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Inhibition of Protease Activity of Periodontopathogens by Purified Lactoferrin and Lactoferrin Supplement

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Abstract

Periodontitis is caused by periodontopathic bacteria such as *Porphyromonas gingivalis* and *Tannerella forsythia*. *P. gingivalis* has several biologic activities such as protease secretion. Inhibition of protease activities of periodontopathogens is one of the most effective strategies to prevent the progression of periodontitis. Lactoferrin, an iron-binding glycoprotein, is considered to be a useful tool for this purpose, and lactoferrin supplements may be effective for health promotion.

In this study, the effect of purified lactoferrin on *P. gingivalis* and *T. forsythia* protease was examined. The effect of lactoferrin supplement on protease activity was also tested. The purified lactoferrin inhibited the protease activity of both periodontopathogens. Lactoferrin supplement also exhibited the inhibitory effect.

From these results, lactoferrin and lactoferrin supplement are considered to contribute to the prevention of periodontitis by inhibiting the protease activity of periodontopathogens.

Keywords: *P. gingivalis*; *T. forsythia;* Protease; Lactoferrin; Supplement

Introduction

Periodontitis is caused by periodontopathic bacteria such as *Porphyromonas gingivalis* [1] and *Tannerella forsythia* [2], which are components of so-called red-complex bacteria [3]. *P. gingivalis* has several biologic activities, and one of the most important properties is protease activity named gingipain [4]. We have previously isolated gingipain and reported its suppressive activity on human neutrophils [5] and confirmed its virulence in a mouse mixed infection model with *P. gingivalis* and *T. forsythia* [6]. Gingipain was also found to be related with the growth promotion of *P. gingivalis* by *T. forsythia* [7].

Inhibition of pathogenic activities of periodontopathogens is one of the most effective strategies to prevent the progression of periodontitis. Lactoferrin, an 80-kDa iron-binding glycoprotein of the transferrin family, is present in the milk, other exocrine secretions such as tears, and synovial fluid, and the secondary granules of neutrophils and blood [8]. In the oral cavity, it is secreted to saliva [9] and gingival crevicular fluid [10]. It is believed to play an important role in innate immunity, exhibiting antibacterial, antifungal, antiviral, antitumor, parasiticidal, immunomodulatory, and anti-inflammatory activities [11]. The iron-binding capacity of lactoferrin sequesters iron from the microbial environment, contributing to its ability to inhibit the growth of bacteria and yeasts [12,13]. In addition, lactoferrin can directly interact with microbial membranes to alter their permeability through dispersion of membrane components, such as lipopolysaccharide, thereby causing cell death [14].

Aguilera et al reported the antibacterial activity of lactoferrin on *P. gingivalis* and other bacteria [15]. Bovine lactoferrin was found to inhibit the protease activity of *P. gingivalis* [16], but the effect on another periodontopathic bacterium *T. forsythia* was not well known. Lactoferrin supplement is widely used for health promotion, but the actual effect on the protease activity of periodontopathogens is not investigated. Therefore, the purpose of this study was to investigate the inhibition of protease activity of periodontopathogens with purified lactoferrin and lactoferrin supplement.

Materials and Methods

Bacterial strains and culture conditions

P. gingivalis was grown as previously reported [5]. *P. gingivalis* ATCC 33277 was maintained on CDC anaerobic blood agar (Becton Dickinson, Cockeysville, MD, USA) in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2), and inoculated into tryptic soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with hemin (5 μg/mL) and menadione (1 μg/mL). *T. forsythia* was grown as previously reported [6]. *T. forsythia* ATCC 43037 was grown on CDC anaerobic blood agar together with *Fusobacterium nucleatum* ATCC 23726 to accelerate its growth, and transferred to Brain Heart Infusion broth containing 0.001% N-acetyl-muramic acid (Sigma Chemical Co., St. Louis, MO, USA) and 5% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA).

Preparation of sonicated extracts of *P. gingivalis* cells

P. gingivalis cells of late logarithmic stage in tryptic soy broth were harvested by centrifugation and washed with phosphate-buffered saline (PBS). Fifty micrograms of these bacteria were suspended in 0.5 mL of PBS, and the cells were disrupted by sonication on ice [17]. Intact cells

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were removed by centrifugation and the protein concentration of the supernatant was measured.

Protease activity of *P. gingivalis* and *T. forsythia* sonicated extract

N_{α}-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) was purchased from Sigma Aldrich (St. Louis, MS, USA). BAPNA is a synthetic substrate, which develops yellow color when the site of arginine is digested by trypsin-like proteases. Enzyme assay was performed according to the method of Potempa et al [18]. Briefly, *P. gingivalis* sonicated extract was added to an aqueous reaction mixture containing 1 mM BAPNA, 0.2 M Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $CaCl_2$, and 10 mM cysteine. For the control, *P. gingivalis* and *T. forsythia* sonicated extract was removed from the reaction mixture. The background color is calculated by measuring the optical density of *P. gingivalis* and *T. forsythia* sonicated extract.

