치의과학박사 학위논문

# Effects of Different Molecular Weights of Hyaluronic Acids on Viscosities and Lysozyme- and Peroxidase-related Enzymatic Activities

Hyaluronic acid의 분자량에 따른 차이가 점도 및
Lysozyme과 Peroxidase 관련 효소 활성에
미치는 영향

2017년 8월

서울대학교 대학원
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이 논문을 치의과학 박사학위논문으로 제출함

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## ABSTRACT

# Effects of Different Molecular Weights of Hyaluronic Acids on Viscosities and Lysozyme- and Peroxidase-related Enzymatic Activities

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**Objectives:** Hyaluronic acid has been considered as a candidate molecule for saliva substitutes. To investigate influences of molecular-weight of hyaluronic acid on its rheological and biological properties, we examined viscosities of hyaluronic acids with different molecular-weights and their effects on lysozyme- and peroxidase-related enzymatic activities both in solution and on hydroxyapatite surface.

**Methods:** Four different-sized hyaluronic acids (10 kDa, 100 kDa, 1 MDa, and 2 MDa), hen egg-white lysozyme (HEWL, 30 μg/mL), bovine lactoperoxidase (bLPO, 25 μg/mL), glucose oxidase-mediated peroxidase (GO-PO), and human whole saliva were used. Viscosity values for hyaluronic acids were measured by a cone-and-plate digital viscometer at six different concentrations (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL) and six different shear rates (11.3, 22.5, 45.0, 90.0, 225, and 450 $s^{-1}$). Enzymatic activities of lysozyme, peroxidase, and GO-PO were examined

by hydrolysis of fluorescein-labelled *Micrococcus lysodeikticus*, oxidation of fluorogenic 2',7'-dichlorofluorescein (LDCF) to fluorescing 2',7'-dichlorofluorescein (DCF), and production of oxidized o-dianisidine, respectively. Lysozyme and peroxidase activity were measured both in solution and on hydroxyapatite surface, and GO-PO activity was examined only in solution. For enzymatic activities, hyaluronic acids at 0.5 mg/mL were used.

**Results:** The 100 kDa-hyaluronic acid at 5 mg/mL, 1 MDa at 0.5 mg/mL, and 2 MDa at 0.2 mg/mL showed similar viscosity values to human whole saliva at the shear rates from 60 to 160 $s^{-1}$. In solution assays, only 2 MDa-hyaluronic acid inhibited lysozyme activities in saliva significantly. In surface assays, high-molecular-weight hyaluronic acids inhibited lysozyme and peroxidase activities and the inhibitory activities were more apparent on saliva than purified enzymes. GO-PO activities were not significantly affected by all the hyaluronic acids experimented.

**Conclusions:** Hyaluronic acids of low-molecular-weight at high concentrations and high-molecular-weight at low concentrations showed similar viscosity values to human whole saliva, and inhibitory effects of hyaluronic acids on lysozyme and peroxidase activities were more significant in high-molecular-weight ones, on-surface than in-solution, and on saliva than purified enzymes.

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**Keywords:** Hyaluronic acid, Molecular weight, Viscosity, Lysozyme, Peroxidase

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## Hyaluronic acid의 분자량에 따른 차이가 점도 및 Lysozyme과 Peroxidase 관련 효소 활성에 미치는 영향

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# I. INTRODUCTION

Hyaluronic acid is one of the most common glycosaminoglycans in human body consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units, which mostly exists in the vitreous humor of the eye, in the synovial fluid of articular joints, and in the extracellular matrix (Almond, 2007). The intrinsic biocompatibility of hyaluronic acid and its unique physical properties make it important for medical fields such as drug delivery (Luo et al., 2000), production of biomaterials (Collier et al., 2000), artificial tears (Doughty and Glavin, 2009), and substances for the symptomatic relief of osteoarthritis (Balazs, 1985; Moreland, 2002). Hyaluronic acids exist in biological tissues and fluids as different molecular sizes and weights, and hyaluronic acids with different molecular weights are commercially available. Hyaluronic acid molecules may have different rheological and biological properties according to their molecular weights, which information is essential for the proper usage of hyaluronic acid in the development of biomaterials and medications.

