Running title: Thermo-Stable Catalytic Activity of Prion-Derived Peptides

Thermo-Stable Nature of Aromatic Monoamine-Dependent Superoxide-Generating Activity of Human Prion-Derived Cu-Binding Peptides

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Abbreviations: CLA, *Cypridina* luciferin analog; $O_2^{\bullet -}$, superoxide anion; PrP, prion protein; PrP^C, intrinsic cellular PrP; PrP^{Sc}, scrapie isoform of PrP; rlu, relative luminescence units

Abstract:

Human prion protein has four distinct Cu-binding motifs that catalyze the generation of superoxide coupled to oxidation of phenols and amines. Here, the thermostability of the superoxide-generating prion-derived peptides was tested. Among the peptides tested, two maintained high catalytic activity even after heating and repeated freezing/thawing cycles. The biological roles for these thermostable catalysts are discussed.

Key words: prion protein; reactive oxygen species; heat tolerance; freezing tolerant

Prion proteins (PrPs) are well recognized as causative molecules in development of neurodegenerative 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.[1)] Recently, our group has reported that the Cu-bound form of PrP-derived oligopeptides corresponding to the four distinct Cu-binding regions catalyze the generation of superoxide anions in the presence of hydrogen peroxide and neurotransmitters,[2)] and of natural amino phenols such as tyramine[2)] and tyrosine.[3)] It is well known that prion-infected brain tissues or homogenates hardly lose their infectivity even after severe heat treatment[1)] and repeated freezing and thawing.[4)] Since it has been suggested that redox reactions likely play important roles in the development of protein conformational diseases,[5,6)] we hypothesized that redox activities (reflected by the generation of reactive oxygen species) in PrP-derived Cu-binding peptides can be also the thermostable under both heating and repeated freezing and thawing cycles.

Here, the Cu-binding oligo-peptides of interest, corresponding to the Cu-binding motifs in PrP (PHGGGWGQ, GGGTH, KTNMKHMA, and VNITKQHTVTTTT) were chemically synthesized and used in demonstrations. The thermostability contributing to both the heat tolerance and freezing/thawing tolerance of the superoxide-generating

activity in each peptide before and after the heating or freezing/thawing treatment was assessed by superoxide-specific chemiluminescence-based measurement.[2,3] This is the first experimental demonstration connecting the thermostability of the Cu-binding regions of PrP and their redox activities.

