Copper-Binding Peptides from Human Prion Protein and Newly Designed Peroxidative Biocatalysts

Tomoko Kagenishi[a,b], Ken Yokawa[a], Takashi Kadono[a,b], Kazuya Uezu[a], and Tomonori Kawano[a,*]

[a] Laboratory of Chemical Biology and Bioengineering, Faculty and Graduate School of Environmental Engineering, The University of Kitakyushu, Kitakyushu, 808-0135, Japan. E-mail: kawanotom@env.kitakyu-u.ac.jp
[b] Fukuoka Industry, Science and Technology Foundation, Fukuoka, 814-0001, Japan

* Author for correspondence and reprint requests

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A previous work suggested that peptides from the histidine-containing copper-binding motifs in human prion protein (PrP) function as peroxidase-like biocatalysts catalyzing the generation of superoxide anion radicals in the presence of neurotransmitters (aromatic monoamines) and phenolics such as tyrosine and tyrosyl residues on proteins. In this study, using various phenolic substrates, the phenol-dependent superoxide-generating activities of PrP-derived peptide sequences were compared. Among the peptides tested, the GGGTH pentapeptide was shown to be the most active catalyst for phenol-dependent reactions. Based on these results, we designed a series of oligoglycyl-histidines as novel peroxidative biocatalysts, and their catalytic performances including kinetics, heat tolerance, and freezing tolerance were analysed.

Key words: Artificial Enzyme, Peroxidase, PrP

Introduction

Prion proteins (PrPs) are causative agents for transmissible spongiform encephalopathies in mammalian brains (Jeffray *et al*., 2000). 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 (Tabner *et al*., 2001). In the cases of β-amyloid accumulation in Alzheimer's disease and of α-synuclein accumulation in Parkinson's disease, evidence for involvement of reactive oxygen species (ROS) such as H_2O_2 and hydroxyl radicals ($HO^{\bullet}$) in the toxic mechanisms has 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 stimulate the formation of abnormal protein aggregates (Tabner *et al*., 2001; Allsop *et al*., 2008).

Key involvement of metals (especially copper) in prion diseases has been well documented by a number of works (Watt *et al*., 2005; Wong *et al*., 2001; Sauer *et al*., 1999). However, two opposing roles for copper-bound PrPs have been proposed, namely as anti-oxidants and contrary as pro-oxidants enhancing the neurodegenerative process (Opazo *et al*., 2003). The likely factors associated with generation (Kawano, 2007) or removal of ROS (Wong *et al*., 2001) within PrPs are Cu-binding sequences highly preserved in PrPs.

Our previous works suggested that four distinct peptide sequences corresponding to the putative copper-binding sites containing metal anchoring histidine residues (His61, His69, His77, His85, His96, His111, and His187) in human PrP function as putative biocatalysts catalyzing the generation of superoxide anion radicals ($O_2^{\bullet-}$) in the presence of aromatic monoamines (Kawano, 2007) and phenolics such as tyrosine and tyrosyl residues on proteins (Yokawa *et al.*, 2009a). Since copper and H_2O_2 are required as co-factors, the reactions were considered to be peroxidase-like reactions catalyzed by the copper-centered peptides (Kawano, 2007).

In the present study, the phenol-dependent $O_2^{\bullet-}$ generation catalyzed by several PrP-derived copper-binding peptides was assessed using various phenolics as substrates. Based on the results with PrP-derived peptides, we designed a series of novel peroxidative biocatalysts and their catalytic behaviours were analysed.

