Direct evaluation of the antioxidant properties of salivary proline-rich proteins

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Proline-rich proteins are associated with the formation of an acquired protein layer overlying the tooth enamel surface. Previous studies have described the antioxidant activity of salivary histatin against the hydroxyl radical from Fenton’s reaction, acting as the critical reactive oxygen species. However, the role of proline-rich proteins in mitigating the oxidative stress caused by reactive oxygen species in the oral cavity remains unclear. In this study, we investigated the antioxidant effects of proline-rich proteins 2 on direct reactive oxygen species using electron spin resonance spectroscopy. For the first time, we demonstrated that proline-rich proteins 2 exhibits antioxidant activity directly against the hydroxyl radical produced by hydrogen peroxide with ultraviolet. Considering that identical results were obtained when assaying 30 residues of proline-rich proteins 2, the direct antioxidant effects against the hydroxyl radical by proline-rich proteins 2 may be related to these specific 30 residues.

Key Words: saliva, proline-rich proteins, oxidative stress, reactive oxygen species, antioxidant

Oxidative stress due to the activity of reactive oxygen species (ROS) such as the hydroxyl radical (HO•) or superoxide (O2•−), and antioxidant imbalances, is related to various lifestyle-related diseases including hypertension, arteriosclerosis, diabetes, myocardial infarction, cerebral infarction, and cancer.1–3 However, antioxidant systems comprising antioxidant enzymes and antioxidants, play a protective role by removing ROS.4–6 To maintain homeostasis of the oral cavity, an adaptive balance is sustained between oxidative stress caused by ROS and the antioxidant system. An imbalance in this environment due to an increase in oxidative stress augments the risk of systemic diseases including various oral diseases.7,8 We have previously reported that a failure to balance antioxidant activity with oxidative stress levels can further exacerbate diabetes mellitus, a disease that is linked to the oral disease periodontitis.9–11 Excess oxidative stress in the synovial fluid was also observed in human and animal models of temporomandibular disorders.12 In addition, ROS release has been reported to cause a decrease in the antioxidant capacity of saliva, leading to the development of oral cancer in smokers.13,14 Interestingly, the antioxidant activity of salivary vitamins has been reported to be effective against the oxidative stress caused by the oral lichen, planus.15 Studies have established that antioxidant activity, including that of the saliva and salivary proteins, is crucial in maintaining homeostasis, influencing the progression of systemic diseases, and sustaining the overall health of the oral cavity.16,17 Salivary proteins are reported to be involved in homeostatic processes, lubrication, antibacterial activity, and control of tooth demineralization and remineralization.18 These proteins are comprised of a mixture of protein and peptide derived from the salivary glands, oral exudates, and cell debris, and have a protective effect in the oral cavity.19–23 Thirty percent of these mixtures are made up of small proteins called saliva-derived peptides.24,25 The four major saliva-derived peptides are histatin, cystatin, statherin, and proline-rich proteins. It is well established that saliva contains high concentrations of proline-rich proteins (PRPs), secreted from the salivary glands.26–29 PRPs have been reported to play a role in the formation of the acquired enamel pellicle, a thin film that is formed by the selective binding of salivary proteins onto the enamel surface of the teeth. Therefore, PRPs function as a part of this protective protein layer that covers the tooth surface, with acidic PRPs having a high-affinity binding site in their phosphorylated domain for hydroxyapatite.30 PRPs interact with several oral bacteria, can inhibit the formation of hydroxyapatite, and suppresses crystal growth of calcium phosphate, by binding calcium ions from a supersaturated solution of hydroxyapatite, thereby protecting the tooth enamel.

Studies have found that measuring the levels of the antioxidant protein SOD30,31 or relative lipid peroxidation32 of salivary proteins are effective tests to assess oxidative stress in the oral cavity. However, there are only a few reports on the antioxidant activity of saliva, and specifically salivary proteins. Salivary PRPs are characterized by high amounts of the amino acids proline, glycine, glutamine, and glutamic acid, and make up over 60% of total salivary peptides.33,34 These proteins are typically grouped into the following categories: acidic (aPRP), basic (bPRP), and glyco- sylated (gPRP).35 We recently confirmed that histatin could reduce ROS-induced oxidative stress, especially that caused by HO• (via Fenton’s reaction), using electron spin resonance (ESR) spectroscopy.36 However, there are few, if any studies that assess the direct effects of PRPs on ROS generation. Therefore, in this study, we investigated the effect of PRPs, especially PRP2, on the generation of ROS, using ESR. These findings provide novel evidence characterizing the antioxidant properties of salivary PRPs.