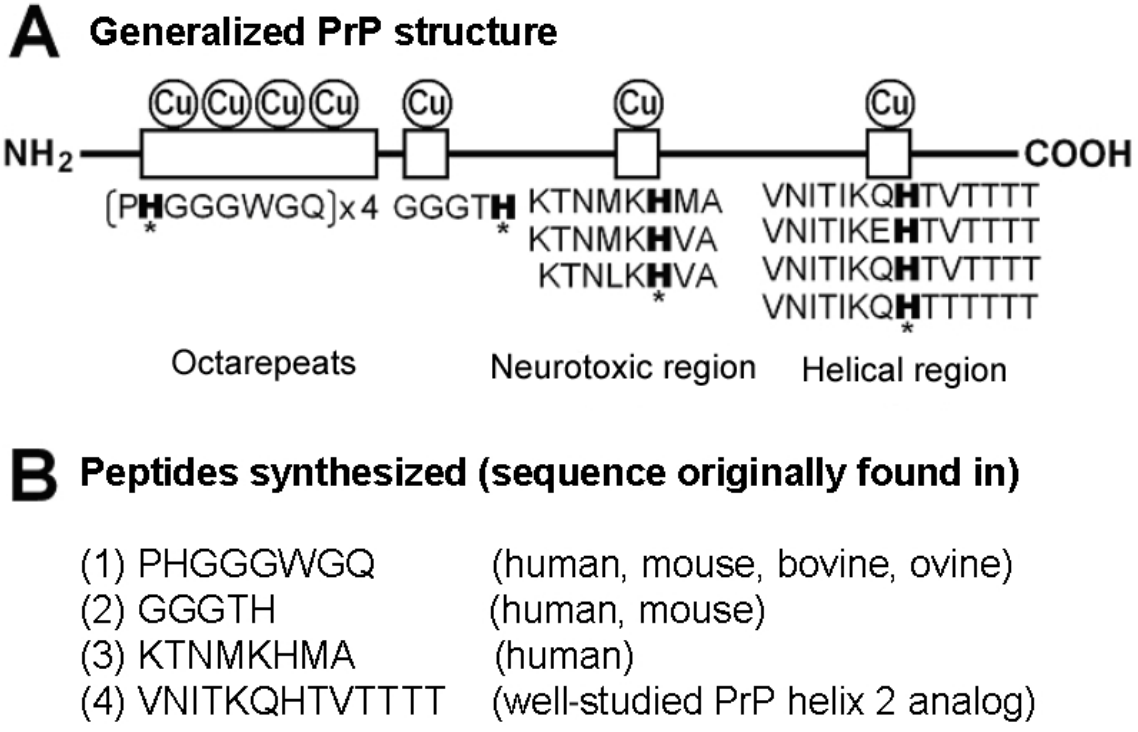


Fig. 1. Copper-Binding Motifs in Mammalian PrPs.
A, Generalized PrP structure. Asterisks indicate the His residues putatively anchoring copper. B, Sequence of synthesized peptides. This figure was made based on our previous work.[2]

Figure 1A shows seven copper-binding sites made of four distinct amino acid sequences found in mammalian PrPs: (1) the four time-repeated octarepeat regions, immediately followed by (2) a short sequence, (3) the neurotoxic region, and (4) the helical Cu-binding region. The newly synthesized peptide sequences corresponding to four distinct regions were (1) PHGGGWGQ, (2) GGGTH, (3) KTNMKHMA, and (4) VNITIKQHTVTTTT (Fig. 1B). To assess the activity of the helical Cu-binding

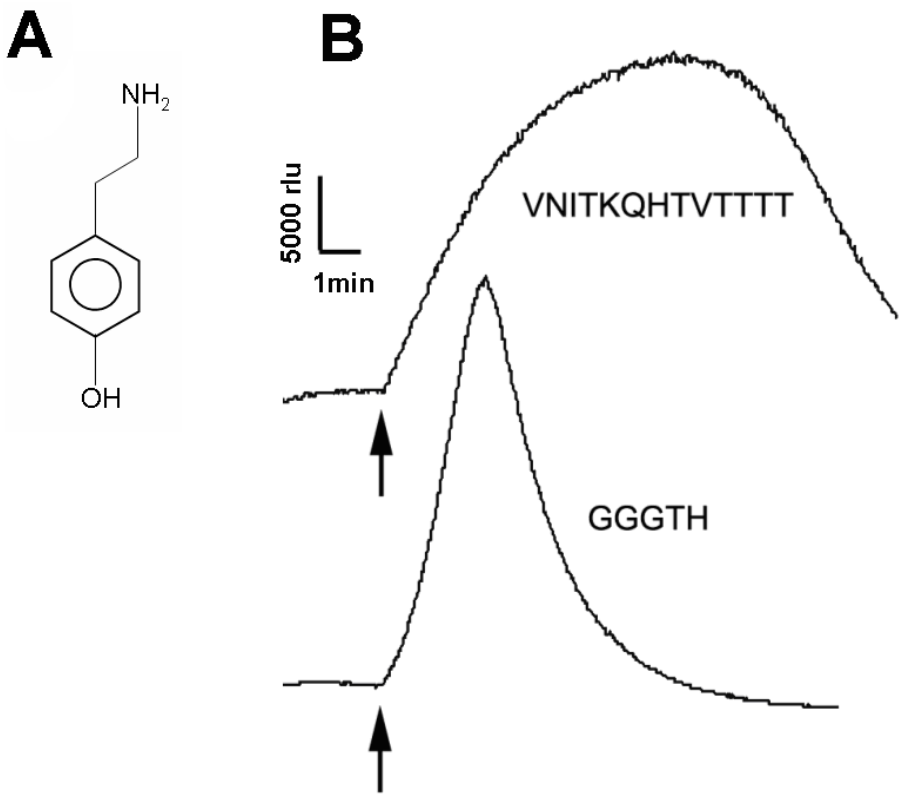
sequences, a well-studied model sequence, VNITKQHTVTTTT, proposed by Brown *et al.*,[7] which almost entirely encompasses the PrP^C's helix 2 in the C-terminal region, was used. Circular dichroism measurements in aqueous solution under physiological pH revealed that this peptide sequence forms α-helical conformation as in native PrP.[7] We have found that this sequence has high catalytic activity for the generation of superoxide in the presence of certain aromatic monoamines[2] and tyrosine.[3] The peptide, chemically synthesized and purified on high pressure liquid chromatography, was obtained from the custom peptide service department of Sigma Genosys Japan (Ishikari, Japan). The purities of the four peptide sequences, PHGGGWGQ, GGGTH, KTNMKHMA, and VNITKQHTVTTTT, were 95.72%, 99.40%, 98.85% and 98.10%.