Lactoferrin preparation

Purified lactoferrin was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and it was dissolved in distilled water. The lactoferrin supplement "Morinaga Lactoferrin Original" was purchased from Morinaga Milk Industry Co. Ltd (Tokyo, Japan). The tablet was crushed into powder and dissolved in distilled water. Insoluble precipitate was removed by centrifugation. To analyze the effect of lactoferrin on protease activity, several concentration of lactoferrin was mixed with bacterial suspension and pre-incubated at 37°C for 30 min. Then, equal amount of BAPNA solution was added to the reaction mixture. The enzyme assay mixture was incubated at 37°C for 30 min or 60 min, and a colorimetric assay was performed (OD 405 nm) to determine colored metabolites. Lactoferrin solution had a light pink color and the optical density (OD) of lactoferrin solution was subtracted from the BAPNA OD.

Results

Inhibition of *P. gingivalis* protease activity by lactoferrin

Lactoferrin was found to inhibit the protease activity of *P. gingivalis* in a dose-dependent manner (Figure 1). The inhibitory effect was observed both after 30 minutes and 60 minutes incubation. The lactoferrin supplement also inhibited the *P. gingivalis* protease activity, but the inhibition rate was lower than that of purified lactoferrin (Figure 2).

Inhibition of *T. forsythia* protease activity by lactoferrin

Similar to the result of *P. gingivalis*, degradation of BAPNA by *T. forsythia* was increased with the prolonged incubation time, but the enzyme activity was much lower than *P. gingivalis* (Figure 3). Lactoferrin was found to inhibit the protease activity in a dose-dependent manner, but the inhibition was not so drastic as that of *P. gingivalis*. Significant inhibition was observed only when 20.4 mg/ml of lactoferrin was added and incubated for 60 min. Time course experiment indicated that protease activity of *T. forsythia* was weakly suppressed in the presence of lactoferrin, but significant inhibition was observed when the reaction mixture was incubated for more than 60 min (Figure 4).

Statistical analysis

Statistical analysis was performed with multiple comparisons (Tukey's method and Bonferroni's method) after one-way analysis of variance for the concentration-dependent inhibition. For the time-course experiment, Student's t test was applied.

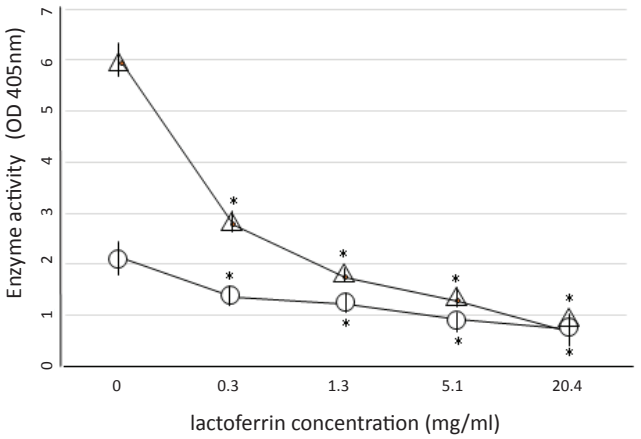

Figure 1: Dose-dependent nhibition of BAPNA hydrolyzing activity of *P. gingivalis* by purified lactoferrin.
Triangle: 60 min-incubation, Circle: 30 min-incubation.
Asterisk indicates significantly different from the OD at 0 mg/ml ($p<0.05$).

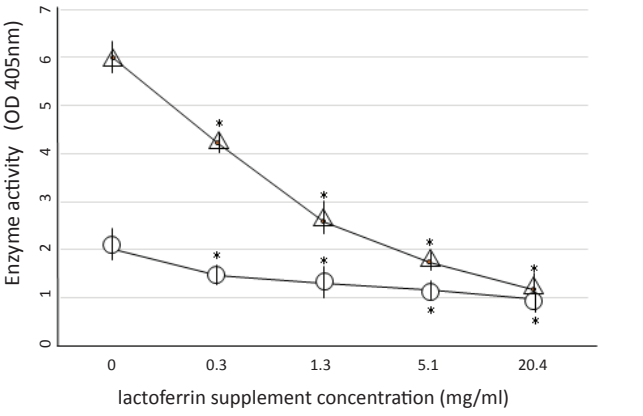

Figure 2: Dose-dependent inhibition of BAPNA hydrolyzing activity of *P. gingivalis* by lactoferrin supplement.
Triangle: 60 min-incubation, Circle: 30 min-incubation.
Asterisk indicates significantly different from the OD at 0 mg/ml ($p<0.05$).