The presence of hyaluronic acid in human saliva has been reported, and salivary hyaluronic acid may contribute to the lubricating and healing properties of saliva, thereby assisting in protection of the oral mucosa (Pogrel et al., 1996, 2003). The anti-*Candida* activity of hyaluronic acid has also been reported (Kang et al., 2011; Sakai et al., 2007). Because of its viscoelastic properties, biocompatibility, and fungistatic activity, hyaluronic acid has been considered as a candidate molecule for saliva substitutes for patients with dry mouth whose susceptibility to candidiasis is increased. Interestingly, the relationship between the level of hyaluronic acid in saliva and the occurrence of dry mouth symptoms has been reported (Higuchi et al., 2009).

The development of effective saliva substitutes is the mimicry process of human saliva in the aspects of rheological and biological properties, which could be

influenced by molecular interactions. It has been reported that hyaluronic acid of a specific molecular weight showed similar viscosity values to human whole saliva at specific concentration ranges (Park et al., 2010). The influences of hyaluronic acid with a specific molecular weight on the enzymatic and candidacidal activities of lysozyme and peroxidase have also been demonstrated (Kang et al., 2011). Therefore, when hyaluronic acids of different molecular weights are involved, rheological and biological properties could be affected by their molecular weights.

The formation of complex molecules between hyaluronic acid and salivary antimicrobial enzymes such as lysozyme and peroxidase have already been reported and ionic interaction has been suggested between hyaluronic acid and lysozyme or peroxidase (Green et al., 1990; Kang et al., 2011; Moss et al., 1997; Park et al., 2010; Van Damme et al., 1991, 1994). Therefore, the amount of anionic charge that is directly related to the molecular weight of hyaluronic acid could affect the strength of ionic interaction and resulting enzymatic activities. Additionally, considering the possibility of molecular conformational changes on solid surfaces compared with in solution, the results of these interactions could be different on the tooth surface compared to in salivary fluid. Therefore, the purpose of this study was to investigate the influence of molecular weight of hyaluronic acid on its rheological and biological properties important for the development of oral health care products. Viscosity values of hyaluronic acids of four different molecular weights and their effects on lysozyme and peroxidase-related enzymatic activities both in solution and on hydroxyapatite surface were examined.

# II. REVIEW OF LITERATURE

## 1. Saliva for oral health

(1) Function of saliva

Saliva is an essential oral fluid, which is a clear and slightly acidic mucoserous exocrine secretion from major and minor salivary glands (Humphrey and Williamson, 2001).Whole saliva contains glandular saliva, gingival crevicular fluid, desquamated oral epithelial cells, and microorganisms. Saliva has an important role in protecting, lubrication, buffering action, remineralization of teeth, antimicrobial activity, taste, and digestion (Mandel, 1987). By coating the oral mucosa with seromucous components, saliva protects and lubricates oral mucosal tissues against many irritants including proteolytic and hydrolytic enzymes from plaque and neutrophils, potential carcinogens from smoking, and desiccation from mouth breathing (de Almeida et al., 2008). The major components for lubricating effects are mucins which are secreted from mainly submandibular and sublingual salivary glands. Mucins are complex glycoprotein molecules that have the properties of low solubility, high viscosity, high elasticity, and strong adhesiveness (Humphrey and Williamson, 2001). Most of oral functions such as mastication, speech, and swallowing are helped by lubrication of mucins (Tabak, 1990). Buffering action of saliva is performed by bicarbonate, phosphate, urea, and amphoteric proteins and enzymes. Among them, bicarbonate is the most important buffering system, which diffuses into plaque and neutralizes acidic conditions. Remineralization of teeth could be done by salivary calcium and phosphate, which are maintained as a supersaturation state by salivary proteins. Salivary proteins, such as proline-rich proteins and statherins, have important roles to remineralize the enamel by stabilizing salivary calcium and phosphate, protecting the teeth from wear and mineral egress, and allowing the penetration of minerals for remineralization into the enamel. Saliva also has antimicrobial functions, which are performed by both immunologic components, such as secretory IgA, IgG, and IgM, and nonimmunologic components including mucins, peptides, and enzymes. The hypotonicity of saliva contributes to taste, which is also affected by salivary

proteins and gustin. Salivary amylases help digestion in early stage by breaking down starch, and salivary lubrication aid swallowing the food bolus.

