Original Article

JOMP
Journal of Oral Medicine and Pain

ISSN 2288-9272
J Oral Med Pain 2014;39(3):90-95
http://dx.doi.org/10.14476/jomp.2014.39.3.90

# Viscosity and Wettability of Hyaluronic Acid according to Antimicrobial Supplementation, Ionic Strength, and pH

Hong-Seop Kho[1,2], Ji-Youn Chang[1], Yoon-Young Kim[1], Moon-Soo Park[3]

[1]Department of Oral Medicine and Oral Diagnosis, School of Dentistry, Seoul National University, Seoul, Korea
[2]Dental Research Institute, Seoul National University, Seoul, Korea
[3]Department of Oral Medicine and Diagnosis, Oral Science Institute, College of Dentistry, Gangneung-Wonju National University, Gangneung, Korea

**Received** May 28, 2014
**Revised** June 19, 2014
**Accepted** June 30, 2014

**Correspondence to:**
Moon-Soo Park
Department of Oral Medicine and Diagnosis, Oral Science Institute, College of Dentistry, Gangneung-Wonju National University, 7, Jukheon-gil, Gangneung 210-702, Korea
Tel: +82-33-640-2466
Fax: +82-33-640-3113
E-mail: mpark@gwnu.ac.kr

This work was supported by the National Research Foundation of Korea Grant through the Oromaxillofacial Dysfunction Research Center for the Elderly (No. 2013-070465) at Seoul National University in Korea.

**Purpose:** To investigate viscosity and wettability of hyaluronic acid (HA) solutions according to supplementation of lysozyme and/or peroxidase, and different ionic strength and pH conditions.

**Methods:** Solutions containing HA were prepared using distilled deionized water (DDW) and simulated salivary buffer (SSB) in different conditions. Different concentrations of hen egg-white lysozyme and bovine lactoperoxidase was added into HA solutions. HA solutions with antimicrobials in different ionic strength and pH conditions were prepared. Viscosity was measured using cone-and-plate digital viscometer at six different shear rates and wettability on acrylic resin and Co-Cr alloy was determined by contact angle.

**Results:** The viscosity values of HA dissolved in DDW were decreased in order of HA, HA containing lysozyme, HA containing peroxidase, and HA containing lysozyme and peroxidase. The viscosity values for HA in DDW were decreased as the concentration of lysozyme and/or peroxidase increased. However, the viscosity values for HA in SSB showed no significant changes according to the concentration of lysozyme and/or peroxidase. The viscosity values of HA solutions were inversely proportional to ionic strength and pH. The contact angle of HA solutions showed no significant differences according to tested surface materials, addition of lysozyme and/or peroxidase, and different ionic strength and pH conditions. Contact angles on acrylic resin by HA solutions in all tested conditions were much higher than those by human saliva.

**Conclusions:** The rheological properties of HA supplemented with lysozyme and/or peroxidase in different ionic strength and pH conditions were objectively confirmed, indicating the possibility of HA with lysozyme and/or peroxidase as main components in the development of effective saliva substitutes.

**Key Words:** Hyaluronic acid; Lysozyme; Peroxidase; Saliva substitute; Viscosity; Wettability

## INTRODUCTION

An understanding of both the rheological and biological properties of human saliva is required for the development of effective saliva substitutes, and an ideal saliva substitute should imitate the rheological and biochemical properties of human saliva.[1,2] However, few objective data of saliva substitutes exist regarding viscosity and film-forming properties essential to the proper function. It has been reported that hyaluronic acid (HA) in saliva may contribute to the lubricating and healing properties of saliva, and assisting in protecting the oral mucosa.[3,4] The previous study provides an objective observation on the properties of HA solutions as a candidate molecule for saliva substitutes in terms of their rheological and biological aspects.[5]

The oral cavity offers an environment in which ingredients in saliva substitutes and various molecules in saliva exist at the same time. Therefore, HA molecules in saliva

substitutes may also interact with antimicrobial molecules in human saliva. The previous reports has already suggested the formation of complex molecules between HA and lysozyme[6-8] and between HA and peroxidase.[9] However, there is no information as to the rheological change of complex molecules between HA and lysozyme and between HA and peroxidase.