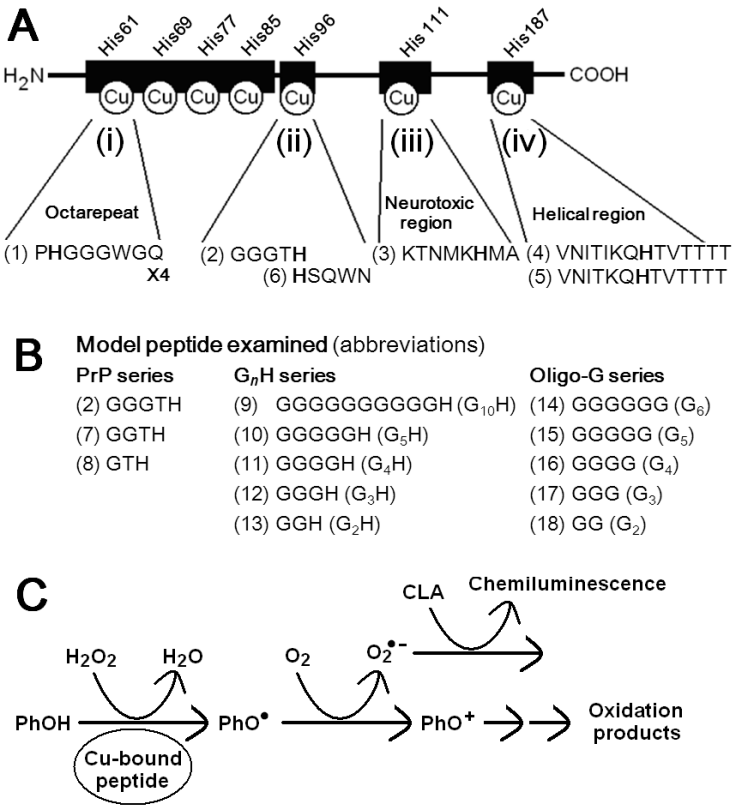

Fig. 1. PrP-related redox-active peptides and model derivatives. (A) Histidine-containing Cu-binding motifs (i–iv) found in human PrP. Positions of histidine residues and PrP-related peptides (1–6) synthesized and tested are shown. (B) Model peptides synthesized (peptides 7–18, to be compared with peptide 2). (C) Model mechanism for monitoring the $O_2^{\bullet-}$-generating peroxidative reactions catalyzed by the Cu-bound PrP-derived peptides, involving both H_2O_2 and phenolics as substrates. The $O_2^{\bullet-}$-generating activity can be assessed by detecting the CLA-CL.

Material and Methods

Peptide synthesis

As shown in Fig. 1, seven histidine-containing copper-binding sites consisting of four distinct amino acid sequences are found in mammalian PrPs, namely the four time-repeated octarepeat regions (PHGGGWGQ x 4, motif i), immediately followed by a short sequence (GGGTH-SQWN, motif ii), the neurotoxic region (motif iii), and the helical Cu-binding region (motif iv). The PrP-derived peptide sequences chemically synthesized and purified by high-performance liquid chromatography (Sigma Genosys Japan, Ishikari, Hokkaido, Japan) for the present study correspond to four distinct regions mentioned above: (1) PHGGGWGQ (motif i), (2) GGGTH (upper half of the motif ii), (6) HSQWN (lower half of the motif ii), (3) KTNMKHMA (motif iii), (4) VNITIKQHTVTTTT (motif iv, native helical sequence), and (5) VNITKQHTVTTTT (motif iv, a well studied model analogue sequence often employed in biochemical studies; Brown *et al.*, 2004). In addition, three series of peptides consisting of G_nTH (peptides 7 and 8; oligoglycyl-threonyl-histidines referred to as PrP series, where $n = 1–3$), G_nH (peptides 9–13; oligoglycyl-histidines, where $n = 2–5$ and 10), and G_n (peptides 14–18; oligoglycyl-glycine referred to as oligo-G series, where $n = 2–6$), respectively, were synthesized (Fig. 1B). The purities of these peptides were all above 90%.

Chemicals

An $O_2^{\bullet-}$-specific chemiluminescence (CL) probe, the *Cypridina* luciferin analogue (2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one), designated as CLA, was purchased from Tokyo Kasei

Kogyo Co. (Tokyo, Japan), and used for monitoring of $O_2^{\bullet-}$ as described below. Tyramine, tyrosine, benzoic acid (BA), 2-hydroxybenzoic acid (2-HBA), 3-hydroxybenzoic acid (3-HBA), 4-hydroxybenzoic acid (4-HBA), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), 2,6-dihydroxybenzoic acid (2,6-DHBA), phenol, catechol, resorcinol, and hydroquinone were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals were purchased from Sigma-Life Science Japan (Tokyo, Japan).

Monitoring of $O_2^{\bullet-}$ generation

The phenol-dependent generation of $O_2^{\bullet-}$ was monitored by CL of CLA (CLA-CL) with a photometer (Luminescensor PSN AB-2200-R, Atto Corp., Tokyo, Japan) and expressed as relative luminescence units (rlu) as previously described (Kawano, 2007). CLA-CL specifically indicates the generation of $O_2^{\bullet-}$ (and singlet oxygen to a lesser extent) but not that of H_2O_2 or $HO^{\bullet}$ (Nakano *et al.*, 1986).

Calibration of the CLA-CL photometer reflecting the changes in the $O_2^{\bullet-}$ level was carried out by dropping a solution of potassium superoxide (KO_2) dissolved in DMSO onto the CLA-containing media as previously reported (Kawano *et al.*, 1998).

The peptides and other chemicals were dissolved in phosphate buffer (pH 7). Molar ratios among the components in the reaction mixture (totally 200 μl), namely peptides, Cu^{2+}, H_2O_2, and a model substrate (phenolics) were approx. 1:3:3:10 (*i.e.*, each reaction mixture contained 0.15 mM peptides, 0.5 mM $CuSO_4$, 0.5 mM H_2O_2, and 1.5 mM phenols such as tyramine), unless otherwise indicated.

Thermostability tests

The thermostability of the model peptides was examined by measuring the $O_2^{\bullet-}$-generating activity following heating or repeated freezing and thawing treatments. For heat treatment, peptide solutions (0.2 ml, 3 mM) in 1.5-ml Eppendorf tubes were incubated in a hot water bath (90 °C). Following incubation for up to 100 min, the tubes were immediately cooled down on ice. Then peptides were used for the $O_2^{\bullet-}$-generating reaction.