(2) Rheological properties of saliva

Saliva is a non-Newtonian fluid, which has viscoelastic properties (Kho, 2014). Salivary glycoproteins, such as mucins, have a major role for viscoelasticity, mainly contributing to lubrication (Johansson et al., 1994). The viscosity of saliva may determine whether saliva could adhere to the oral mucosal surfaces in order to protect and lubricate the mucosal surfaces. Because the surfaces of oral cavity are in moving contact continuously, lubricating property of saliva is essential to protect the oral mucosa from irritation and damage (Schwarz, 1987). Salivary macromolecules contribute to forming a lubricant film on the oral tissues, which smoothens the oral surfaces, reduces the friction between food and mucosa, and makes the food bolus swallowed easily (Mandel, 1989). This film-coating property is mainly attributed to salivary mucins (Tabak et al., 1982). The wettability of saliva is also important property for retention and lubrication of oral removable appliances especially in xerostomic patients (Monsenego et al., 1989).

(3) Biological properties of saliva

Antimicrobial activities of saliva are performed by various components, such as lysozyme, peroxidase, mucins, lactoferrin, and immunoglobulins (Humphrey and Williamson, 2001). Among them, lysozyme and peroxidase are the most commercially used components for antibacterial or antifungal activities (Kho, 2014). Lysozyme, which is derived from major and minor salivary glands, phagocytic cells, and gingival crevicular fluid, performs antimicrobial activities mainly by a muramidase activity and a cationic property (Laible and Germaine, 1985). Furthermore, it has been reported that lysozyme might have antiviral properties, contributing to lysis of tumor cells (Lee-Huang et al., 1999, Sava et al.,

1989). While most peroxidase activity in whole saliva is originated from salivary peroxidase secreted from major and minor salivary glands, some peroxidase activity is from myeloperoxidase of white blood cells exudated from gingival crevice. Peroxidase molecules perform antimicrobial activities by producing hypothiocyanite ($OSCN^-$), resulting from oxidation of thiocyanate ($SCN^-$) by hydrogen peroxide ($H_2O_2$) (Ashby, 2008). Glucose oxidase-mediated peroxidase (GO-PO) system, which does not exist in human saliva, is another peroxidase system, usually included in health care products to exert antimicrobial activities. For example, one GO-PO system which consists of bovine lactoperoxidase (bLPO), glucose oxidase, and $SCN^-$ exhibits candidacidal activities by utilizing glucose at a physiological glucose concentration in human saliva (Kho et al., 2012). Salivary mucins perform antimicrobial functions by controlling bacterial and fungal colonization via modulating adhesion of microbes (Humphrey and Williamson, 2001). Lactoferrin, produced by intercalated ductal cells, adheres to salivary ferric irons, resulting in starvation of microorganisms which need ferric irons to survive (Mandel, 1976). Secretory IgA, which is the largest component among various immunologic contents in saliva, is produced by plasma cells in connective tissues, acting as an antibody to microbial antigens, aggregating bacteria to inhibit host tissue attachment, and neutralizing viruses (Dowd, 1999; McNabb, 1981).

## 2. Dry mouth

(1) Etiology, signs, and symptoms

Xerostomia, usually defined as subjective discomfort of patients with dry mouth, has a variety of etiologies, including medications, Sjögren syndrome, radiotherapy in the head and neck region, and other systemic conditions (Guggenheimer and Moore, 2003). The most common etiological factor of dry mouth is taking various medications, and it has been reported that hundreds of medications contribute to

oral dryness of the patients (Scully, 2003). Sjögren syndrome is an autoimmune disease that characterized by lymphocytic infiltration of exocrine glands and epithelia in multiple sites, especially lacrimal and salivary glands, resulting in dryness of eye and oral cavity (Vitali et al., 2002). The radiation therapy of the head and neck cancers could injure major and minor salivary glands, resulting in decrease of salivary flow rate and changes in salivary composition (Shiboski et al., 2007)

Patients with dry mouth often complain of difficulties in oral functions such as eating, swallowing, or speaking, as well as dry sensation of oral cavity, resulting in degradation of quality of life (Kho, 2014). Some patients with xerostomia might complain of burning sensation of tongue and oral mucosa, dysgeusia, or halitosis. Moreover, oral dryness could cause oral candidiasis or other infectious diseases and tend to increase the risk of dental caries or periodontitis.

