Among benzoic acid (BA) and hydroxybenzoic acids (HBAs) tested as $O_2^{\bullet-}$-generating substrates, BA and salicylic acid (2-HBA) was shown to be poor substrates for G-G-G-T-H pentapeptide and G_nH series metalloenzymes [8]. Results with BA were common to plant natural peroxidase. In plant system, removal or masking of hydroxyl group on HBA results in lowered generation of $O_2^{\bullet-}$ [69].

In contrast, 3-HBA and 4-HBA were shown to be good substrates for G-G-G-T-H pentapeptide and G_nH series metalloenzymes. These results suggest that the presence of phenolic moieties with *meta*- or *para*-positioned hydroxyl group is required for generation of $O_2^{\bullet-}$. This is a notable difference from the plant peroxidase which favors 2-HBA as $O_2^{\bullet-}$-generating substrates [18].

Kagenishi *et al.* [8] also tested the effects of dihydroxybenzoic acids (DHBAs). No generation of $O_2^{\bullet-}$ was observed after addition of 2,6-DHBA to G-G-G-T-H pentapeptide. Thus, the inactiveness of the *ortho*-positioned hydroxyl group was further confirmed. However, The G-G-G-T-H pentapeptide and G_nH series metalloenzymes showed $O_2^{\bullet-}$ generating activity upon addition of 2,3-DHBA, 2,4-DHBA, and 2,5-DHBA, suggesting that the presence of *ortho*-positioned hydroxyl group does not interfere with the roles for active hydroxyl groups at *meta*- and *para*-positions. Similar substrate specificity favoring hydroxyl groups on aromatic molecules with *meta*- and *para*-positions was also observed for the helical V-N-I-T-K-Q-H-T-V-T-T-T-T tridecapeptide derived from human PrP [10] and ozone-induced peptides from plants [7] (Fig. 7).

Since the actions of phenol moieties in HBA-derivatives were suggested, effects of phenol and benzendiols were also examined [8]. As expected, phenol and the *meta*-positioned benzendiol (resorcinol) were shown to be active while *ortho*-positioned benzendiol (catechol) was shown to be inactive. Interestingly, *para*-positioned benzendiol (hydroquinone) was shown to be inactive in generation of $O_2^{\bullet-}$.

In summary, generalized structures of phenolic substrates favored by the V-N-I-T-K-Q-H-T-V-T-T-T-T tridecapeptide, the G-G-G-T-H pentapeptide, and the G_nH series metalloenzymes are shown in Fig. 7.

Tyrosine-rich peptide sequences as possible targets

Above studies revealed that the common structural feature required to be active substrates for Cu-bound peptides derived from PrP was shown to be the presence of phenolic moiety. Thus, in case of tyrosine, the presence of phenolic moiety, but not the -NH_2 or -COOH groups, was shown to be important in interaction with Cu-loaded PrP peptide [10]. Therefore, it is natural to consider the tyrosine residues on peptides or proteins as putative targets of this type of reaction.

As the tertiary structure of human PrP is shown in Fig. 8A suggests, the human PrP possesses several Tyr-resides exposed to the external media. Recent reports suggested that helix H1 of PrP and its two flanking loops (highly rich in tyrosine residues) undergo a conformational transition into a β sheet-like structure during conformational conversion of the PrP^C into PrP^{SC}, which is a fundamental event in the onset of transmissible spongiform encephalopathies [70]. In this section, we describe our recent attempt to testify if the Tyr residues on PrP or derived peptides, in addition to free tyrosine, can be the putative targets of Cu-loaded catalytic PrP peptide [10]. Tested tyrosine-containing peptide was a tripeptide sequence (tyrosyl-tyrosyl-arginine, Y-Y-R) which can be found two times in the PrP's Tyr-rich region (DYEDR-YYR-ENMHRYPNQV-YYR-PMDEY). Our working

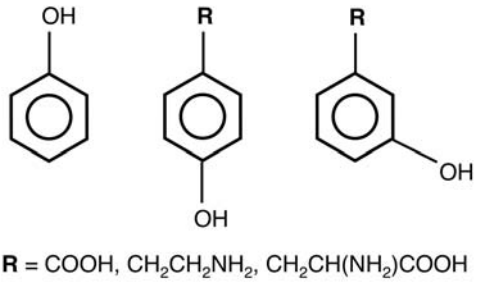

Fig. 7 Generalized structures of phenolic substrates favored by V-N-I-T-K-Q-H-T-V-T-T-T-T tridecapeptide, G-G-G-T-H pentapeptide, and G_nH series metalloenzymes.