Similarly, peptide solutions (0.2 ml, 3 mM) kept in 1.5-ml Eppendorf tubes were frozen by immersing in liquid nitrogen and thawed by incubating in a water bath (40 °C) for *ca.* 1 min. Moreover, peptide solutions in 1.5-ml Eppendorf tubes were autoclaved for 20 min at 121 °C. Then peptides were used for $O_2^{\bullet-}$-generating reactions.

Results and Discussion

Tyramine-dependent and tyrosine-dependent oxidative burst

The $O_2^{\bullet-}$-generating catalytic activities of five peptides (0.15 mM) from four Cu-binding motifs (i–iv, Fig. 1A) were compared according to the reported protocol. For the motif iv (helical region), both native (peptide 4) and model analogue (peptide 5) were tested. Since previous studies suggested that tyramine in neuronal tissues (Kawano, 2007), free tyrosine, and tyrosine residues on PrP protein (Yokawa *et al.*, 2009a) can be natural substrates for PrP, the $O_2^{\bullet-}$-generating activities were assessed with tyramine and tyrosine as substrates (each 1.5 mM). As co-factors both Cu^{2+} (0.5 mM) and H_2O_2 (0.5 mM) were added to phosphate buffer (pH 7.0). Following addition of tyramine (Fig. 2A) or tyrosine (Fig. 2B) as a substrate, an increase in CLA-CL was observed. Among the peptides tested, the GGGTH pentapeptide (peptide 2) from motif ii was found to be most active.

As the $O_2^{\bullet-}$-generating activity of GGGTH pentapeptide was lost when a co-factor such as Cu^{2+} or H_2O_2 was missing (Figs. 2C, D), both Cu^{2+} and H_2O_2 were considered as the key co-factors required for the generation of $O_2^{\bullet-}$. This is in support of our view that the complex formed between copper and the pentapeptide is acting as minimal biocatalyst for peroxidative reactions.

Although involvement of copper and generation of $O_2^{\bullet-}$ are analogous to tyrosinase which oxidizes tyrosine and polyphenols with concomitant release of $O_2^{\bullet-}$ (Koga *et al.*, 1992), the roles played by H_2O_2 are largely different. While the reaction studied here requires the presence of H_2O_2 as a co-factor (or co-substrate) (Figs. 2C, D), H_2O_2 is often regarded as an inhibitor of the tyrosinase reaction (Wood and Schallreuter, 1991). On the other hand, plant peroxidases such as horseradish peroxidase (HRP) were shown to be active in generation of $O_2^{\bullet-}$ upon oxidation of various phenolics and monoamines in the presence of H_2O_2 (Kawano and Muto, 2000; Kawano, 2003). Therefore, we can conclude that the mode of reactions catalyzed by PrP-derived peptides is analogous to that of the the plant peroxidase reaction.

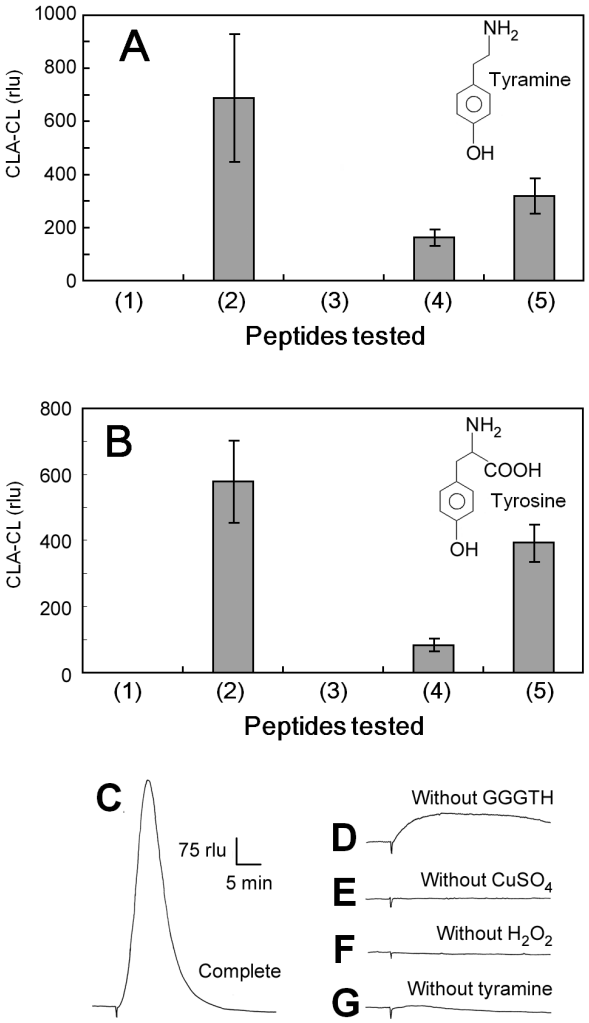

Fig. 2. Effects of PrP-derived Cu-binding peptides, $CuSO_4$, H_2O_2, and tyramine on superoxide generation. (A) Effect of tyramine as a substrate. (B) Effect of tyrosine as a substrate. Numbers (1)–(5) specify the peptides used (as listed in Fig. 1A). Error bars in (A) and (B) represent S.E. (n = 3, each). (C–G) Requirement of the co-factors, substrate, and catalytic peptide (GGGTH pentapeptide) for generation of $O_2^{\bullet-}$. The $O_2^{\bullet-}$-generating activity can be assessed by detecting the CLA-CL, *Cypridina* luciferin analogue-dependent chemiluminescence.