Typically, diagnostic evaluation of patients with dry mouth is performed by measuring salivary flow rates of whole saliva. The most common methods for measuring salivary flow rates are spitting or draining whole saliva into a test tube, and these methods have been identified to be reproducible and reliable (Navazesh, 1993). Salivary flow rate could be measured at rest (unstimulated) or during chewing gum or wax (stimulated). Diagnostic criteria for hyposalivation is usually defined by a flow rate of unstimulated whole saliva (UWS) less than 0.1 mL/min, or that of stimulated whole saliva (SWS) less than 0.7 mL/min (von Bültzingslöwen et al., 2007). The patients with decreased flow rate of both UWS and SWS are considered as non-responders, while the patients with decrease flow rate of UWS only are regarded as responders (Sreebny and Broich, 1987).

(2) Treatments

1) Intrinsic approach

The intrinsic approach for the management of dry mouth patients is the prescription of parasympathomimetic sialogogue medications such as pilocarpine

and cevimeline, stimulating the residual capacity of hypofunctional salivary glands (Porter et al., 2004). It has been reported that these medications could help the patients with Sjögren syndrome (Al-Hashimi, 2005) and with a history of head and neck radiotherapy (Shiboski et al., 2007). However, sialogogues are not effective for all the patients with xerostomia, especially for non-responders, and have some degrees of side effects such as hypersalivation of other glands such as sweat glands and exacerbation of asthma, bronchitis, and cardiac symptoms.

2) Extrinsic approach

For the patients who are not responsive to saliva stimulants or show side effects, extrinsic approach could be a help, which use artificial salivas or saliva substitutes. Most widely used saliva substitutes are sodium carboxymethylcellulose(CMC)- or animal mucin-based solutions, and these substitutes have been evaluated in terms of subjective improvements (Duxbury et al., 1989; Momm et al., 2005; Visch et al., 1986; Vissink et al., 1983, 1987). Other saliva substitutes using other polymers or natural products have been also reported (Epstein et al., 2017; Morales-Bozo et al., 2017; Ship et al., 2007). While these commercially used saliva substitutes may alleviate dry symptoms in some degrees, the effects are usually short-lasting and limited (Oh et al., 2008). In addition, electrostimulation has been suggested to be effective for xerostomic patients (Alajbeg et al., 2012; Strietzel et al., 2007). However, these stimulation therapies need more studies with longer-term and larger-size for clinical application.

## 3. Hyaluronic acid

(1) Properties of hyaluronic acid

Hyaluronic acid is a linear polymer consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units, which belongs to the glycosaminoglycans

(Pogrel et al., 1996). Hyaluronic acid is abundant in the eye vitreous humor and in soft connective tissues (Almond, 2007), having important roles such as lubrication of joints and tissues (Arrich et al., 2005; Forsey et al., 2006; Moreland, 2003; Swann et al., 1974) or organizing framework of cartilage (Heinegård and Oldberg, 1989). The existence of hyaluronic acid in human whole saliva has been found, and salivary hyaluronic acid has been considered to contribute to wound healing and lubricating properties of saliva (Pogrel et al., 1996, 2003). Additionally, it has been suggested that hyaluronic acid also contributes to anti-*Candida* activity (Sakai et al., 2007)

(2) Biomedical use of hyaluronic acid

Due to intrinsic biocompatibility of hyaluronic acid, it has been used in a variety of biomedical fields. Since it was reported that hyaluronic acid injection into arthritic joints of horses reduced clinical symptoms effectively (Balazs and Denlinger, 1985), sodium hyaluronate preparations have been used to relieve the symptoms from osteoarthritis in medical and dental fields. Hyaluronic acid is also used during cataract surgery to facilitate surgical manipulation of ocular tissues and protect eye cells from damage (Almond, 2007). For this reason, it has been used in various medical surgeries for lubrication or hydration. The wound healing property of hyaluronic acid makes it useful in tissue engineering with ability to enhance keratinocyte proliferation and migration, as well as the angiogenesis in wound sites (Price et al., 2005). Also, hyaluronic acid derivatives have been used as diagnostic markers for intraperitoneal inflammation (Edelstam et al., 1994).