In the present study, we have investigated physical properties of complex molecules between HA and lysozyme and between HA and peroxidase in different concentrations of antimicrobial supplements and different ionic strength and pH conditions. For physical properties the viscosity and film-forming property of HA solutions were examined.

## MATERIALS AND METHODS

### 1. Hyaluronic Acid Solution

HA (1,630 kDa; Sigma-Aldrich, St. Louis, MO, USA) was solubilized with distilled deionized water (DDW) or simulated salivary buffer (SSB, 0.021 M $Na_2HPO_4/NaH_2PO_4$, containing 36 mM NaCl and 0.96 mM $CaCl_2$)[10] at 0.05 mg/mL in DDW and 0.5 mg/mL in SSB, to verify the influence of ionic strength on viscosity and wettability.

### 2. Lysozyme and Peroxidase

Hen egg-white lysozyme (HEWL) and bovine lactoperoxidase (bLPO) (Sigma-Aldrich) dissolved in SSB with phenylmethylsulphonylfluoride (a final concentration of 1.0 mM) served as lysozyme or peroxidase sources, respectively. The final concentrations of 10 μg/mL, 20 μg/mL, and 40 μg/mL HEWL or 12.5 μg/mL, 25 μg/mL, and 50 μg/mL bLPO were used for the assay. And 20 μg/mL-HEWL and 25 μg/mL-bLPO were used for the assay for influence on physical properties according to ionic strength and pH.

### 3. Measurement of Viscosity

Viscosity measurements were performed with a model LVT Wells-Brookfield cone-and-plate digital viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA). Shear rates were varied incrementally from 11.3 to 450.0 $s^{-1}$ at six different speeds. All measurements were carried out at 37°C, and 0.5 mL volume of fluid was used in each test. The experiment was duplicated and performed 6 times.

### 4. Measurement of Contact Angle

Heat-cured acrylic resin, Paladent 20 (Herareus Kulzer, Wehrheim, Germany), and cobalt-chromium alloy, Biosil F (DeguDent, Hanau, Germany) were used as surface phases. Ten specimens of each material (30×30×1.5 mm) were prepared to have highly flat surfaces.

Measurement of contact angle and surface tension was performed with a Phoenix 300 (Surface Electro Optics Co., Ansan, Korea). Contact angles were measured on the photographs as follows: 10 μL droplets of each liquid were positioned on the test specimens by means of a 1 mL syringe with a blunt point. After 30 seconds, a tangent to the droplet was drawn from the point of air-fluid-solid phase intersection. Contact angles between this tangent line and the dental material surface were calculated from enlarged photonegatives of the droplets. Measurements of contact angle also was duplicated and performed 6 times.

### 5. Ionic Strength and pH

HA solution with 20 μg/mL-HEWL and 25 μg/mL-bLPO was used for the assay for influence on physical properties according to ionic strength and pH. Measurement of viscosity and contact angle were performed at different levels of ionic strength (0 mM, 50 mM, and 100 mM) and pH (pH 5, pH 7, and pH 9).

### 6. Statistics

The Mann-Whitney U and Kruskal-Wallis tests were used to compare the mean values of viscosity and contact angle. The Wilcoxon signed rank test was used to analyze the effects of variables, as compared with their controls. p-values less than 0.05 were considered statistically significant.

## RESULTS

### 1. Physical Properties of HA with Lysozyme and/or Peroxidase

The viscosity values for HA with lysozyme and/or peroxidase dissolved in DDW followed a pattern typical of a non-Newtonian fluid, whereas the viscosity values for HA with lysozyme and/or peroxidase dissolved in SSB at low concentrations displayed decreased viscoelastic properties at low shear rates.

The viscosity values for HA with lysozyme and/or peroxidase

dissolved in DDW followed a pattern typical of a non-Newtonian fluid, whereas the viscosity values for HA with lysozyme and/or peroxidase dissolved in SSB at low concentrations of supplemented antimicrobials displayed decreased viscoelastic properties at low shear rates (Figs. 1, 2).