To detect the superoxide anion radical, a super-oxide-specific chemiluminescence agent, *Cypridina* luciferin analog (designated CLA; 2-Methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) purchased from Tokyo Chemical Industry (Tokyo), was used. A model substrate, tyramine hydrochloride, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The other chemicals used in this study were of reagent grade, and were purchased from Sigma (St. Louis, MO).

The peptides and other chemicals were dissolved in 50 mM potassium phosphate buffer (pH 7.0). The molar ratios among the components in the reaction mixture (200 μl in total), viz., the peptides of interest, Cu^{2+}, H_2O_2, and tyramine (a model substrate) were approximately 1 : 3 : 3 : 10 (that is each reaction mixture contained 0.15 mM peptides, 0.5 mM $CuSO_4$, 0.5 mM H_2O_2, and 1.5 mM tyramine). For heating treatment, peptide solutions in 1.5-ml plastic tubes with lids were immersed in a hot water bath (95˚C) for 1, 3, 10, 30, and 100 min. In the end, the peptide solutions were autoclaved for 20 min at 121˚C to examine the ultimate heat tolerance of the peptides. On the other hand, peptide solutions (in 1.5-ml plastic tubes with lids) were frozen in liquid nitrogen and thawed at room temperature (23˚C). This procedure was repeated as indicated.

Generation of superoxide was monitored by the chemiluminescence of CLA with a luminometer (Luminescensor PSN AB-2200-R, Atto, Tokyo), and the

chemiluminescence obtained was expressed as relative luminescence units (rlu), as previously described.[8] CLA-chemiluminescence specifically indicates the generation of superoxide (and singlet oxygen to a lesser extent), but not that of H_2O_2 or the hydroxyl radical.[9] According to our previous study, the signal for singlet oxygen can be minimized by avoiding the use of high concentrations of organic solvents such as ethanol not exceeding 2% (v/v) in the reaction mixture.[10] Thus the induced chemiluminescence recorded here reflects the generation of superoxide rather than singlet oxygen.



