Interactions between Hyaluronic Acid, Lysozyme, Peroxidase, and Glucose Oxidase in Enzymatic Activities at Low pH

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Purpose: Many substances in saliva or oral health care products interact with each other. The aim of this study was to investigate interactions between hyaluronic acid (HA), lysozyme, peroxidase, and glucose oxidase (GO) in enzymatic activities at low pH levels.

Methods: HA (0.5 mg/mL), hen egg-white lysozyme (HEWL, 30 μg/mL), bovine lactoperoxidase (bLPO, 25 μg/mL), and GO (50 μg/mL) were used. The influences of HA, bLPO, and GO on HEWL activity were determined by measuring the turbidity of a Micrococcus lysodeikticus suspension. The influences of HA and HEWL on bLPO activity were determined by the NbsSCN assay, measuring the rate of oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs)2. The influences of HA and HEWL on GO activity were determined by measuring oxidized o-dianisidine production. All experiments were performed at pH 4, 5, and 6.

Results: HA and GO did not affect the enzymatic activity of HEWL at pH 4, 5, and 6. bLPO enhanced the enzymatic activity of HEWL at pH 5 (p<0.05) and pH 6 (p<0.05) significantly. The enzymatic activity of bLPO was not affected by HA and HEWL at pH 4, 5, and 6. HA and HEWL did not affect the enzymatic activity of the GO at pH 4, 5, and 6.

Conclusions: Peroxidase enhances lysozyme activity at low pH, otherwise there were no significant interactions in enzymatic activities between HA, lysozyme, peroxidase, and GO at low pH levels.

Key Words: Glucose oxidase; Hyaluronic acid; Low pH; Lysozyme; Peroxidase

INTRODUCTION

Hyaluronic acid (HA) is a glycosaminoglycan consisting of alternating D−glucuronic acid and N-acetyl-D-glucosamine units. Due to the intrinsic biocompatibility of HA and its viscoelastic physical properties,1 HA has been suggested as a candidate substance for saliva substitutes for patients with dry mouth, and it displays similar viscosity to human saliva at certain concentrations.2 HA has lubricating, wound repair, and fungistatic activities.3−5 A inverse relationship between concentration of salivary HA and dry mouth symptoms has been reported.6

Lysozyme and peroxidase are the most common antimicrobial host proteins incorporated in oral health care products.7 Hen egg-white lysozyme (HEWL) and bovine lactoperoxidase (bLPO) are commonly used as lysozyme and peroxidase sources, respectively. The muramidase activity of lysozyme is well known and lysozyme also provides antimicrobial activity through its cationic property and structure-related mechanisms.8−10 Peroxidase is usually incorporated as the glucose oxidase-mediated bLPO (GO-bLPO) system, which comprises bLPO, glucose oxidase (GO), and thiocyanate (SCN−). GO in the GO-bLPO system utilizes glucose in whole saliva and produces hydrogen peroxide (H2O2), which
serves as a substrate for activating bLPO and peroxidase in saliva.

It is well known that antimicrobial proteins interact with each other and the results could be enhancing or inhibitory effects on microorganisms. The previous studies related to lysozyme and peroxidase include between lysozyme and lactoferrin, lysozyme and histatins, lysozyme and the peroxidase system, lysozyme and the GO-bLPO system, peroxidase and secretory immunoglobulin A, and peroxidase and lactoferrin. Antimicrobials could also interact with candidate substances of artificial saliva such as HA. The results could also be enhancing or inhibitory effects.

Salivary pH varies widely and depends on salivary flow, food taking, bacterial load, etc. Patients with dry mouth usually have relatively lower salivary pH and buffering capacity compared with normosalivators. Therefore, when they use oral health care products such as artificial saliva and mouth rinses, interactions between various components occur in low pH and the results of these interactions might be different from those in neutral pH. The purpose of this study was to investigate interactions between HA, lysozyme, peroxidase, and GO in enzymatic activities at low pH levels.

MATERIALS AND METHODS

1. HA, Lysozyme, Peroxidase, and GO

HA (1,630 kDa, final concentration of 0.5 mg/mL), HEWL (final concentration of 30 μg/mL), bLPO (final concentration of 25 μg/mL), and GO from Aspergillus niger (final concentration of 50 μg/mL) (Sigma-Aldrich Chemical Co., St Louis, MO, USA) were used in the experiments. All components were solubilized with simulated salivary buffer (SSB, 0.021 M Na₂HPO₄/NaH₂PO₄, containing 36 mM NaCl and 0.96 mM CaCl₂) at different pH levels (pH 4, 5, and 6).