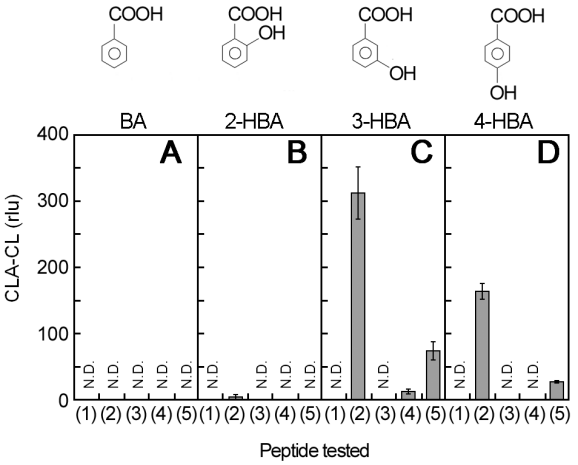

Fig. 3. Effects of benzoic acid (BA) and hydroxybenzoic acids (HBAs) as substrates for superoxide generation catalyzed by PrP-derived Cu-binding peptides. Effects of (A) BA, (B) 2-HBA, (C) 3-HBA, and (D) 4-HBA are compared. Numbers (1)–(5) specify the peptides used (as listed in Fig. 1A). N.D., not detected. Error bars represent S.E. (n = 3, each).

Effects of hydroxylated benzoic acid derivatives

Effects of benzoic acid (BA) and hydroxybenzoic acids (HBAs) were tested (Fig. 3). No increase in CLA-CL was observed after addition of BA to any peptide (Fig. 3A). Salicylic acid (2-HBA), known as a suicide substrate for plant peroxidases such as HRP transiently producing robust $O_2^{\bullet-}$ (Kawano and Muto, 2000) but concomitantly inactivating the enzyme (by forming so-called verdohemoprotein) (Kawano *et al.*, 2002), was shown to be a poor substrate for PrP-derived peptides (Fig. 3B). In contrast, 3-HBA and 4-HBA were shown to be good substrates for the GGGTH pentapeptide and the helical model peptide (Figs. 3C, D). These results suggest that the presence of phenolic moieties with a *meta*- or *para*-positioned hydroxy group is required for generation of $O_2^{\bullet-}$.

Effects of dihydroxybenzoic acids (DHBAs) were also tested (Fig. 4). No increase in CLA-CL was observed after addition of 2,6-DHBA to the reaction mixture (Fig. 4D), further confirming the inactivity of the *ortho*-positioned hydroxy group. The GGGTH pentapeptide was shown to be active towards 2,3-DHBA, 2,4-DHBA, and 2,5-DHBA (Figs. 4A–C). Therefore, it is evident that the presence of an *ortho*-positioned hydroxy group does not interfere with the roles for active hydroxy groups at *meta*- and *para*-positions.

Effects of phenol and diphenols

Since a role for phenolic moieties in BA derivatives was suggested, effects of phenol and benzenediols were examined. As expected, phenol

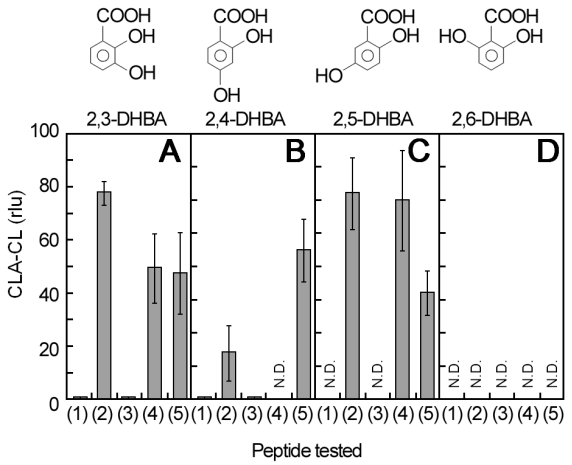

Fig. 4. Effects of dihydroxybenzoic acids (DHBAs) as substrates for superoxide generation catalyzed by PrP-derived Cu-binding peptides. Effects of (A) 2,3-DHBA, (B) 2,4-DHBA, (C) 2,5-DHBA, and (D) 2,6-DHBA are compared. Numbers (1)–(5) specify the peptides used (as listed in Fig. 1A). N.D., not detected. Error bars represent S.E. (n = 3, each).

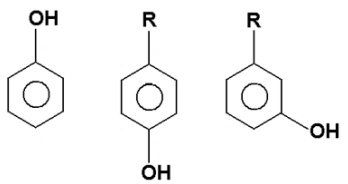

Fig. 5. Generalized chemical structures of phenolic substrates favoured by GGGTH pentapeptide.