Materials and Methods

Reagents. Reagents were purchased or sourced from the following: xanthine (X) and xanthine oxidase (XO) (grade III) were obtained from a chromatographically purified suspension of buttermilk in 2.3 M (NH₄)₂SO₄ and 10 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 1 mM sodium salicylate, respectively. Superoxide dismutase was obtained from Sigma

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(St. Louis, MO), hydrogen peroxide (H₂O₂) and FeSO₄ were purchased from Wako Chemical (Osaka, Japan), and 5,5-dimethyl-1-pyrroline-N-Oxide (DMPO) was sourced from Labotech (Tokyo, Japan).

Synthetic PRP2 was obtained from the American Peptide Company (Sunnyvale, CA) and from Quality Controlled Biochemicals (Hopkinton, MA). Human parotid secretion (HPS) was collected from five healthy volunteers aged 25 to 38 years. Informed consent was obtained and approved by all participants, according to the protocol from the Institutional Review Board at Boston University Medical Center. The HPS was collected using a Curby cup device that fits into the opening of the Stensen duct. The flow of HPS and salivary secretions stimulated by sour candy were collected in a graduated cylinder on ice. A 25 µl aliquot of parotid secretion (PS) was seeded on blood agar (Hardy Diagnostics), to confirm that the collected PS was sterile and free of whole saliva contamination. The PS protein was dialyzed free of whole saliva contamination. The PS protein was dialyzed.

**Diagram:**

- **ESR Spin Resonance (ESR) Spin-Trap Method.**

- **O₂⁻** was generated via the X/XO reaction system, as previously reported.**O₂⁻** was generated from xanthine oxidase (0.1 U/ml) and xanthine (362 µM) in 0.1 M phosphate-buffered saline (pH 7.2), containing 50 mM DMPO, with or without pretreatment by PRP2 or 30rPRP. The mixture was transferred to a flat cell, and the DMPO-OH spin adduct was measured using the X-band electron spin resonance (ESR) spin-trap method.

**Statistical analysis.** Statistical analysis using Dunnett’s test (OMS, Saitama, Japan) showed that the results from the experiments conducted in this study were normally distributed. Experimental results are expressed as mean ± SD. The relative mean from the 3 or 4 different protein concentration levels were compared using a two-way analysis of variance. P values less than 0.05 were considered statistically significant.

**Results**

- **Effects of PRP2 on HO²⁻ generation by the Fenton’s reaction system.** The amino acid sequence of the salivary protein PRP2 used in this study is shown in Fig. 1. The only basic residue in this peptide is arginine, and there are no aromatic residues present. Conversely, the other major salivary protein, histatin 5, consists of 24 residues, of which none are proline, with 14 basic residues and 3 aromatic residues. This could indicate that histatin displays different antioxidant properties when compared to PRP2 or other PRPs.

- **Effects of PRP2 on HO²⁻ generation by ultraviolet irradiation of H₂O₂.** The effects of PRP2 on HO²⁻ generated using the UV/H₂O₂ reaction system were examined by ESR spin trapping, using DMPO as the spin trap. As previously reported, after adding H₂O₂ to FeSO₄, a characteristic spin adduct spectrum was observed, with hyperfine splitting resulting in four typical peaks. However, in the reaction with FeSO₄ and PRP2 (6.7, 20, and 33.3 µM) pretreatment at different concentrations, and the subsequent addition of H₂O₂, the DMPO-OH signal did not change at each concentration of PRP2 (Fig. 2).