(3) Hyaluronic acid as a base molecule as artificial saliva

The viscoelastic properties and non-immunogeneity of hyaluronic acid make it possible to be recommended as a base molecule for saliva substitutes. To alleviate discomfort of the patients with xerostomia, most commonly used saliva substitutes

are CMC- or mucin-based mouth rinse solutions, and the efficacy of these solutions reducing subjective symptoms has been reported (Levine, 1993; Oh et al., 2008; Olsson and Axéll, 1991). However, most of previous studies have focused on the relief of subjective symptoms without evaluation of objective rheological or biological properties. In fact, rheological and biological properties of traditional saliva substitutes, such as mucin-based or CMC-based ones, are not so similar to those of human whole saliva. On the other hand, hyaluronic acid has been reported to have similar rheological properties to those of human whole saliva at a certain range of concentration (Park et al., 2010). The correlation of oral dryness symptom with decreased salivary levels of hyaluronic acid has also been reported (Higuchi et al., 2009), which imply that hyaluronic acid has an important role in lubrication and protection of oral mucosa and could be used as an effective saliva substitute. In addition, fungistatic activity of hyaluronic acid could be another advantage as a substance for oral health care products for patients with dry mouth, suggesting playing a role as an antimicrobial agent as well as a base molecule with viscoelastic property (Kang et al., 2011).

## 4. Perspective in the development of artificial saliva

To manage the patients with dry mouth, both intrinsic and extrinsic approaches can be used. However, the intrinsic method including sialogogue, such as pilocarpine and cevimeline, has several side effects and limited effectiveness due to short duration of efficacy and less satisfaction especially for the patients who are non-responders (Fox, 2004; Wiseman and Faulds, 1995). Therefore, development of new drugs with prolonged activity and less side effects is necessary.

Development of effective saliva substitutes as an extrinsic approach is also important. It has been recommended that the addition of antimicrobial substances to base solutions rheologically similar to saliva might be more practical way to

develop artificial saliva (Kho, 2014). To achieve this goal, a thorough understanding of rheological properties of saliva and development of biologically beneficial antimicrobial substances insufficient in patients with dry mouth should be preceded. Molecular interactions of these substances with human saliva or saliva substitutes should also be evaluated. Based on the results of these objective researches, more effective saliva substitutes could be developed to reduce the discomfort of xerostomic patients.

Another thing to be considered is how to provide artificial saliva continuously to the patients. A novel technique that could make artificial saliva remain for a long time in the oral cavity after single use is needed. Moreover, further studies for developing customized saliva substitutes that could satisfy the needs for individual patients or artificial saliva that could enhance salivary function even in patients without dry mouth might provide new directions for development of salivary researches.

Although various polymers have been evaluated in terms of rheological and biological properties and molecular interactions, most studies have been limited to specific molecular weight at a certain range of concentrations (Cho et al., 2013; Kang et al., 2011; Kim et al., 2014; Park et al., 2007, 2010). Further studies on polymers with various molecular weights and a wide range of concentration are needed to determine better conditions. Therefore, to develop more effective saliva substitutes, additional studies on various candidate molecules for saliva substitutes are needed to find optimal conditions of both rheological and biological properties similar to human saliva without negative interactions, to alleviate subjective symptoms of patients with xerostomia.

# III. MATERIALS AND METHODS

## 1. Participants and collection of saliva

Saliva samples were collected from 4 healthy adults (2 males and 2 females, 28.5 ± 2.7 years) between 8 a.m. and 11 a.m. to minimize variability in salivary composition. All the participants had no medical histories for serious illnesses and medications affecting salivation at least for the recent three months. The oral hygiene and periodontal status of all participants were good. At the day of salivary collection, all participants refrained from eating, drinking, and tooth brushing for at least 1 h before the collection. UWS was collected by the spitting method. The collected saliva was placed in a chilled centrifuge tube in which phenylmethylsulfonyl fluoride (PMSF) was added immediately to a final concentration of 1.0 mM. The saliva sample was centrifuged at 3,500 x g for 15 min at 4℃, and the clarified supernatant fluid was used immediately for assays. The research protocol was approved by the Institutional Review Board (IRB) of Seoul National University Dental Hospital (#CRI16010) and informed consent was obtained from all participants.