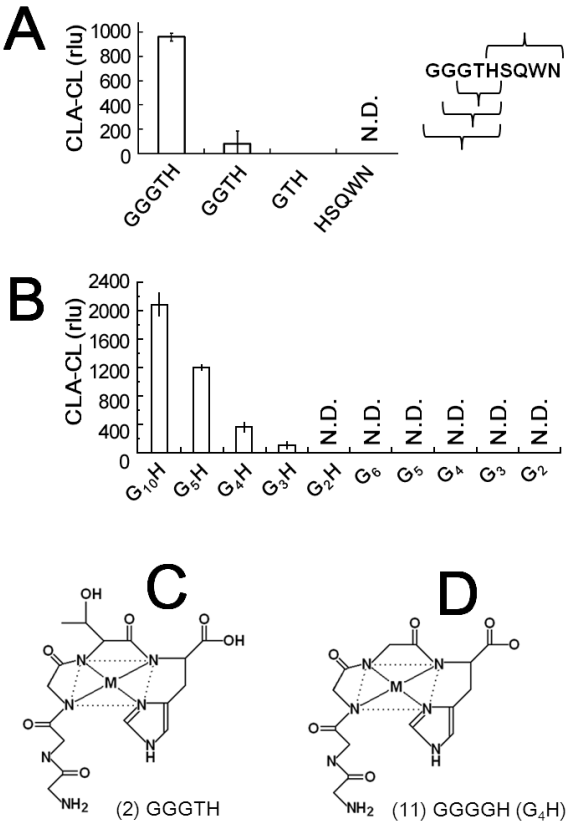

Fig. 6. Effects of various peptides differing in size as catalysts for the tyramine-dependent superoxide-generating reaction. (A) Effects of GGGTH-related peptides. (B) Effects of newly designed G_nH series and oligo-G series peptides. (C, D) Possible chemical structures of metal-bound forms of GGGTH pentapeptide and GGGGH pentapeptide. N.D., not detected. Error bars in (A) and (B) represent S.E. (n = 3, each).

and the *meta*-positioned benzenediol (resorcinol) were found to be active while *ortho*-positioned benzenediol (catechol) was inactive (data not shown). Interestingly, *para*-positioned benzenediol (hydroquinone) was inactive in generation of $O_2^{\bullet -}$ (data not shown). Fig. 5 generalizes the structure of the putative substrates for the GGGTH pentapeptide-dependent reaction. Free phenol and *meta*- and *para*-positioned phenolic moieties can be good substrates for the GGGTH pentapeptide-mediated reactions with exception of hydroquinone.

The neurotoxic sequence (peptide 3) has strong Cu-binding property and, therefore, protection of living plant cells from copper toxicity has been attempted (Kagenishi *et al.*, 2009). However, peptide 3 was shown to be catalytically inactive towards all of the substrates tested here. Therefore, the catalytic activity and neurotoxicity has no or small (if any) correlation.

Effects of peptide chain length and position of histidine residue

Among the Cu-anchoring histidine residues in human PrP, we focused on the role of His96, and thus the effect of amino acid chains around His96 was studied. In human PrP, His96 is located between GGGT sequence and SQWN sequence. Therefore, to see the effect of the histidine po-

sition, HSQWN pentapeptide was used for comparison with the GGGTH pentapeptide. While reaction with tyramine and GGGTH resulted in robust production of $O_2^{\bullet-}$, HSQWN showed no catalytic activity (Fig. 6A). This suggests that the position of the histidine residue would be highly important for designing novel peroxidase-like biocatalysts (artificial enzymes).

Furthermore, comparison of the catalytic activities among the GGGTH pentapeptide and shorter derivatives with C-terminal histidine residues (GGTH and GTH) suggested the importance of the N-terminal glycyl chain elongation for manifesting the maximal redox activity (Fig. 6A).

Designing novel biocatalysts

By analogy to the GGGTH sequence, we designed a series of simplified model peptides composed of oligoglycyl chains and C-terminal histidine (G_nH series, peptides 9–13; Fig. 1B) as putative biocatalysts. Since the above data indicated the importance of the elongation of the N-terminal glycyl chain, the peptides designed differed in the N-terminal glycyl chain length. To test the importance of the C-terminal histidine, an additional series of peptides (peptides 14–18 in the oligo-G series, G_2–G_6) were also prepared for comparison.

Among the newly designed peptides 9–18, catalytic activity was found only within the G_nH series (Fig. 6B). None of the oligoglycyl peptides lacking histidine (G_n) 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 copper ions (Yang *et al*., 2003). However, catalytic activity of the minimal Cu-binding motif was hardly detected, but the G_3H tetrapeptide produced a detectable increase in CLA-CL (Fig. 6B), suggesting that N-terminal elongation of the Cu-binding peptide contributes to catalytic activity. By single amino acid elongation, the catalytic performance was enhanced by *ca*. 3-fold (between G_3H and G_5H), but elongation by further 5 residues from G_5H hexapeptide to $G_{10}H$ undecapeptide merely resulted in *ca*. 3-fold enhancement, suggesting that the chain length effect was nearly saturated. These data suggest that the presence of the C-terminal histidine is the primary requirement for catalytic performance, and elongation of the N-terminal chain contributes to elevation of the catalytic activity.