The viscosity of HA solutions dissolved in DDW was decreased in order of HA, HA containing lysozyme, HA containing peroxidase, and HA containing lysozyme and peroxidase (Fig. 1). Whereas the viscosity of HA solutions dissolved in SSB did not show a constant pattern (Fig. 2). The viscosity values for HA in DDW were decreased significantly ($p<0.01$) as the concentration of lysozyme and/or peroxidase increased, but the viscosity values for HA in SSB did not show any significant changes as the concentration of lysozyme and/or peroxidase increased (Fig. 3).

The contact angle of HA solutions showed no significant differences according to tested materials and used solvents (DDW and SSB). Addition of lysozyme and/or peroxidase with various concentrations did not influence on contact angles. Contact angles on acrylic resin by HA solutions in different experimental conditions were much higher than those by human saliva ($p<0.01$) (The contact angles of

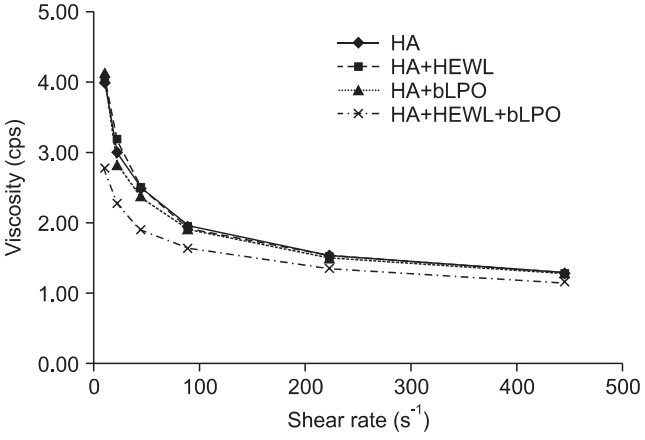


**Fig. 1.** Viscosity values of HA with lysozyme and/or peroxidase in distilled deionized water (20 μg/mL-HEWL and 25 μg/mL-bLPO). HA, hyaluronic acid; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase.

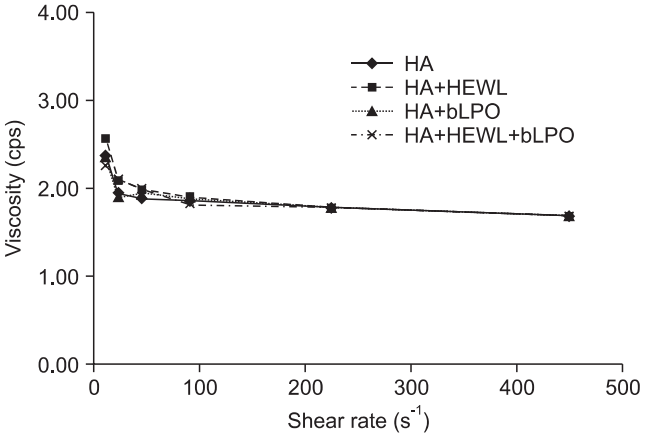


**Fig. 2.** Viscosity values of HA with lysozyme and/or peroxidase in simulated salivary buffer (20 μg/mL-HEWL and 25 μg/mL-bLPO). HA, hyaluronic acid; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase.