hypothesis was that such duplicated YYR motifs conserved in bovine, goat, human and pig PrPs effectively act as potent inducers (substrates) of $O_2^{\bullet-}$-generating reaction catalyzed by Cu-bound catalytic peptide. In this demonstration, the Cu-loaded and H_2O_2-fueled V-N-I-T-K-Q-H-T-V-T-T-T-T helical peptide was used as a model catalyst. As expected, compared to free tyrosine, Y-Y-R tripeptide was shown to be much more active as an oxidative burst inducer. These data indicate that both free form and peptide-integrated forms of tyrosine can be recognized by the Cu-loaded catalytic peptide.

Furthermore, reactivity of longer peptide sequences corresponding to the Tyr-rich region in human PrP (D-Y-E-D-R-Y-Y-R-E-N-M-H-R) or its Y-to-F substitution mutant (D-F-E-D-R-F-F-R-E-N-M-H-R) with the Cu-loaded catalytic peptide were tested. When compared with the Y-to-F substitution mutant sequence, the level of $O_2^{\bullet-}$ production with PrP-derived native Tyr-rich sequence was higher, thus, indicating that the tyrosine residues on intra-, and inter-PrP molecules could be the additional targets of the Cu-bound PrP-catalyzed reaction.

Figs. 8B and C summarize the likely modes of interactions between the Cu-binding catalytic motifs and Tyr-rich motifs on human PrP, namely, intermolecular interaction (Fig. 8B) and intramolecular interactions (Fig. 8B).

Designing novel metalloenzymes directly converting H_2O_2 to superoxide without additional substrates

One of our goals in metallopeptide study is to engineer the novel peroxidase mimics without phenol requirement, thus capable of $O_2^{\bullet-}$ production simply by addition of H_2O_2, as inspired by our recent work demonstrating that tyrosine-containing peptides can participate in the metallopeptide-catalyzed $O_2^{\bullet-}$-generating reactions [10]. Recent reports described that one plant species (salt bush, *Atriplex canescens*) produces a series of peptides so-called ozone-inducible peptides containing PrP-like repeated sequence consisted of a tyrosine-

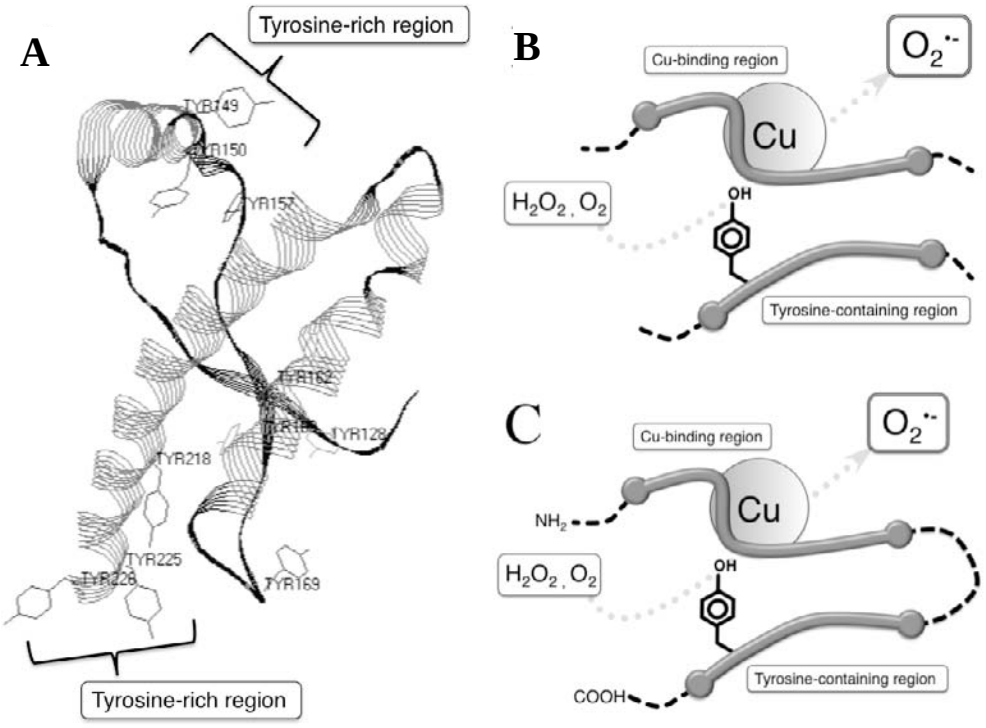

Fig. 8. Tyrosine residues on human PrP and proposed modes of interaction between copper-binding region and tyrosine residues. (A) A tertiary structure of human PrP emphasizing the presence of Tyr residues. (B) Intermolecular interaction model. (C) intramolecular interaction model.