The likely structures of the Cu-peptide complexes shown here (Figs. 6C, D, M = Cu) were estimated according to Fang *et al*. (2004) and briefly confirmed by molecular orbital calculations (unpublished results).

Thermostability of the G_nH peptides

Since most of the known enzymes and proteins are sensitive to high temperature and repeated freezing, 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 thermostability. Since it is well known that prion-infected brain tissues or homogenates hardly lose their infectivity even after severe heat treatments (Kitamoto, 2005) or repeated freezing and thawing (Castilla *et al*., 2005), we have recently tested the thermostability of the $O_2^{\bullet-}$-generating prion-derived peptides (Yokawa *et al*., 2009b). We found that the model helical sequence (identical to peptide 5) and GGGTH pentapeptide maintained their high catalytic activity even after heat incubation (90 °C, 100 min), autoclaving, and repeated freezing/thawing cycles (Yokawa *et al*., 2009b).

Since we have designed the novel biocatalysts based on the structure of GGGTH pentapeptide which is known to be thermostable, we expected that the two active peptides designed here (G_5H hexapeptide and $G_{10}H$ undecapeptide) possess a similar thermostable nature.

In case of G_5H hexapeptide and $G_{10}H$ undecapeptide kept in the absence of copper, loss of catalytic activity following thermal denaturing treatments (both heating and freezing) was hardly detectable (Fig. 7). When the Cu-bound form of peptides was used, some decrease in catalytic activity (*ca*. 20%) was recorded for G_5H hexapeptide after autoclaving (121 °C, 20 min; Fig. 7A), but this peptide tolerated 100 min of heating at 90 °C and a 10 times repeat of freezing and thawing cycles (Figs. 7A, B). The damaging impact of Cu^{2+} during thermal denaturing processes was more obvious in $G_{10}H$ undecapeptide. By heat incubation, autoclaving, and freezing/thawing cycles, Cu-loaded $G_{10}H$ undecapeptide lost its catalytic activity by 64%, 23%, and 60%, respectively (Figs. 7C, D). These comparisons suggested that elongation of the glycyl chains lowers the stability of the peptidic catalysts under high and low temperatures.