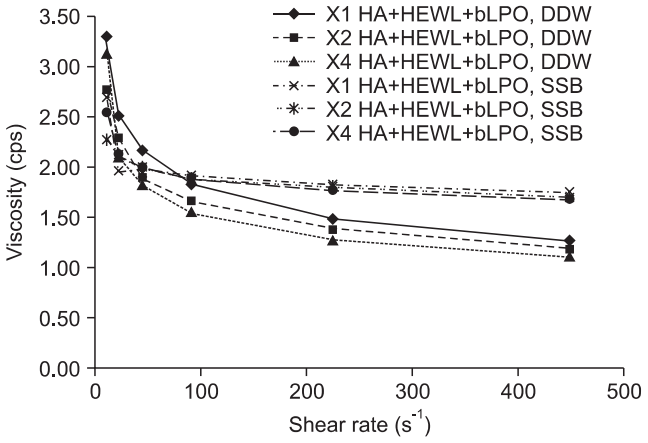


**Fig. 3.** Viscosity values of HA solutions at various concentrations of antimicrobials in DDW and SSB. X1, 10 μg/mL-HEWL and 12.5 μg/mL-bLPO in HA solution; X2, 20 μg/mL-HEWL and 25 μg/mL-bLPO in HA solution; X4, 40 μg/mL-HEWL and 50 μg/mL-bLPO in HA solution. HA, hyaluronic acid; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase; DDW, distilled deionized water; SSB, simulated salivary buffer.

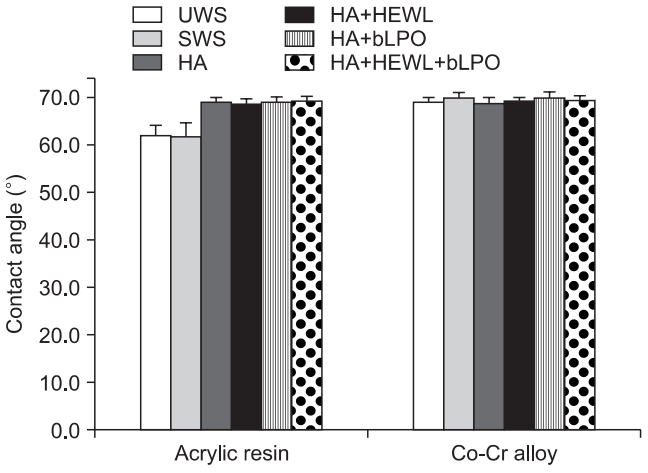


**Fig. 4.** Contact angle of HA with lysozyme and/or peroxidase in distilled deionized water. UWS, unstimulated whole saliva; SWS, stimulated whole saliva; HA, hyaluronic acid; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase. Data from the article of Park, et al. (Oral Dis 2007;13:181-186).[11]

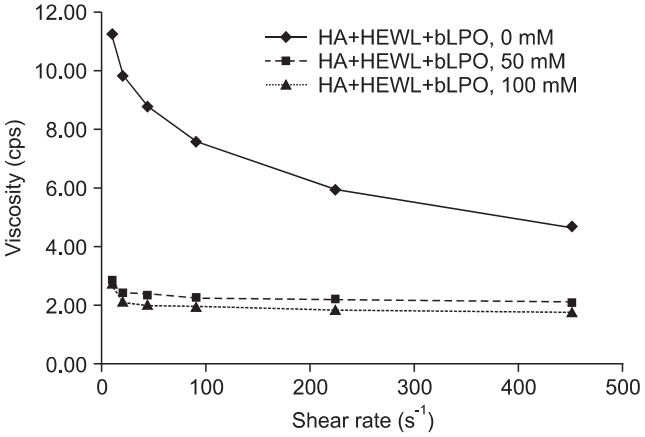


**Fig. 5.** Viscosity values of HA solutions at 0 mM, 50 mM, and 100 mM. Tested HA solutions include 20 μg/mL-HEWL and 25 μg/mL-bLPO. HA, hyaluronic acid; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase.

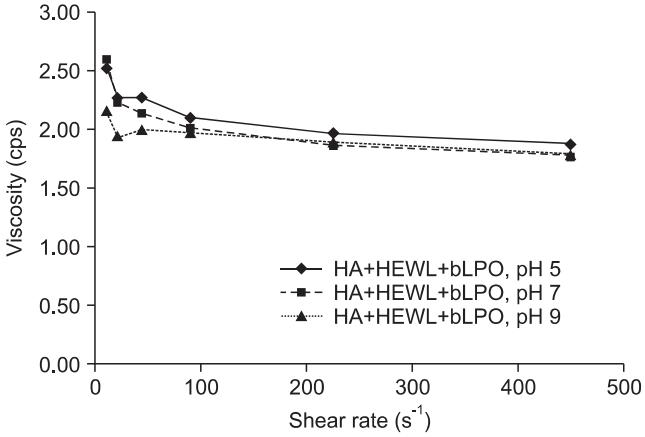


**Fig. 6.** Viscosity values of HA solutions at pH 5, pH 7, and pH 9. Tested HA solutions include 20 μg/mL-HEWL and 25 μg/mL-bLPO. HA, hyaluronic acid; HEWL, hen egg-white lysozyme; bLPO, bovine lactoperoxidase.

human saliva were adopted from Park et al.[11]; Fig. 4).