## 2. Hyaluronic acid solution, lysozyme, peroxidase, and GO-PO

Hyaluronic acids of four different molecular weights (10 kDa, 100 kDa, 1 MDa, and 2 MDa, Lifecore Biomedical, LLC, Chaska, MN, USA), which 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 $CaCl_2$) (Bennick and Cannon, 1978), were used. Hyaluronic acids at six different concentrations (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL) were used for the analysis of viscosity at each molecular weight. For the measurement of enzymatic activities, hyaluronic acids at the concentration of 0.5 mg/mL were used.

Hen egg-white lysozyme (HEWL, final concentration of 30 μg/mL, Sigma–

Aldrich Chemical Co., St. Louis, MO, USA) and bovine lactoperoxidase (bLPO, final concentration of 25 μg/mL, Sigma–Aldrich Chemical Co.) were used as sources of lysozyme and peroxidase, respectively. A glucose assay kit (Sigma-Aldrich Chemical Co.) which included GO-PO reagent and o-dianisidine was used to analyze the effects of hyaluronic acid on the enzymatic activity of glucose oxidase-mediated peroxidase.

**3. Measurement of viscosity**

Viscosity was measured by a model LVT Wells-Brookfield cone-and-plate digital viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA). Shear rates were varied from 11.3 to 450 $s^{-1}$ at six different speeds (11.3, 22.5, 45.0, 90.0, 225, and 450 $s^{-1}$). All measurements were performed at 37℃, and 0.5 mL volume of fluid was used in each test. The viscosity of each sample was measured ten times. The results of viscosity values of hyaluronic acids were compared to those of human whole saliva obtained from the previous study (Park et al., 2010).

**4. Measurement of enzymatic activity of lysozyme**

Lysozyme activity was measured by hydrolysis of fluorescein-labelled *Micrococcus lysodeicticus* (EnzCheck Lysozyme assay kit; Molecular Probes, Eugene, OR, USA). The preparation of substrate solution and incubation procedures was performed according to the manufacturer's instructions. The enzymatic activity was measured by a fluorescence microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek instruments Inc., Winooski, VT, USA) at an excitation of $\lambda = 485$ nm, and an emission of $\lambda = 535$ nm.

(1) Influence of hyaluronic acid on lysozyme activity in solution phase

The effects of hyaluronic acids of four different molecular weights on HEWL and salivary lysozyme in solution were examined by incubating 500 μL of each hyaluronic acid with 500 μL of HEWL or clarified whole saliva for 30 min at room temperature (RT). The incubated mixture was placed in a suspension of fluorescein-labelled *M. lysodeikticus*, and incubated buffer with HEWL or clarified whole saliva served as a control. An incubated mixture of hyaluronic acid with the buffer or an incubated buffer alone was used as a blank.

(2) Influence of hyaluronic acid on lysozyme activity on surface phase

To examine the influence of hyaluronic acid on the enzymatic activity of lysozyme adsorbed to hydroxyapatite surface, experiments were performed in two different ways. First, the effects of the hyaluronic acid which was pre-adsorbed on hydroxyapatite beads were examined on subsequent adsorption of lysozyme (surface assay I). Second, the effects of the hydroxyapatite-adsorbed lysozyme were observed after pre-incubation of hyaluronic acid with lysozyme (surface assay II). Ceramic hydroxyapatite beads (Macro-prep, HA type I) were obtained from Bio-Rad (Hercules, CA, USA) and used as the surface assay. Ten milligrams of hydroxyapatite beads were used in each assay.

In surface assay I, hydroxyapatite beads were coated with 300 μL of hyaluronic acid for 30 min at RT, and the coated beads were washed 5 times with the buffer. The hyaluronic acid-coated beads were incubated with 300 μL of HEWL or clarified whole saliva for 30 min at RT. Unbound HEWL or salivary molecules were removed by 5 washes. The beads were incubated with a suspension of fluorescein-labelled *M. lysodeikticus*. Lysozyme activities of these samples were compared with those of the bare hydroxyapatite surface coated with HEWL or salivary lysozyme without hyaluronic acid coating.