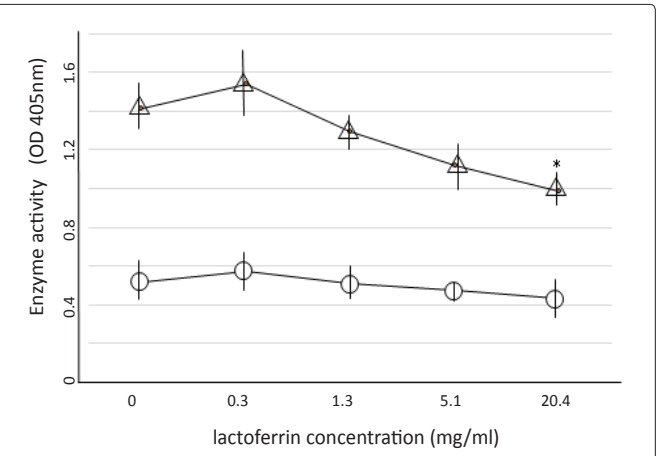

Figure 3: Inhibition of BAPNA hydrolyzing activity of *T. forsythia* by purified lactoferrin.
Triangle: 60 min-incubation, Circle: 30 min-incubation.
Asterisk indicates significantly different from the OD at 0 mg/ml ($p<0.05$).

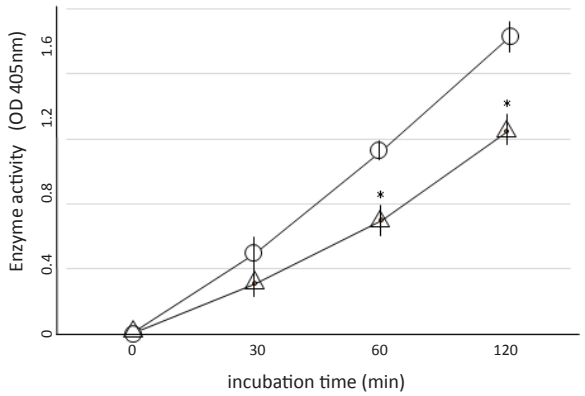

Figure 4: Time course of BAPNA hydrolysis by *T. forsythia* in the presence and absence of purified lactoferrin.
Circle: In the absence of purified lactoferrin, Triangle: In the presence of purified lactoferrin (20.4mg/ml)
Asterisk indicates significantly different from the OD in the absence of lactoferin ($p<0.05$).

Discussion

The importance of protease of periodontopathogens is well known [4]. It is closely associated with the destruction of periodontal connective tissues, disruption of host defense mechanisms, and development and maintenance of inflammation in periodontal pockets. We had previously reported the effect of gingipains on host defense system [5], mixed infection [6], and growth promotion by *T. forsythia* [7]. Inhibition of gingipain activity is considered to be a useful strategy for prevention of periodontitis, and new protease inhibitor was developed [19].

Periodontitis is widely spread among adults, and more easy method to suppress the protease activity is required. Lactoferrin, a multifunctional protein, is known to be safe to even children, and the supplement is widely used for health promotion.

The protease activity of *P. gingivalis* was suppressed by purified lactoferrin in a dose-dependent manner, and it was consistent with the report by Daspher et al [16]. Daspher et al reported the possible mechanism of protease inhibition by lactoferrin. The zinc ion-binding site of lactoferrin can bind the zinc ion in the active site of the gingipains of *P. gingivalis*. They also indicated that lactoferrin is a slow-binding inhibitor, which is consistent with our experiments, because 30 minutes pre-incubation was required for a maximum inhibition [data not shown]. We also examined the effect of lactoferrin supplement. The lactoferrin supplement also inhibited the protease activity of *P. gingivalis*, but the inhibition rate was lower than that of purified lactoferrin (data not shown). It is not surprising because the supplement contains several additives in the tablet such as carbohydrates, fiber, oil, and the lactoferrin concentration is lower than purified lactoferrin reagent. There is also a possibility that such additives may have affected the color development of BAPNA, and further analysis is necessary.

The protease activity of *T. forsythia* was much lower than that of *P. gingivalis*. It may be the reason of low inhibitory activity of lactoferrin on *T. forsythia*. In the mixed infection of *P. gingivalis* and *T. forsythia, P. gingivalis* is considered to be using nutrients from *T. forsythia* by the help of gingipain [7]. So, inhibition of *P. gingivalis* protease will affect the growth promotion of *P. gingivalis* by *T. forsythia*.

Periodontopathic bacteria are also considered to cause oral malodor [20,21], and lactoferrin will be useful in reducing oral malodor. Nakano et al reported that lactoferrin-containing tablet showed a suppressive effect on oral malodor by a clinical examination [22].

From these results, lactoferrin and lactoferrin supplement is considered to improve the quality of life by inhibiting the protease activity of periodontopathogens.

Acknowledgements

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Conflict of Interests

There is no conflict of interests in this work.

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