2. Influence of HA, Peroxidase, and GO on the Enzymatic Activity of Lysozyme

To determine the effects of HA, peroxidase, and GO on lysozyme activity, 250 μL of HA, bLPO, or GO in SSB was incubated with 250 μL of HEWL for 10 min at room temperature (RT). The turbidimetric method was used to measure lysozyme activity. The incubated mixture was placed in a lyophilized cell suspension of Micrococcus lysodeikticus ATCC 4698 as a substrate and an incubated mixture of buffer with HEWL was used as a control. Either an incubated mixture of HA, bLPO, or GO with buffer, or an incubated buffer alone was used as a blank. The experiment was duplicated and performed 10 times. The lysozyme activity was expressed as Units/mL.

3. Influence of HA and Lysozyme on the Enzymatic Activity of Peroxidase

To determine the effects of HA and lysozyme on peroxidase activity, 250 μL of HA or HEWL was incubated with 250 μL of bLPO for 10 min at RT. For the NbsSCN assay, 300 μL of reaction mixture was prepared, in that 15 μL of KSCN (final concentration of 4.2 mM SCN⁻) and 15 μL of sample solution were added, and the reaction was initiated by the addition of 15 μL of H₂O₂ (final concentrations were 50 μM). Peroxidase activity was determined by measuring the rate of oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5´-dithiobis(2-nitrobenzoic acid) (Nbs) by hypothiocyanite (OSC⁻) ions generated during the oxidation of SCN⁻ by bLPO. An incubated mixture of buffer with either bLPO was used as a control. For the blank reaction, an incubated mixture of HA and HEWL with buffer, or an incubated buffer alone was used. The experiment was duplicated and performed 10 times. Peroxidase activities were expressed as mUnits/mL.

4. Influence of HA and Lysozyme on the Enzymatic Activity of GO

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 GO. 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 RT. Glucose (final concentrations of 20, 40, and 60 μg/mL) was added to initiate the reaction and enzymatic activities of the two different GO/peroxidase reagents were measured. Oxidized o-dianisidine production, measured by the optical density (OD) at 540 nm, reflected the enzymatic activity of GO/peroxidase reagents. Experiments were duplicated and
performed 6 times.

5. Statistics
The Wilcoxon signed rank test was used to analyze differences between variables. p-values less than 0.05 were considered statistically significant.

RESULTS

1. Influence of HA, Peroxidase, and GO on the Enzymatic Activity of Lysozyme
The enzymatic activity of HEWL was increased by increase of pH from 4 to 6. The enzymatic activity of HEWL was not affected by HA at pH 4, 5, and 6. bLPO enhanced the enzymatic activity of HEWL, and its effects were statistically significant at pH 5 (p<0.05) and pH 6 (p<0.05). The percentage of enhancement was 3.9±7.4%, 4.6±4.4%, and 5.4±4.9% at pH 4, 5, and 6, respectively. GO did not affect the enzymatic activity of HEWL at pH 4, 5, and 6 (Table 1).

2. Influence of HA and HEWL on the Enzymatic Activity of Peroxidase
The enzymatic activity of bLPO was decreased by increase of pH from 4 to 6. The enzymatic activity of bLPO was not affected by HA and HEWL at pH 4, 5, and 6 (Table 2).

3. Influence of HA and Lysozyme on the Enzymatic Activity of GO
The enzymatic activity of GO was increased by increase

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<thead>
<tr>
<th>Variable</th>
<th>pH</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEWL (Units/mL)</td>
<td>366.8±33.7</td>
<td>760.8±42.1</td>
<td>972.6±59.2</td>
<td>0.307</td>
</tr>
<tr>
<td>HEWL with HA (Units/mL)</td>
<td>358.0±20.7</td>
<td>772.8±22.6</td>
<td>981.0±39.6</td>
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</thead>
<tbody>
<tr>
<td>HEWL (Units/mL)</td>
<td>370.6±23.4</td>
<td>847.2±67.7</td>
<td>976.6±53.0</td>
<td>0.153</td>
</tr>
<tr>
<td>HEWL with bLPO (Units/mL)</td>
<td>384.8±30.4</td>
<td>884.4±54.3</td>
<td>1,029.0±56.5</td>
<td>0.017*</td>
</tr>
</tbody>
</table>

Table 1. Influences of HA, peroxidase, and GO on the enzymatic activity of lysozyme at low pH levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>pH</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEWL (Units/mL)</td>
<td>374.6±25.9</td>
<td>783.0±52.9</td>
<td>989.6±76.2</td>
<td>0.221</td>
</tr>
<tr>
<td>HEWL with GO (Units/mL)</td>
<td>368.4±27.0</td>
<td>784.0±39.5</td>
<td>1,005.4±77.0</td>
<td>0.313</td>
</tr>
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</table>

Table 2. Influences of HA and peroxidase on the enzymatic activity of peroxidase at low pH levels

Values are presented as mean±standard deviation.
HA, hyaluronic acid; GO, glucose oxidase; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase.
Statistical significance was evaluated using the Wilcoxon signed rank test; *p<0.05.
of glucose in a concentration-dependent manner. HA and HEWL did not affect the enzymatic activity of the GO at pH 4, 5, and 6 (Table 3).