### 2. The Changes of Physical Properties in HA Solutions according to Ionic Strength and pH

Reductions in viscosity values were found with increasing ionic strength in all experimental conditions (Fig. 5), and HA solutions at pH 5 displayed the highest viscosity value in all tested conditions (Fig. 6).

The contact angles of HA solutions showed no significant differences according to either ionic strength or pH.

## DISCUSSION

To accomplish a viscoelastic pattern similar to that of human whole saliva is the practical goal of developing effective salivary substitutes for xerostomic patients. According to our previous study, HA displayed the viscoelastic properties, which is characteristic of macromolecular solutions, therefore HA was verified as a strong candidate molecule for saliva substitutes in physical and biological properties.[5]

Effective saliva substitutes are also required to be qualified for antimicrobial activities besides viscoelastic properties, so innate antimicrobial components in human saliva such as lysozyme and peroxidase can be considered as candidate antimicrobial components for saliva substitutes.

HA including other antimicrobial components may be different from HA alone in rheological and biological properties due to interaction between HA and supplemented antimicrobial components. Some reports have suggested an ionic interaction between HA and lysozyme or peroxidase.[6-9] It has been previously reported that HA inhibited lysozyme activity of leucocytic lysosome by 30%.[12] However, the enzymatic activity of HEWL and salivary lysozyme was not affected by HA in our previous study.[5] It has been also suggested that salt concentrations and pH had an effect on HA-lysozyme interaction. High binding affinity was previously confirmed at approximately 10-50 mM salt and at pH 7.5.[6-8] It has also been reported that the myeloperoxidase activity was not affected by the formation of HA-myeloperoxidase ionic complex,[9,12] which was in consistent with our pervious study using bLPO.[5]

Although the enzymatic activity of lysozyme or peroxidase was not affected by the presence of HA, the rheological properties of HA can be affected by complex formation of HA with antimicrobials in human saliva or saliva substitutes. Therefore, the changes of rheological properties of HA-antimicrobial complexes compared with HA alone need to be explored.

It has been reported that depolymerization of HA decreases in HA viscosity following exposure to myeloperoxidase.[9,13,14] It has also been proposed that a specific interaction of lysozyme with HA generates disaggregation of proteoglycans.[15]

In the present study, the viscosity values of HA dissolved in DDW were decreased in order of HA, HA containing lysozyme, HA containing peroxidase, and HA containing

lysozyme and peroxidase. These were expected findings according to the aforesaid previous studies, and it was thought that bLPO is stronger than HEWL in disaggregation of HA in the tested conditions.

Although HA with lysozyme and/or peroxidase can have an effect on wettability in extremely high concentrations, the contact angles of HA solutions showed no significant differences according to either the tested materials or addition of lysozyme and/or peroxidase in the present study. Contact angles on acrylic resin by HA solutions in all tested conditions were much higher than those by human saliva (The contact angles of human saliva were adopted from Park et al.[11]; Fig. 4).

According to our present study, reductions in viscosity values were confirmed with increasing ionic strength in all conditions. These findings have been previously reported by a study on the relationship between ionic strength and viscosity wherein decreases in intrinsic viscosity of bovine submaxillary mucin or canine tracheal mucin were verified upon increasing the ionic strength.[11,16] Further, it was reported elsewhere that an approximately 50% decrease in specific viscosity was observed upon increasing the ionic strength from 35 to 235 mM.[17] In another study, the pH-optimum was almost completely abolished by the increase of ionic strength up to 200 mM.[17] And we also confirmed the difference in viscosity between HA-DDW and HA-SSB in our previous study.[5]

The pH is another important intraoral environment that can effect on HA solution. Under lower ionic strength conditions, the maximum viscosity of mucin solutions was identified at pH 4,[17] and profound increase in viscosity and aggregation of pig gastric mucin at low pH was reported in another study.[18] Because these pH effects generally took some time, these may be attributed to conformational changes in the proteins.[19] In the present study, the highest viscosity was observed at pH 5. However, considering that very low pH might cause proteolysis, leading to structural collapse,[19] there is a further investigation to check viscosity values at extremely low pH.