- **Effects of PRP2 on O²⁻ generation.** As reported previously, following the X/XO reaction, a characteristic DMPO-OH spin adduct spectrum was observed, with hyperfine splitting giving rise to 12 resolved peaks. These signals were quenched by 150 U/ml superoxide dismutase, thus confirming that they were derived from O²⁻ (data not shown). However, with XO and PRP2 (6.7, 20 and 33.3 µM) pretreatment at different concentrations, and the subsequent addition of xanthine, the DMPO-OH signal did not change at each concentration of PRP2 (Fig. 4).

**Fig. 1.** The amino acid sequence of proline-rich protein 2 (PRP2) and 30rPRP.
Effects of 30rPRP on ROS generation. Finally, to establish and clarify the correlation between the structure of PRP2 and antioxidant activity, we investigated the antioxidant activity of 30rPRP, a 30-residue structure contained in PRP2. With different concentrations of 30rPRP (6.7, 20 and 33.3 μM) pretreatment of H₂O₂ from Fenton’s reaction (FeSO₄/H₂O₂) system, it was apparent that the DMPO-OH signal did not show any change at each concentration of 30rPRP (6.7, 20 and 33.3 μM) (Fig. 5A). When H₂O₂ was pretreated with different concentrations of 30rPRP, followed by UV irradiation, we observed that the DMPO-OH signal was reduced (Fig. 5B). When O₂•− was generated from the X/OX reaction system with 30rPRP pretreatment at different concentrations (6.7, 20 and 33.3 μM), the DMPO-OOH signal did not change at any concentration of 30rPRP (Fig. 5C).

Discussion

PRPs are comprised of approximately 25–40% of the amino acid proline and are the main salivary proteins produced by the parotid and submandibular glands, accounting for almost 70% of total protein in human saliva. PRPs have a phosphorylated domain that has a high affinity for hydroxyapatite. These proteins play an important role in the formation of enamel film, which functions as a protein covering on the surface of the tooth. It has been shown that salivary peptides such as histatin show antibacterial activity. We have recently reported the direct antioxidant activity of histatin using the ESR method, suggesting that salivary proteins display both antioxidant activity and oral antibacterial activity. However, previous studies have not investigated the actual effect of PRPs on the generation of ROS.
Therefore, we used ESR to examine the direct antioxidant effects of PRPs, especially PRP2, on the production of ROS such as \( \text{HO}^\cdot \) and \( \text{O}_2^\cdot \). This study provides the first evidence that PRP2 directly suppresses \( \text{HO}^\cdot \) production, though it did not reduce the level of \( \text{O}_2^\cdot \) generation from the X-XO reaction system (Fig. 1–4).

We studied the effects of PRP2 on \( \text{HO}^\cdot \) generation in both Fenton’s reaction system and the UV/\( \text{H}_2\text{O}_2 \) reaction system. PRP2 did not affect \( \text{HO}^\cdot \) generation by Fenton’s reaction (Fig. 1), but significantly suppressed \( \text{HO}^\cdot \) generation from the UV/\( \text{H}_2\text{O}_2 \) system (Fig. 2). Therefore, this suggested that PRP2 may directly eliminate \( \text{HO}^\cdot \) without iron chelation. We have already confirmed that histatin suppresses the production of \( \text{HO}^\cdot \) generated by Fenton’s reaction system, without affecting \( \text{HO}^\cdot \) generated from the UV/\( \text{H}_2\text{O}_2 \) reaction system.\(^{34}\) It is interesting to note that the same salivary protein had different effects on \( \text{HO}^\cdot \) production\(^{34}\) (Fig. 2 and 3). In addition to Fenton’s reaction, the generation of highly reactive \( \text{HO}^\cdot \) in vivo is important in a variety of ROS-induced diseases, including oral diseases.\(^{12,36,40}\) These findings suggest that PRPs and other salivary proteins such as histatin, are coupled, and have an antioxidant effect on \( \text{HO}^\cdot \).

**Fig. 4.** Effects of different concentrations of PRP2 (6.7, 20.0, and 33.3 \( \mu \text{M} \)) on \( \text{O}_2^\cdot \) generation from xanthine (X)/xanthine oxidase (XO) reaction system. (A) Dose-response of PRP2 or the control on \( \text{O}_2^\cdot \) generation from X and XO. (B) ESR spin trapping measurement of \( \text{O}_2^\cdot \) generated from XO (0.1 \( \text{U/ml} \)) and X (362 \( \text{mM} \)) in 0.1 M PBS, using 440 mM DMPO as the spin trap, in the absence of PRP2 (control), or under conditions of PRP2 (33.3 \( \mu \text{M} \)) pretreatment, respectively. *Significantly different (\( p<0.05 \)) from the corresponding control value.

