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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 (H_2O_2), which

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serves as a substrate for activating bLPO and peroxidase in saliva.[11]

It is well known that antimicrobial proteins interact with each other and the results could be enhancing or inhibitory effects on microorganisms.[12-14] The previous studies related to lysozyme and peroxidase include between lysozyme and lactoferrin,[15] lysozyme and histatins,[16] lysozyme and the peroxidase system,[17,18] lysozyme and the GO-bLPO system,[19] peroxidase and secretory immunoglobulin A,[20] and peroxidase and lactoferrin.[21,22] Antimicrobials could also interact with candidate substances of artificial saliva such as HA.[2,5,19] 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 Na_2HPO_4/NaH_2PO_4, containing 36 mM NaCl and 0.96 mM $CaCl_2$)[23] 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.[24] 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 H_2O_2 (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) $(Nbs)_2$ by hypothiocyanite ($OSCN^-$) ions generated during the oxidation of SCN^- by bLPO.[25] 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

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

Variable	pH		
	4	5	6
Influence of HA (n=10)			
HEWL (Units/mL)	366.8±33.7	760.8±42.1	972.6±59.2
HEWL with HA (Units/mL)	358.0±20.7	772.8±22.6	981.0±39.6
p-value	0.307	0.343	0.575
Influence of bLPO (n=10)			
HEWL (Units/mL)	370.6±23.4	847.2±67.7	976.6±53.0
HEWL with bLPO (Units/mL)	384.8±30.4	884.4±54.3	1,029.0±65.5
p-value	0.153	0.017*	0.013*
Influence of GO (n=10)			
HEWL (Units/mL)	374.6±25.9	783.0±52.9	989.6±76.2
HEWL with GO (Units/mL)	368.4±27.0	784.0±39.5	1,005.4±77.0
p-value	0.221	0.919	0.313

Values are presentd 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; *$p<0.05$.

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

Variable	pH		
	4	5	6
Influence of HA (n=10)			
bLPO (mUnits/mL)	8.223±0.523	7.267±0.615	6.051±0.666
bLPO with HA (mUnits/mL)	8.369±0.562	7.349±0.472	6.226±0.579
p-value	0.167	0.594	0.173
Influence of HEWL (n=10)			
bLPO (mUnits/mL)	8.417±0.664	7.734±0.435	5.762±0.599
bLPO with HEWL (mUnits/mL)	8.651±0.607	7.887±0.443	5.981±0.711
p-value	0.333	0.206	0.260

Values are presentd as mean±standard deviation.
HA, hyaluronic acid; bLPO, bovine lactoperoxidase; HEWL, hen egg-white lysozyme.
Statistical significance was evaluated using the Wilcoxon signed rank test.

Table 3. Influences of HA and lysozyme on the enzymatic activity of GO at low pH levels

Variable	Glucose (μg/mL), pH 4			Glucose (μg/mL), pH 5			Glucose (μg/mL), pH 6		
	20	40	60	20	40	60	20	40	60
Influence of HA (n=6)									
GO (OD)	0.334±0.010	0.668±0.028	0.980±0.038	0.350±0.004	0.688±0.008	1.007±0.018	0.345±0.003	0.686±0.010	1.000±0.014
GO with HA (OD)	0.335±0.011	0.669±0.028	0.977±0.030	0.352±0.002	0.685±0.005	1.013±0.036	0.346±0.003	0.689±0.009	1.000±0.015
p-value	0.480	1.000	0.833	0.223	0.249	0.917	0.279	0.078	0.916
Influence of HEWL (n=6)									
GO (OD)	0.351±0.003	0.690±0.009	1.005±0.013	0.346±0.007	0.691±0.016	1.003±0.024	0.348±0.007	0.687±0.004	0.998±0.010
GO with HEWL (OD)	0.350±0.003	0.691±0.007	1.005±0.011	0.348±0.007	0.690±0.013	1.014±0.029	0.347±0.003	0.687±0.002	0.998±0.008
p-value	0.194	0.686	0.500	0.344	0.917	0.249	0.916	1.000	1.000

Values are presentd as mean±standard deviation.
HA, hyaluronic acid; GO, glucose oxidase; HEWL, hen egg-white lysozyme; OD, optical density.
Statistical significance was evaluated using the Wilcoxon signed rank test.

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.[7] 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.[12-14]

Most previous studies were implemented under neutral or close to neutral pH condition, but actual environment of the oral cavity could represents lower pH state in the case of hyposalivation or following intake of foodstuffs.[26] The increase of geriatric population and the frequent use of medications due to chronic illnesses lead to higher prevalence of individuals with dry mouth.[27] 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.[2] Another study suggested that enzymatic activity of lysozyme was inhibited by HA at acidic pH.[28] 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.[28] 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.[17] GO alone is not supposed to affect

enzymatic activity of HEWL, but depend on the activity of bLPO, which supports the evidence that GO-bLPO enhanced the enzymatic activity of HEWL.[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,[5,19] the specific interaction between HA and bLPO might disaggregate HA and attenuate the inhibitory effect of HA.[29,30] Apart from the result of this study, lysozyme and the salivary peroxidase system had a synergistic effects in inhibition of glucose metabolism by *Streptococcus mutans*.[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.[2,19] 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,[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 from dry mouth and related 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