containing hexa-repeat unit repeated for 8-10 times in tandem [37]. According to Yokawa *et al.* [7], the repeat unit found in the ozone-induced peptides shows H_2O_2-dependent $O_2^{\bullet-}$ producing activity by using self-tyrosine residues as phonolic substrates required for peroxidative process, thus simply converting H_2O_2 to $O_2^{\bullet-}$ without requirement for additional phenolics or amine as substrates. By analogy to the plant peptides, it is tempting to test the possibility for inventing modified artificial peroxidase available simply by merging a Tyr-containing short peptide with the G_nH series metallopeptides.

For materializing a novel biocatalyst fulfilling above two purposes, our approach chosen was to fuse G_5H, a PrP-inspired copper-binding catalytic peptide sequence [8] with tyrosine-containing Erk1/2 MAP kinase kinase (MAPKK) substrate sequence (erk1/2 residues, 182-187 MAPKK phosphorylation sites) consists of F-L-T-E-Y-V-A [71], to form a novel chimeric bio-catalyst designated as ErkG_5H [72]. Due to the presence of a single tyrosine-residue vicinal to Cu-binding catalytic motif (G_5H), self-phenol-assisted conversion of H_2O_2 to $O_2^{\bullet-}$ was successfully achieved.

ErkG$_5$H is the first phosphorylation-sensitive artificial enzyme

Protein phosphorylation is associated with most cell signaling and developmental processes in eukaryotes. Owing to the introduction of a very large, strongly charged group, phosphate; the phosphorylating events often leads to a drastic changes in the characteristics of the proteins, resulting in alterations in enzyme activity or protein-protein interaction properties [73]. Our second goal in engineering of metalloenzymes was to create an artificial biocatalyst which can be used as novel probes for assessing the cellular protein phosphorylation/dephosphorylation events possibly available for future clinical applications. Such novel type of biocatalysts could be integrated within the regulatory mechanisms under cellular signaling networks or protein-protein interactions by replacing the role of natural proteins such as protein kinases and down-stream redox enzymes.

The novel metalloenzyme described in the above section designated as ErkG_5H, consisting of the Tyr-containing substrate mimic region and the catalytic region was used as a model for phosphorylation-regulated artificial enzyme [72]. Within the peptide sequence F-L-T-E-Y-V-A-G-G-G-G-G-H, two amino acid residues, namely, threonine (T) and tyrosine (Y) residues, can be the sites of phosphorylation since the original sequence was derived from the phosphorylation domain within MAPKK [71]. In order to demonstrate that ErkG_5H is a phosphorylation-sensitive catalyst, preliminary data was obtained with non-phosphorylated and phosphorylated peptides at two phosphorylation sites (Fig. 9). As expected, model phosphorylations effectively lowered the catalytic activity of the peptide. Notably, there were positional impacts of phosphorylation on the $O_2^{\bullet-}$-generating activity. The catalytic activity of the peptide was most effectively lowered by phosphorylation at tyrosine residue, and phosphorylation at threonine residue resulted in partial inhibition. The catalytic activity in the fully phosphorylated peptide was completely lost.

This is the first implication that phosphorylation-controllable enzyme mimics could be artificially invented. In addition, we wish to propose a

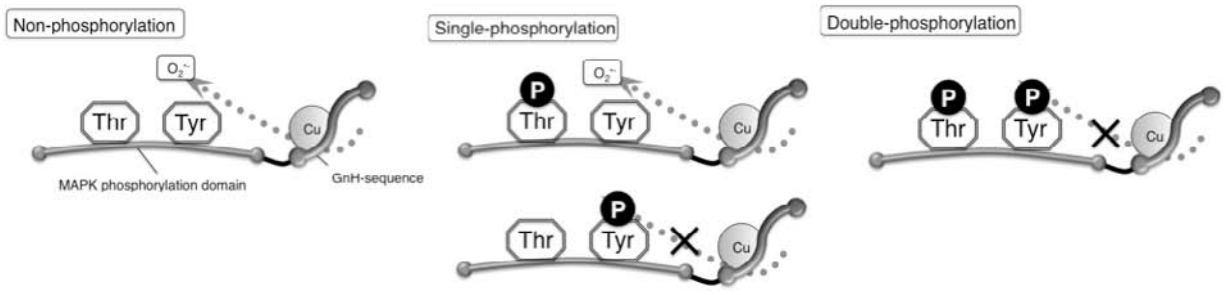

Fig. 9. A novel phosphorylation-sensitive chimeric biocatalyst designated as ErkG_nH.