Although extremely strong ionic strength and/or extremely low pH conditions can have an effect on wettability, the contact angles of HA solutions showed no significant differences in our study.

Collectively, the present study provides an objective observation on the rheological properties of HA supplemented with different concentrations of lysozyme and/or peroxidase in different ionic strength and pH conditions. These results could be utilized for the development of effective saliva substitutes in the aspect of rheological properties. Considering the complexity of rheological properties, additional studies using other rheological parameters will be needed for the development of effective saliva substitutes.

## CONFLICT OF INTEREST

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

## REFERENCES

1. Vissink A, Waterman HA, s-Gravenmade EJ, Panders AK, Vermey A. Rheological properties of saliva substitutes containing mucin, carboxymethylcellulose or polyethylenoxide. J Oral Pathol 1984;13:22-28.
2. Levine MJ. Development of artificial salivas. Crit Rev Oral Biol Med 1993;4:279-286.
3. Pogrel MA, Lowe MA, Stern R. Hyaluronan (hyaluronic acid) in human saliva. Arch Oral Biol 1996;41:667-671.
4. Pogrel MA, Low MA, Stern R. Hyaluronan (hyaluronic acid) and its regulation in human saliva by hyaluronidase and its inhibitors. J Oral Sci 2003;45:85-91.
5. 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.
6. Van Damme MP, Moss JM, Murphy WH, Preston BN. Binding of hyaluronan to lysozyme at various pHs and salt concentrations. Biochem Int 1991;24:605-613.
7. Van Damme MP, Moss JM, Murphy WH, Preston BN. Binding properties of glycosaminoglycans to lysozyme--effect of salt and molecular weight. Arch Biochem Biophys 1994;310:16-24.
8. Moss JM, Van Damme MP, Murphy WH, Preston BN. Dependence of salt concentration on glycosaminoglycan-lysozyme interactions in cartilage. Arch Biochem Biophys 1997;348:49-55.
9. Green SP, Baker MS, Lowther DA. Depolymerization of synovial fluid hyaluronic acid (HA) by the complete myeloperoxidase (MPO) system may involve the formation of a HA-MPO ionic complex. J Rheumatol 1990;17:1670-1675.
10. Bennick A, Cannon M. Quantitative study of the interaction of salivary acidic proline-rich proteins with hydroxyapatite. Caries Res 1978;12:159-169.
11. Park MS, Chung JW, Kim YK, Chung SC, Kho HS. Viscosity and wettability of animal mucin solutions and human saliva. Oral Dis 2007;13:181-186.

12. Avila JL, Convit J. Inhibition of leucocytic lysosomal enzymes by glycosaminoglycans in vitro. Biochem J 1975;152:57-64.
13. 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.
14. 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.
15. Blanco LN, Pita JC. Light-scattering study on the influence of link-glycoproteins and lysozyme on the hyaluronate molecular conformation in solution. Arch Biochem Biophys 1985;239:296-304.
16. Litt M, Khan MA, Shih CK, Wolf DP. The role of sialic acid in determining rheological and transport properties of mucus secretions. Biorheology 1977;14:127-132.
17. Veerman EC, Valentijn-Benz M, Nieuw Amerongen AV. Viscosity of human salivary mucins: effect of pH and ionic strength and role of sialic acid. J Biol Buccale 1989;17:297-306.
18. Bhaskar KR, Gong DH, Bansil R, et al. Profound increase in viscosity and aggregation of pig gastric mucin at low pH. Am J Physiol 1991;261:G827-G832.
19. Nordbö H, Darwish S, Bhatnagar RS. Salivary viscosity and lubrication: influence of pH and calcium. Scand J Dent Res 1984;92:306-314.