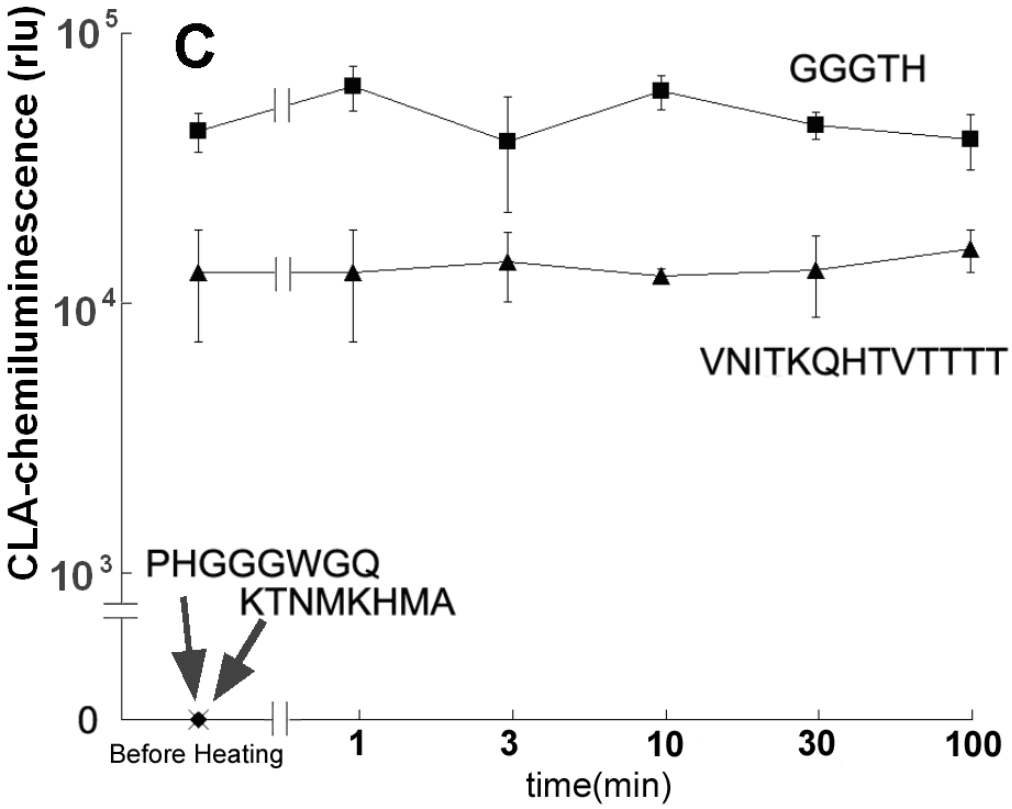


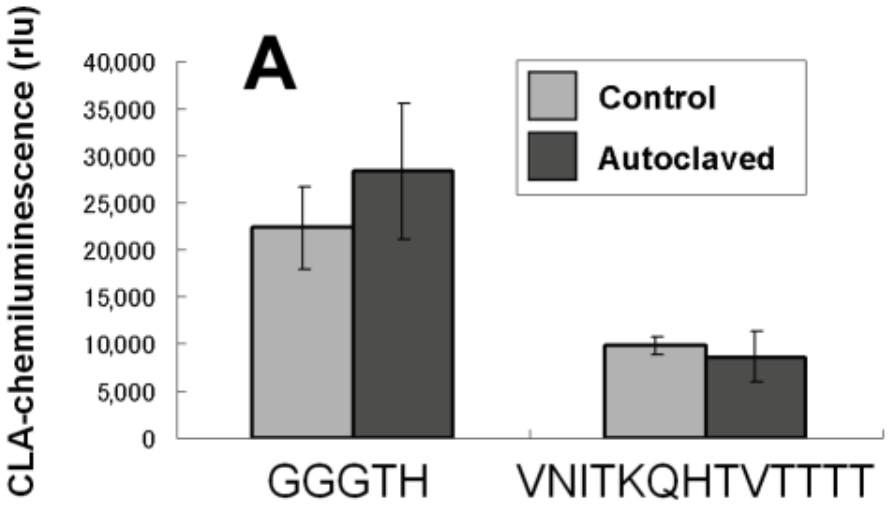
Fig. 2. Heat Tolerance of the Superoxide-Generating Activities Found in Two Distinct Copper-Binding Motifs.

A, Chemical structure of a model substrate, tyramine. B, Typical traces of superoxide generation detected with a superoxide-specific chemiluminescence probe, CLA. These traces represent the temporal profiles of the reactions catalyzed by two peptides prior to thermal treatment. Arrows indicate the timing of tyramine addition. C, Heat tolerance of the catalytic activity found in the two peptides. Peptide solutions were incubated at 95˚C for 1, 3, 10, 30, or 100 min. Then a chemiluminescence probe (CLA), co-factors ($CuSO_4$ and H_2O_2), and the model substrate (tyramine) were added to the reaction mixture containing buffer and peptides. Vertical bars indicate the range of errors.

When tyramine (Fig. 2A) was used as a model substrate for the generation of superoxide, only GGGTH and VNITKQHTVTTTT were found to be active (Fig. 2B). Gradual increases in chemiluminescence, reflecting the generation of superoxide, were

observed after the addition of tyramine to reaction mixtures containing GGGTH or VNITKQHTVTTTT (Fig. 2B). Although the activity of PHGGGWGQ and of KTNMKHMA was hardly detectable even before the thermal denaturing treatments when tyramine was employed as the model substrate, our previous work indicates that peptides corresponding to the octarepeat and the neurotoxic region were active in the oxidation of other substrates, such as phenylethylamine.[2)] Interestingly, compared to tyramine, phenylethylamine was found to be a poor substrate for the helical sequence.[2)]