**DISCUSSION**

There are many antimicrobial host proteins in saliva and they play significant roles in protecting the oral cavity from noxious agents. Caries, periodontal diseases, mucosal lesions, and even halitosis are regarded to be associated with the impairment of these antimicrobial molecules. The lack of antimicrobial components often results from hyposalivation. Therefore, individuals with dry mouth usually need some commercialized oral health care products compensating for the decreased antimicrobial functions. In this case, interactions between antimicrobial host proteins and oral health care products may occur. These interactions have been investigated in previous studies, which proved additive, synergistic or inhibitory effects on target systems.

Most previous studies were implemented under neutral or close to neutral pH condition, but actual environment of the oral cavity could represent lower pH state in the case of hyposalivation or following intake of foodstuffs. The increase of geriatric population and the frequent use of medications due to chronic illnesses lead to higher prevalence of individuals with dry mouth. For this reason, it is worthwhile to evaluate the interactions between antimicrobial components at low pH values.

HA and GO did not affect the enzymatic activity of HEWL under low pH, while bLPO increased the one in the present study. The results of the previous studies about the influence of HA on lysozyme activity were not the same. One study reported that HA did not affect the enzymatic activity of HEWL at pH 7. Another study suggested that enzymatic activity of lysozyme was inhibited by HA at acidic pH. These mean that the interaction between HA and lysozyme would be affected by experimental conditions such as ionic strength and pH, in which HA may modify lysozyme activity through the formation of complexes with lysozyme molecule. On the contrary, bLPO was proved to enhance the enzymatic activity of lysozyme even at low pH, because ionic interaction between two enzymes with cationic nature is dominant. GO alone is not supposed to affect

<table>
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<tr>
<th>Variable</th>
<th>Glucose (μg/mL), pH 4</th>
<th>Glucose (μg/mL), pH 5</th>
<th>Glucose (μg/mL), pH 6</th>
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<td></td>
<td>GO (OD)</td>
<td>GO with HA (OD)</td>
<td>p-value</td>
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<tr>
<td>Influence of HA (n=6)</td>
<td>0.33±0.01</td>
<td>0.33±0.01</td>
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<td>20</td>
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<td>40</td>
<td>0.98±0.038</td>
<td>0.97±0.030</td>
<td>0.233</td>
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<td></td>
<td>GO with HA (OD)</td>
<td>GO with HEWL (OD)</td>
<td>p-value</td>
</tr>
<tr>
<td>Influence of HEWL (n=6)</td>
<td>0.35±0.003</td>
<td>0.34±0.003</td>
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Values are presented as mean±standard deviation. Statistical significance was evaluated using the Wilcoxon signed rank test.
enzymatic activity of HEWL, but depend on the activity of bLPO, which supports the evidence that GO-bLPO enhanced the enzymatic activity of HEWL.\textsuperscript{[19]}

On the other hand, neither HA nor HEWL had an effect on the enzymatic activity of bLPO in the present study. Although concentrated HA contain viscoelastic properties and could inhibit the enzymatic properties of bLPO by diffusion-controlled limit,\textsuperscript{5,13}\textsuperscript{9} the specific interaction between HA and bLPO might disaggregate HA and attenuate the inhibitory effect of HA.\textsuperscript{29,30}\textsuperscript{14} Apart from the result of this study, lysozyme and the salivary peroxidase system had a synergistic effects in inhibition of glucose metabolism by \textit{Streptococcus mutans}.\textsuperscript{18}

The enzymatic activity of GO-mediated peroxidase was not inhibited by HA as well as HEWL at low pH in the present study. The previous study suggested that HA inhibited the enzymatic activity of GO-mediated peroxidase, but it did not affect that of bLPO at pH 7.\textsuperscript{5,13}\textsuperscript{9} This suggests that HA might compromise the capacity of GO-mediated peroxidase by inhibiting the enzymatic activity of GO. However, the results were different at lower pH values. On the contrary, there were no evidence that HEWL enhance or inhibit the enzymatic activity of GO,\textsuperscript{19} which means ionic interaction between HEWL and GO might be negligible.

There were some limitations to extrapolate these results into those of in vivo system. Specific interactions between several antimicrobial components were too simplified to assess the real interactions in the oral cavity, because there are diverse innate molecules and multivariate factors which could influence on the real in vivo system. In spite of these limitations, the search for the interactions between antimicrobial host proteins in low pH must have extended our knowledge for the evaluation and treatments of symptoms and diseases.

In conclusion, there were no significant enhancing or inhibitory effects among HA, lysozyme, peroxidase, and GO at low pH values except that bLPO enhanced the enzymatic activity of HEWL.

**CONFLICT OF INTEREST**

No potential conflict of interest relevant to this article was reported.

**REFERENCES**

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