1. Almond A. Hyaluronan. Cell Mol Life Sci 2007;64:1591-1596.
2. Park MS, Chang JY, Kang JH, Park KP, Kho HS. Rheological properties of hyaluronic acid and its effects on salivary enzymes and candida. Oral Dis 2010;16:382-387.
3. Sakai A, Akifusa S, Itano N, et al. Potential role of high molecular weight hyaluronan in the anti-Candida activity of human oral epithelial cells. Med Mycol 2007;45:73-79.
4. Chen WY, Abatangelo G. Functions of hyaluronan in wound repair. Wound Repair Regen 1999;7:79-89.
5. Kang JH, Kim YY, Chang JY, Kho HS. Influences of hyaluronic acid on the anticandidal activities of lysozyme and the peroxidase system. Oral Dis 2011;17:577-583.
6. Higuchi Y, Ansai T, Awano S, et al. Salivary levels of hyaluronic acid in female patients with dry mouth compared with age-matched controls: a pilot study. Biomed Res 2009;30:63-68.
7. Tenovuo J. Clinical applications of antimicrobial host proteins lactoperoxidase, lysozyme and lactoferrin in xerostomia: efficacy and safety. Oral Dis 2002;8:23-29.
8. Laible NJ, Germaine GR. Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against Streptococcus sanguis and Streptococcus faecalis: inhibition by chitin oligosaccharides. Infect Immun 1985;48:720-728.
9. Ibrahim HR, Matsuzaki T, Aoki T. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett 2001;506:27-32.
10. Ihalin R, Loimaranta V, Tenovuo J. Origin, structure, and biological activities of peroxidases in human saliva. Arch Biochem Biophys 2006;445:261-268.
11. Hannig C, Hannig M, Attin T. Enzymes in the acquired enamel pellicle. Eur J Oral Sci 2005;113:2-13.
12. Tenovuo J, Lumikari M, Soukka T. Salivary lysozyme, lactoferrin and peroxidases: antibacterial effects on cariogenic bacteria and clinical applications in preventive dentistry. Proc Finn Dent Soc 1991;87:197-208.
13. Tenovuo J. Antimicrobial function of human saliva--how important is it for oral health? Acta Odontol Scand 1998;56:250-256.
14. Lenander-Lumikari M, Loimaranta V. Saliva and dental caries. Adv Dent Res 2000;14:40-47.
15. Soukka T, Lumikari M, Tenovuo J. Combined bactericidal effect of human lactoferrin and lysozyme against Streptococcus mutans serotype c. Microbial Ecol Health Dis 1991;4:259-264.
16. Murakami Y, Nagata H, Amano A, et al. Inhibitory effects of human salivary histatins and lysozyme on coaggregation between Porphyromonas gingivalis and Streptococcus mitis. Infect Immun 1991;59:3284-3286.
17. Lee JY, Kim YY, Chang JY, Park MS, Kho HS. The effects of peroxidase on the enzymatic and candidacidal activities of lysozyme. Arch Oral Biol 2010;55:607-612.
18. Lenander-Lumikari M, Månsson-Rahemtulla B, Rahemtulla F. Lysozyme enhances the inhibitory effects of the peroxidase system on glucose metabolism of Streptococcus mutans. J Dent Res 1992;71:484-490.
19. Cho MA, Kim YY, Chang JY, Kho HS. Interactions between hyal-

uronic acid, lysozyme, and the glucose oxidase-mediated lactoperoxidase system in enzymatic and candidacidal activities. Arch Oral Biol 2013;58:1349-1356.
20. Tenovuo J, Moldoveanu Z, Mestecky J, Pruitt KM, Rahemtulla BM. Interaction of specific and innate factors of immunity: IgA enhances the antimicrobial effect of the lactoperoxidase system against Streptococcus mutans. J Immunol 1982;128:726-731.
21. Lassiter MO, Newsome AL, Sams LD, Arnold RR. Characterization of lactoferrin interaction with Streptococcus mutans. J Dent Res 1987;66:480-485.
22. Soukka T, Lumikari M, Tenovuo J. Combined inhibitory effect of lactoferrin and lactoperoxidase system on the viability of Streptococcus mutans, serotype c. Scand J Dent Res 1991;99:390-396.
23. Bennick A, Cannon M. Quantitative study of the interaction of salivary acidic proline-rich proteins with hydroxyapatite. Caries Res 1978;12:159-169.
24. Grossowicz N, Ariel M. Methods for determination of lysozyme activity. Methods Biochem Anal 1983;29:435-446.
25. Mansson-Rahemtulla B, Baldone DC, Pruitt KM, Rahemtulla F. Specific assays for peroxidases in human saliva. Arch Oral Biol 1986;31:661-668.
26. Luke GA, Gough H, Beeley JA, Geddes DA. Human salivary sugar clearance after sugar rinses and intake of foodstuffs. Caries Res 1999;33:123-129.
27. Turner MD, Ship JA. Dry mouth and its effects on the oral health of elderly people. J Am Dent Assoc 2007;138 Suppl:15S-20S.
28. Avila JL, Convit J. Inhibition of leucocytic lysosomal enzymes by glycosaminoglycans in vitro. Biochem J 1975;152:57-64.
29. Baker MS, Green SP, Lowther DA. Changes in the viscosity of hyaluronic acid after exposure to a myeloperoxidase-derived oxidant. Arthritis Rheum 1989;32:461-467.
30. Lindvall S, Rydell G. Influence of various compounds on the degradation of hyaluronic acid by a myeloperoxidase system. Chem Biol Interact 1994;90:1-12.