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Interactions between hyaluronic acid, lysozyme, and the glucose oxidase-mediated lactoperoxidase system in enzymatic and candidacidal activities

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ABSTRACT

Objective: To investigate interactions between hyaluronic acid (HA), lysozyme, and the glucose oxidase-mediated lactoperoxidase (GO-LPO) system in enzymatic and candidacidal activities.

Design: The influences of HA (0.5, 1.0, and 2.0 mg/mL) and lysozyme (30 μg/mL hen egg white lysozyme) on the enzymatic activity of GO-LPO system (25 μg/mL bovine LPO, 1 mM KSCN, 10 units/mL GO, and 30 μg/mL glucose) were determined by measuring oxidized o-dianisidine production. The influence of the GO-LPO system on lysozyme activity was determined by measuring the turbidity of a *Micrococcus lysodeikticus* suspension. The effects of interactions between HA, lysozyme, the GO-LPO system on candidacidal activity were examined by pre-incubating various combinations of components. Candidacidal activity was determined by comparing the numbers of colony forming units using *Candida albicans* ATCC strains 10231, 18804, and 11006.

Results: HA inhibited the enzymatic activity of the GO-LPO system in a dose-dependent manner. HA inhibited the candidacidal activities of the GO-LPO system. However, the inhibitory activity of HA was not significantly different according to concentration of HA. The GO-LPO system enhanced the enzymatic activity of lysozyme, though lysozyme did not affect the enzymatic activity of the GO-LPO system. The candidacidal activities of the GO-LPO system and lysozyme were not additive.

Conclusions: HA inhibited the enzymatic and candidacidal activity of the GO-LPO system. The GO-LPO system enhanced the enzymatic activity of lysozyme, but the candidacidal activities were not additive.

1. Introduction

Hyaluronic acid (HA) is present in human saliva and may contribute to the lubricating and healing properties of saliva, thereby assisting in protecting the oral mucosa.[1,2] HA also has anti-*Candida* activity.[3] Due to its viscoelastic properties and non-immunogeneity,[4] HA can be a candidate substance for effective saliva substitutes for patients with dry mouth as a solution containing HA has rheological properties which

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might mimic the viscosity of human saliva at shear rates for routine oral functions.[5] A relationship between decreased levels of salivary HA and dry mouth symptoms has also been reported,[6] suggesting that HA is important in protecting and lubricating the oral mucosa. In addition, the wound repair and potential anti-*Candida* activities of HA[3,7] can provide additional benefits to patients with dry mouth, who are susceptible to oral mucosal injuries and candidiasis.[8]

The most widely used antimicrobial host proteins in oral health care products are lysozyme and the peroxidase system.[9] One lysozyme supplement in these products is hen egg white lysozyme (HEWL). The bactericidal activity of lysozyme is muramidase-dependent and uses cation-dependent or structure-related mechanisms.[10–12] Its antifungal activity is also well known.[13–15] The peroxidase system supplements are the glucose oxidase-mediated lactoperoxidase (GO-LPO) system, which comprises bovine lactoperoxidase (bLPO), glucose oxidase (GO), and SCN^-. The GO-LPO system interacts with peroxidases (salivary peroxidase and myeloperoxidase), SCN^-, H_2O_2, and glucose in whole saliva. GO degrades glucose and produces H_2O_2, which serves as a substrate for bLPO and peroxidase in saliva to form antimicrobial $OSCN^-$ from SCN^-.[16] Furthermore, by using glucose, GO can hamper the carbohydrate metabolism of glycolytic cariogenic bacteria.[16–18] In addition, the GO-LPO system has antifungal activities.[19]

Many antimicrobial proteins in saliva interact with each other *in vitro*. These interactions can result in additive, synergistic, or inhibitory effects on mutans streptococci, lactobacilli, or fungi.[20–22] For example, interactions have been reported between sIgA and peroxidase,[23] lactoferrin and peroxidase,[24,25] lactoferrin and lysozyme,[26] lysozyme and histatins,[27] and lysozyme and the peroxidase system.[15,28]

Although these observations are *in vitro*, such concerted effects likely exist in mixed saliva or oral health care products, where all the components are present simultaneously. Therefore, HA molecules may also interact with antimicrobial molecules in human saliva or saliva substitutes. HA and peroxidase have been suggested to form complex molecules,[29] and HA affects the enzymatic and candidacidal activities of lysozyme and the peroxidase system.[5,30] However, there is no information as to how HA affects the enzymatic and anticandidal activities of the GO-LPO system or how lysozyme and the GO-LPO system affect each other. In this study, we investigated the influences of HA on the enzymatic and anticandidal activities of the GO-LPO system and the interactions between lysozyme and the GO-LPO system.