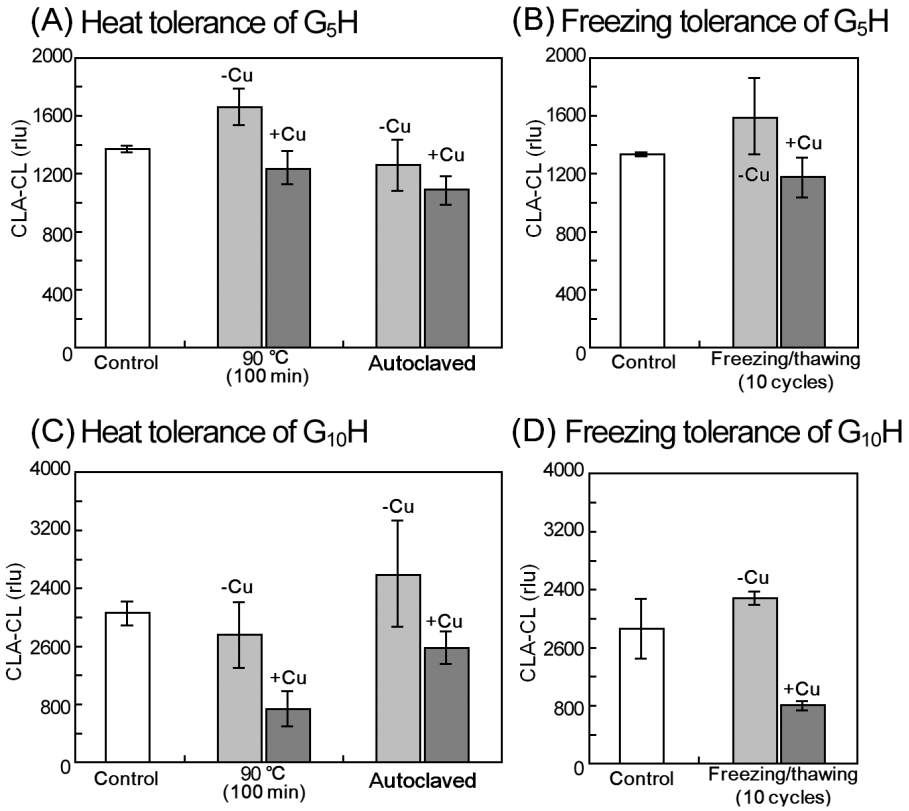

Fig. 7. Effects of heat treatment, autoclaving, and freezing/thawing cycles on the superoxide-generating activities of G_5H and $G_{10}H$ peptides. (A) Tolerance of the catalytic activity of G_5H hexapeptide to heat treatment and autoclaving. (B) Tolerance of the catalytic activity of G_5H hexapeptide to freezing/thawing cycles. (C) Tolerance of the catalytic activity of $G_{10}H$ undecapeptide to heat treatment and autoclaving. (D) Tolerance of the catalytic activity of $G_{10}H$ undecapeptide to freezing/thawing cycles. In the presence or absence of Cu, the peptide solutions (in phosphate buffer, pH 7.0) were incubated at 90 °C for 100 min, autoclaved for 20 min at 121 °C, or exposed to 10 times repeated cycles of rapid freezing in liquid nitrogen and thawing at 40 °C. Following heat treatments, the catalytic activity was assessed by addition of CLA, $CuSO_4$ (only to Cu-lacking samples), H_2O_2, and tyramine to the reaction mixture in this order. Concentrations of peptides and other chemicals were identical with other experiments described above. Vertical bars indicate the range of errors (n = 3).

Requirement for co-factors and kinetic evaluation of G_5H hexapeptide

The above demonstration showed that G_5H hexapeptide possesses both high catalytic activity and thermostable nature; thus this peptide was selected for further analyses. Similarly to the PrP-derived GGGTH pentapeptide, the G_5H hexapeptide showed a requirement for co-factors such as Cu^{2+} and H_2O_2 (Fig. 8A).

By calculating the production of $O_2^{\bullet-}$ using KO_2 as standard for evaluating the yield of CLA-CL, the counts of the tyramine-dependent yield of CLA-CL were converted to the rate of $O_2^{\bullet-}$ production (μmol $O_2^{\bullet-}$ mg peptide^{-1} min^{-1}; Fig. 8B). By means of Lineweaver-Burk analysis, K_m and V_{max} values for the G_5H hexapeptide-catalyzed production of $O_2^{\bullet-}$ in the presence of tyramine were determined to be 0.24 mM and 52.91 mmol mmol-peptide^{-1} min^{-1}, respectively (Fig. 8C). Thus the V_{max} value based on weight (0.12 mmol mg peptide^{-1} min^{-1}) obtained is comparable to that of natural enzymes such as plant peroxidase (*ca.* 1/6 of purified type VI-A peroxidase from horseradish; Sigma-Aldrich).

In the present study, we showed the possibility for the development of a novel class of enzyme mimics with thermostable nature thus tolerant to extreme heating and repeated freezing

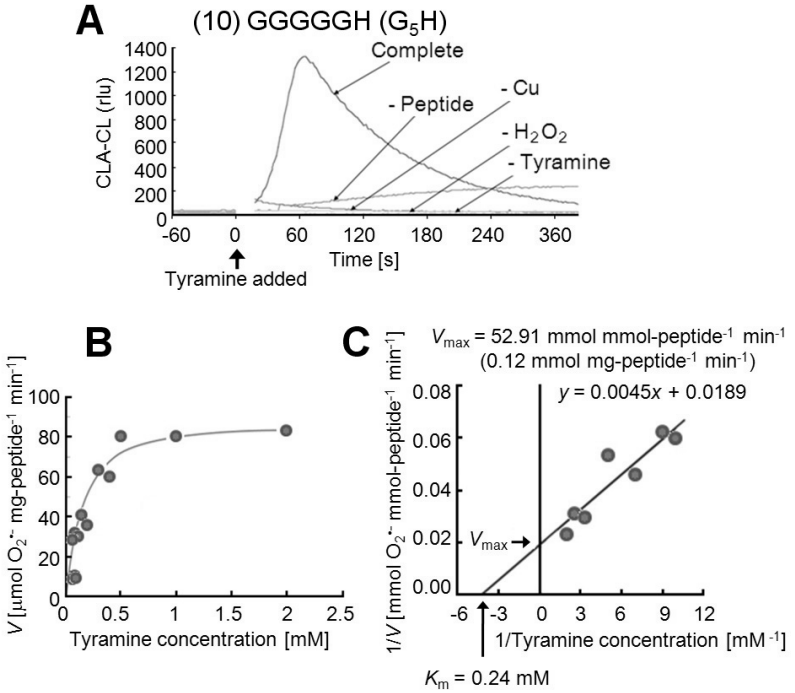

Fig. 8. Requirements for co-factors and kinetic evaluation of G_5H hexapeptide. (A) Requirements for co-factors. (B) Effect of tyramine concentration on the rate of superoxide production by Cu-loaded and H_2O_2-fueled G_5H hexapeptide. (C) Double reciprocal plot for assessing the K_m and V_{max} values.

and thawing cycles. This unusual thermostability of the peptides designed here may allow us to use or preserve these biocatalysts under extreme conditions which natural enzymes are unlikely to tolerate, both at high and low temperatures. One of the possible applications of these peptides is the development of weather-proof biosensors for outdoor uses. We are now engaged in the national program for the development of such engineered catalytic products as acknowledged below.