possible application of this type of peptides as the tools or components for constructing a simplified *in vitro* signaling system processing the phosphorylation signals into the oxidative signals possibly affecting the fate of the living cells.

Designing thermostable metalloenzymes

As discussed earlier, PrPs are well recognized as causative molecules in development of neuro-degenerative diseases such as bovine spongiform encephalopathy (BSE), showing massive accumulation of the scrapie form of PrP (PrP^{sc}) formed from the intrinsic cellular form of PrP (PrP^{c}). However, the biochemical events required for the conformational changes in PrP^{c} leading to the formation of PrP^{sc} are not fully understood [74].

It is well known that prion-infected brain tissues or homogenates hardly lose their infectivity even after severe heat treatment [74] and repeated freezing and thawing [75]. Since it has been suggested that redox reactions likely play important roles in the development of protein conformational diseases [42, 76], Yokawa *et al.* [77] hypothesized that redox activities reflected by the generation of ROS in PrP-derived Cu-binding peptides would also be thermostable under both heating and repeated freezing and thawing cycles.

According to the demonstration using the Cu-binding oligo-peptides of interest, corresponding to the Cu-binding motifs in PrP, the thermo-stability contributing to both the heat tolerance and freezing/thawing tolerance of the tyramine-dependent $O_2^{\bullet-}$-generating activity was confirmed in the G-G-G-T-H peptide and V-N-I-T-K-Q-H-T-V-T-T-T-T peptide. This was the first experimental demonstration connecting the thermostability of the Cu-binding regions of PrP and their redox activities.

Since most of known enzymes and proteins are sensitive to high temperature and repeated freezing, the industrial applications and storage capability of enzymes and functional proteins are largely restricted by the narrow range of temperature. Therefore, it is eagerly requested to develop novel biocatalysts with enhanced thermostablity.

As discussed above, we have recently tested the thermo-stability of the $O_2^{\bullet-}$-generating PrP-derived peptides [77]. By analogy to the fact that the model helical sequence (V-N-I-T-K-Q-H-T-V-T-T-T-T) and the pentapeptide (G-G-G-T-H) derived from human PrP successfully maintained their high catalytic activity even after heat-incubation (90°C, 100 min), autoclaving, and repeated freezing/thawing cycles (Yokawa *et al.* 2009), we have newly designed the novel biocatalysts which are oligo-glycyl His peptides, namely, G_5H hexapeptide and $G_{10}H$ undecapeptide [8]. Notably, when the G_5H hexapeptide and $G_{10}H$ undecapeptide were incubated in the absence of copper, any loss of the catalytic activity following thermal denaturing treatments, both heating and freezing/thawing cycles, was detected. When the Cu-bound form of peptides were incubated, some decrease in catalytic activity (*ca.* 20%) was recorded for G_5H hexapeptide after autoclaving (121°C, 20 min) but this peptide tolerated the 100 min of heating at 90°C and 10-time repeated freezing and thawing cycles.

On the other hand, the damaging impact of Cu during thermal denaturing processes was much severely observed in $G_{10}H$ undecapeptide. By the heating incubation, autoclaving and freezing/ thawing cycles, Cu-loaded $G_{10}H$ undecapeptide has lost its catalytic activity by 64%, 23%, and 60%, respectively. These comparisons suggested that elongation of glycyl chains lowers the stability of the peptidic catalysts under high and low temperatures.