2. Materials and methods

2.1. HA, lysozyme, and glucose oxidase-mediated lactoperoxidase system

HA (1630 kDa, Sigma–Aldrich Chemical Co., St Louis, MO, USA) was used at three concentrations: 0.5, 1.0, and 2.0 mg/mL. HEWL (final concentration of 30 μg/mL) (Sigma–Aldrich) served as the lysozyme source. The GO-LPO system included bLPO (final concentration of 25 μg/mL), potassium thiocyanate (KSCN, final concentration of 1 mM), GO (final concentration of 10 units/mL), and glucose (final concentration of 30 μg/mL) (Sigma–Aldrich). All components were solubilized with simulated salivary buffer (SSB, 0.021 M Na_2HPO_4/NaH_2PO_4, pH 7.0, containing 36 mM NaCl and 0.96 mM $CaCl_2$).[31]

2.2. Influence of HA and lysozyme on the enzymatic activity of the glucose oxidase-mediated lactoperoxidase system

A glucose assay kit (Sigma–Aldrich) which included GO/peroxidase reagent and o-dianisidine, was used to investigate the influence of HA and HEWL on the enzymatic activity of the GO-LPO system. The GO/peroxidase reagent was divided into two parts, one dissolved in SSB and the other dissolved in SSB containing HA or HEWL, and pre-incubated for 30 min at room temperature (RT). Enzymatic activity of the two different GO/peroxidase reagents was measured using samples with known glucose concentrations (20, 40, and 60 μg/mL). Oxidized o-dianisidine production, measured by the ODs at 540 nm, reflected the enzymatic activity of GO/peroxidase reagents. Experiments were duplicated and performed three times.

2.3. Influence of the GO-LPO system on the enzymatic activity of lysozyme

The turbidimetric method was used to determine lysozyme activity. Samples were placed in a suspension of lyophilized *Micrococcus lysodeikticus* (ATCC 4698), starting at OD_{450} = 0.65–0.70, so the lysozyme could degrade the substrate. Lysozyme activity was expressed as units/mL. The effects of the GO-LPO system on the enzymatic activity of HEWL were examined by incubating 500 μL of the GO-LPO system with 500 μL of HEWL for 10 min at RT. The incubated mixture was placed in a suspension of *M. lysodeikticus*. HEWL was incubated with buffer as a control. The GO-LPO system components incubated with buffer or buffer alone was used as a blank. The experiment was duplicated and performed eight times.

2.4. Influence of HA on the candidacidal activities of the GO-LPO system

The influence of HA on the candidacidal activity of the GO-LPO system was investigated two ways: (1) HA was pre-incubated with candidal cells and (2) HA was pre-incubated with the GO-LPO system. Both experiments were performed six times.

Three kinds of *Candida albicans* strains, ATCC strains 18804, 10231, and 11006, were used for the candidacidal assay, because these strains have been used in various fields of dental studies and each strain shows different behaviour.[30]

2.4.1. HA pre-incubated with candidal cells then added to the GO-LPO system

One colony of *C. albicans* grown on Sabouraud dextrose agar (SDA) was inoculated into 10 mL Sabouraud dextrose broth and incubated with shaking at 37 °C for 18 h. Cells were harvested, washed, and resuspended to 1×10^5 cells per mL in SSB. Twenty microliters of cell suspension was added to an equal volume of HA (final concentrations of 0.5, 1.0, or 2.0 mg/mL). The samples were incubated with shaking at 37 °C for 1 h. Forty microliters of cell suspension was mixed with 20 μL of