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Allsop D., Mayes J., Moore S., Masad A., and Tabner B. J. (2008), Metal-dependent generation of reactive oxygen species from amyloid proteins implicated in neurodegenerative disease. Biochem. Soc. Trans. **36**, 1293–1298.

Brown D. R., Guantieri V., Grasso G., Impellizzeri G., Pappalardo G., and Rizzarelli E. (2004), Copper(II) complexes of peptide fragments of the prion protein. Conformation changes induced by copper(II) and the binding motif in C-terminal protein region. J. Inorg. Biochem. **98**, 133–143.

Castilla J., Saa P., and Soto C. (2005), Detection of prions in blood. Nat. Med. **11**, 982–985.

Fang Y. Y., Ray B. D., Claussen C. A., Lipkowitz K. B., and Long E. C. (2004), Ni(II)·Arg-Gly-His-DNA interactions: Investigation into the basis for minor-groove binding and recognition. J. Am. Chem. Soc. **126**, 5403–5412.

Jeffray M., McGovern G., Goodsir C. M., Brown K. L., and Bruce M. E. (2000), Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy. J. Pathol. **191**, 323–332.

Kagenishi T., Yokawa K., Kuse M., Isobe M., Bouteau F., and Kawano T. (2009), Prevention of copper-induced calcium influx and cell death by prion-derived peptide in suspension-cultured tobacco cells. Z. Naturforsch. **64c**, 411–417.

Kawano T. (2003), Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Rep. **21**, 829–837.

Kawano T. (2007), Prion-derived copper-binding peptide fragments catalyze the generation of superoxide anion in the presence of aromatic monoamines. Int. J. Biol. Sci. **3**, 57–63.

Kawano T. and Muto S. (2000), Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco suspension culture. J. Exp. Bot. **51**, 685–693.

Kawano T., Sahashi N., Takahashi K., Uozumi N., and Muto S. (1998), Salicylic acid induces extracellular generation of superoxide followed by an increase in cytosolic calcium ion in tobacco suspension culture: The earliest events in salicylic acid signal transduction. Plant Cell Physiol. **39**, 721–730.

Kawano T., Muto S., Adachi M., Hosoya H., and Lapeyrie F. (2002), Spectroscopic evidence that salicylic acid converts a temporal inactive form of horseradish peroxidase (compound III) to the irreversibly inactivated verdohemoprotein (P-670). Biosci. Biotechnol. Biochem. **66**, 646–650.

Kitamoto T. (2005), Prions. Food and Drug Safety. Springer-Verlag, Tokyo, pp. 1–271.

Koga S., Nakano M., and Tero-Kubota S. (1992), Generation of superoxide during the enzymatic action of tyrosinase. Arch. Biochem. Biophys. **292**, 570–575.

Nakano M., Sugioka K., Ushijima Y., and Goto T. (1986), Chemiluminescence probe with *Cypridina* luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]-pyrazin-3-one, for estimating the ability of human granulocytes to generate O_2^-. Anal. Biochem. **159**, 363–369.

Opazo C., Inés-Barría M., Ruiz F. H., and Inestrosa N. C. (2003), Copper reduction by copper binding proteins and its relation to neurodegenerative diseases. Biometals **16**, 91–98.

Sauer H., Dagdanova A., Hescheler J., and Wartenberg M. (1999), Redox-regulation of intrinsic prion expression in multicellular prostate tumor spheroids. Free Radic. Biol. Med. **27**, 1276–1283.

Tabner B. J., Turnbull S., El-Agnaf O., and Allsop D. (2001), Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. Curr. Top. Med. Chem. **1**, 507–517.

Watt N. T., Taylor D. R., Gillott A., Thomas D. A., Perera W. S., and Hooper N. M. (2005), Reactive oxygen species-mediated β-cleavage of the prion protein in the cellular response to oxidative stress. J. Biol. Chem. **280**, 35914–35921.

Wong B. S., Brown D. R., Pan T., Whiteman M., Liu T., Bu X., Li R., Gambetti P., Olesik J., Rubenstein R., and Sy M. S. (2001), Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities. J. Neurochem. **79**, 689–698.

Wood J. M. and Schallreuter K. U. (1991), Studies on the reactions between human tyrosinase, superoxide anion, hydrogen peroxide and thiols. Biochim. Biophys. Acta **1074**, 378–385.

Yang W., Chow E., Willett G. D., Hibbert D. B., and Gooding J. J. (2003), Exploring the use of the tripeptide Gly-Gly-His as a selective recognition element for the fabrication of electrochemical copper sensors. Analyst **128**, 712– 718.

Yokawa K., Kagenishi T., Goto K., and Kawano T. (2009a), Tyrosine-dependent superoxide generation catalyzed by a peptide fragment corresponding to the helical copper-binding sequence in prion protein. Int. J. Biol. Sci. **5**, 53–63.

Yokawa K., Kagenishi T., and Kawano T. (2009b), Thermo-stable and freeze-tolerant nature of aromatic monoamine-dependent superoxide-generating activity of human prion-derived Cu-binding peptides. Biosci. Biotechnol. Biochem. **73**, 1218–1220.