Scoring the catalytic activity of G_nH series metallopeptides

Above demonstration showed that G_5H hexapeptide possesses both high catalytic activity and thermo-stable nature, thus this peptide was selected for further analyses [8]. By calculating the production of $O_2^{\bullet-}$ using KO_2 as standard for evaluating the yield of chemiluminescence of $O_2^{\bullet-}$-specific probe, *Cypridina* luciferin analog [78], yield of the tyramine-dependent chemiluminescence was converted to the rate of $O_2^{\bullet-}$ production (mmol • mg $peptide^{-1}$ • min^{-1}). With Lineweaver-Burk analysis, K_m and V_{max} for the G_5H hexapeptide-catalyzed production of $O_2^{\bullet-}$ in the presence of tyramine were determined to be 0.42 mM and 0.12 mmol • mg $peptide^{-1}$ • min^{-1}, respectively. The V_{max} obtained is comparable to that of natural enzyme such as plant peroxidase (*ca*. 1/6 of purified Type IV-A peroxidase from horseradish; Sigma-Aldrich).

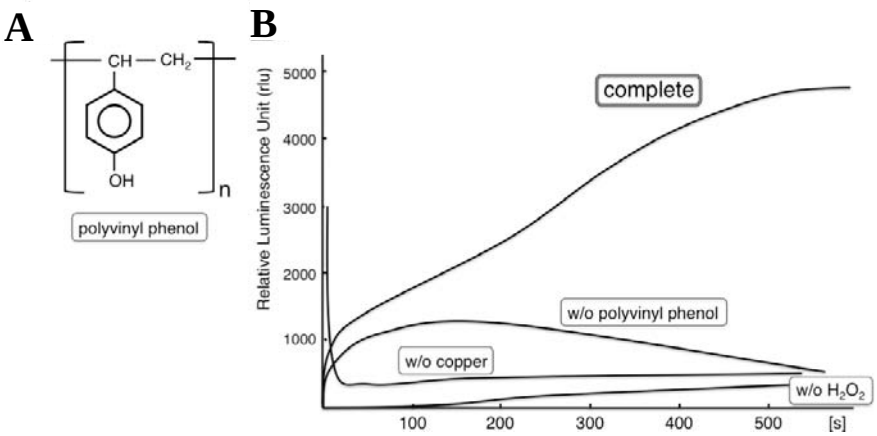

Fig. 10. The use of polyvinyl phenol as a substrate. (A) Chemical structure of polyvinyl phenol. (B) A trace of chemiluminescence reflecting the amount of superoxide in the reaction mixture.

Hybrid materials with catalytic acitiviy

According to the definition of enzymes expressed as an algebra of sets, as shown in the introduction, $\{E\text{s}\} = \{\{E_n\text{s}\}, \{E_a\text{s}\}\}$ [5]; novel artificial materials could be considered as members of enzymes in a broad sense. Therefore, novel hybrid materials covalently connecting peptides onto the plastic materials could be classified in the artificial enzyme category.

Okobira *et al.* [79] has demonstrated that copper-bound peptide with the X-X-H motif chemically conjugated to organic materials could be a novel class of biosensing tool. Accordingly, a tripeptide, glycyl-glycyl-histidine (G-G-H), was introduced to glycidyl methacrylate-grafted porous hollow fiber membrane made on the polyethylene platform by radiation-induced graft polymerization, at the G-G-H tripeptide density on the membrane of 0.352 mmol/g-membrane. Then copper sulfate solution was permeated outward through the peptide-conjugated membrane for loading Cu(II) on the membrane. Chemiluminescence assay proved that the membrane has catalytic activity generating $O_2^{\bullet-}$ upon addition of H_2O_2 and tyramine as the pair of substrates.

There has been another type of hybrid material as the complex system for robust and long-lasting generation of $O_2^{\bullet-}$, which was recently prepared (unpublished results). Usually, the substrates for enzyme reactions are simple small molecules, but this system employed the polyvinyl phenol which is a polymer with multiple phenolic groups (chain length, 12 - 58 mers) as the phenolic substrate for peroxidative $O_2^{\bullet-}$-generating reactions catalyzed by PrP-derived pentapeptide, GGGTH (Fig. 10). We observed that the production of $O_2^{\bullet-}$ in this system can be highly long lasting compared to other models using free phenolics. Although this approach requires further modification before actual trials, this system may be possibly applied for detection of catalytic molecules or PrPs for engineering and medical purposes, respectively.

PERSPECTIVE

Today, we simply imagine that enzymes are of biological origins. However, in the near future, synthetic biologists and chemical biologists might provide a wide variety of artificial enzymes around us, and we are quite sure that the most of which could be considered as novel metalloenzymes and derived hybrid materials. We hope that much creative attempts could be achieved by researchers with inter-discipline background, and the works covered in this review could be the basis for future study.

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