**Fig. 5.** Effects of 30rPRP (6.7, 20.0, and 33.3 \( \mu \text{M} \)) on ROS generation. (A) Dose-response of 30rPRP or the control on \( \text{HO}^\cdot \) generated from the Fenton reaction. (B) The dose-response of 30rPRP and the control on \( \text{HO}^\cdot \) generated from the UV/\( \text{H}_2\text{O}_2 \) reaction system is represented. (C) Dose-response of 30rPRP and the control on \( \text{O}_2^\cdot \) generated from the X-XO reaction system.
this might be important evidence for the physiological defense capabilities of salivary proteins in the oral cavity.

In general, homeostatic balance is maintained by ensuring equilibrium in the generation and removal of ROS in biological systems. The breakdown of this balance due to increased production of ROS in the oral cavity is known to lead to an augmented risk of oral disease. Additionally, we have reported that oxidative stress caused by this disruption increased the risk of specific oral diseases such as periodontitis, and systemic diseases such as diabetes and cardiovascular diseases. In periodontal diseases, ROS-induced oxidative stress is associated with a chronic inflammatory response caused by bacteria, resulting in alveolar bone resorption. In fact, neutrophils from the peripheral blood of patients with acute apical periodontitis (AAP) have been reported to increase ROS production, particularly in response to treatment of chronic periapical granulomas. Furthermore, antioxidant vitamins that exhibit antioxidant activity are known to be effective against oral diseases such as the oral lichen planus. It was confirmed that PRP2 directly suppressed the generation of HO• (Fig. 3). These results are the first direct evidence of the antioxidant effects of PRPs on ROS. Previously, we conducted similar antioxidant research tests on all PRPs (PRP1–4). The reason PRP2 was chosen from all PRPs for this study was that the antioxidant effects of HO• by the Cu ion used in Fenton’s reaction showed different results among the PRPs (data not shown). Further, PRP2 was used because it showed clear suppression of HO• production independent of Fenton’s reaction, which is the main result of this study. Indeed, suppression of HO• production, independent of the Fenton’s reaction, was confirmed for all PRPs (data not shown). In histatin research previously reported, an issue was also reported surrounding HO• suppression by Fenton’s reaction using Cu ions. It is, therefore, important to understand the role of Cu ions in the biological system, especially in oral function, and the mechanism of the HO• scavenging effects on salivary protein such as histatin and PRPs. Accordingly, further experiments on these effects should be conducted in the future.

HO• is produced by the biological Harbor Weiss Fenton reaction (Equation 1–3) and is very important in mitigating oxidative stress in diseases, including oral disease. Since PRPs account for 70% of salivary protein, the evidence that PRPs inhibit HO• produced UV/H2O2 reaction system independent of Fenton’s reaction (Equation 1, 2), may indicate novel evidence for saliva as part of the oral defense system.

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\begin{align*}
\text{Fe}^{3+} + \text{O}_2^- & \rightarrow \text{Fe}^{2+} + \text{O}_3^- \quad (1) \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^+ \quad (2) \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \rightarrow \text{OH}^- + \text{HO}^+ + \text{O}_2 \quad (3)
\end{align*}
\]

We also investigated the effects of salivary proteins on other HO• generation systems using the UV/H2O2 reaction system, a well-known Fe2+-independent system. Since PRPs account for 70% of salivary protein, the evidence that PRPs inhibit HO• produced UV/H2O2 reaction system independent of Fenton’s reaction (Equation 1, 2), may indicate novel evidence for saliva as part of the oral defense system.

In conclusion, we have demonstrated for the first time that PRP directly exhibits antioxidant activity against HO• using the ESR method for our analysis. We also confirmed that 30rPRP is involved in this process. Evidence of the antioxidant effect of PRPs suggests that the antioxidant activity of salivary proteins is important for oral defense against ROS-induced oxidative stress.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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