Figure 2C shows the effect of heating treatments (up to 100 min of incubation at 95°C) on the superoxide-generating reaction catalyzed by two active sequences, GGGTH and VNITKQHTVTTTT. The data suggested that both peptides were active after incubation at 95°C. To further assess the heat-stable nature of the catalytic activity found in the two Cu-binding sequences derived from PrP, the peptide solutions were exposed to autoclaving at 121˚C for 20 min. Figure 3A compares the activities of the two peptides prior to and after autoclaving. We observed no significant change in catalytic activity even after subjection to such extreme conditions.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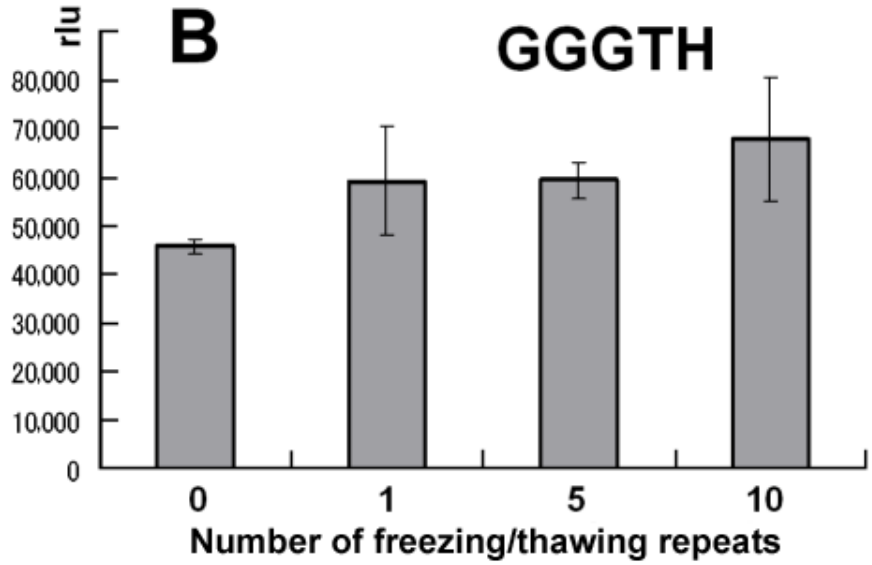
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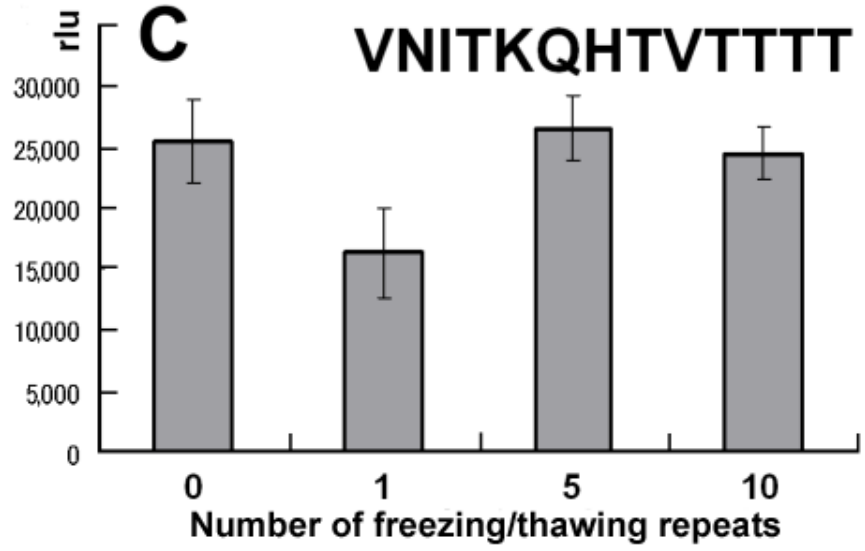
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Fig. 3. Tolerance of Two Peptides to Autoclaving and Freezing/Thawing Cycles.

A, Catalytic activity remained after autoclaving for 20 min at 121˚C. CLA, $CuSO_4$, H_2O_2, and tyramine were added after cooling of the peptide solution. B, C, Effects of repeated freezing and thawing on the catalytic activities of the two peptide sequences. CLA, $CuSO_4$, H_2O_2, and tyramine were added to the peptide solutions only after completion of thermal cycling. Vertical bars indicate the S.E. (n = 3).

In the next series of demonstrations, the peptide solutions were frozen in liquid nitrogen, and a gradual thawing process was allowed at room temperature (23˚C). This cycle was repeated for 0, 1, 5, or 10 times. After the freezing/thawing cycles, the superoxide generating activities in GGGTH (Fig. 3B) and helical peptide (Fig. 3C) were assessed. No significant decay of catalytic activity was observed even after 10 cycles of freezing and thawing (except for the first cycle). For unknown reasons, it appears that catalytic activity slightly lowered by the first single cycle of freezing and thawing could be recovered following further freezing and thawing cycles (Fig. 3C).

In the present study, we tested the thermostability of the superoxide-generating enzyme-like activities found in PrP-derived Cu-binding peptide sequences. Among four peptides tested, only two sequences were found to be active. These active sequences were used to study the impact of thermal changes on the catalytic activity. In conclusion, both GGGTH and VNITKQHTVTTTT remained catalytically active even after extreme heating and repeated thermal cycling. It is tempting to speculate that the thermostable nature of prion infectivity/toxicity can be attributed to thermostable redox-activity within the Cu-binding regions in PrPs.

In strong contrast to common features of enzymes and proteins which readily lose their catalytic activities or physiological functions following heating and freezing-thawing processes, the thermostable nature of the superoxide-generating activities found in PrP-derived Cu-binding peptides is of special interest. Further study to uncover the biological roles of the Cu-binding motifs in PrPs is required.

Acknowledgement

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