In surface assay II, 300 μL of hyaluronic acid was pre-incubated with 300 μL of HEWL or clarified whole saliva for 30 min at RT. After that, hydroxyapatite beads

were incubated with 600 μL of the hyaluronic acid-lysozyme mixture for 30 min at RT, and then washed 5 times with buffer to remove unbound molecules. Lysozyme activities of these samples were compared with those of the hydroxyapatite samples coated with the pre-incubated mixture of HEWL or clarified whole saliva with buffer. Equal amounts of hydroxyapatite beads incubated with hyaluronic acid, or an incubated buffer alone, were used as blanks.

The experiments for measuring enzymatic activities of HEWL were performed eight times each in duplicate. The experiments for salivary lysozyme were also performed eight times in duplicate using pooled salivary samples from four participants.

**5. Measurement of enzymatic activity of peroxidase**

Peroxidase activity was determined by the method which was previously described by Proctor and Chan and Hannig et al. (Hannig et al., 2008, 2010a, 2010b; Proctor and Chan, 1994). In the presence of peroxidase and hydrogen peroxidase, fluorogenic 2',7'-dichlorofluorescein (LDCF) is oxidized to fluorescent dichlorofluorescein (DCF). Stock solutions of the stable reagent 2',7'-dichlorofluorescein diacetate (LDADCF, Molecular Probes) were stored at -80℃ ($5 \times 10^{-5}$ M in absolute ethanol). The fluorogenic substrate LDCF was prepared freshly every day from LDADCF. One part of LDADCF solution was admixed to 9 parts of 0.01 M sodium hydroxide, and incubated for 30 min. The reaction was stopped by addition of an equal amount of phosphate buffer (0.15 M, pH 6.0). The fluorescence of DCF was measured by a fluorescence microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek instruments Inc.) at an excitation of $\lambda = 488$ nm and an emission of $\lambda = 530$ nm. One unit of peroxidase activity was defined as 1 μmol DCF released per minute.

(1) Influence of hyaluronic acid on peroxidase activity in solution phase

The effects of hyaluronic acid on the enzymatic activity of bLPO or salivary peroxidase in solution were examined by incubating 500 μL of hyaluronic acid with 500 μL of bLPO or clarified whole saliva for 30 min at RT. Four μL of the incubated mixture was added to 200 μL phosphate buffer (0.15 M, 1 mM KSCN, pH 6.0) and incubated for 10 min at 37℃. In the following, 20 μL of 2.2 mM hydrogen peroxide solution and 20 μL of the LDCF reagent were added. Following incubation for 4 min, the reaction was stopped by adding 100 μl of 1 M sodium hydroxide. An incubated mixture of buffer with either bLPO or clarified whole saliva was used as a control, and an incubated mixture of hyaluronic acid with buffer or an incubated buffer alone was used as a blank.

(2) Influence of hyaluronic acid on peroxidase activity in surface phase

As the lysozyme assays, experiments on hydroxyapatite surfaces were performed in two different ways. In assay I, the effects of pre-adsorbed hyaluronic acid on subsequent absorption of peroxidase were examined. In assay II, hydroxyapatite-adsorbed peroxidase activity was examined after pre-incubation of hyaluronic acid with peroxidase. The experiments were also performed eight times each in duplicate for measuring enzymatic activities of bLPO and salivary peroxidase.

**6. Measurement of enzymatic activity of GO-PO**

The GO-PO activity was measured by the glucose assay kit measuring oxidized o-dianisidine production. Oxidized o-dianisidine production, measured by OD at 540 nm, reflected the enzymatic activity of GO-PO reagents. The GO-PO reagent in the glucose assay kit was divided into two parts, one dissolved in salivary simulated buffer and the other dissolved in the buffer containing hyaluronic acid, and pre-incubated for 30 min at RT. Enzymatic activity of the two different reagents

was measured using samples with known glucose concentrations (0.02, 0.04, and 0.06 mg/mL). Experiments for enzymatic activity of glucose oxidase-mediated peroxidase were performed six times in duplicate. The glucose oxidase-mediated peroxidase activity was measured only in solution phase.

### 7. Statistics

The Wilcoxon signed rank test was used to analyze statistical differences between the experimental groups with hyaluronic acid and the control groups without hyaluronic acid. *P*-values less than 0.05 were considered as statistically significant.

## IV. RESULTS

### 1. Viscosity of hyaluronic acid of different molecular weights

The viscosity values for hyaluronic acids followed a pattern of non-Newtonian fluid at all four different molecular weights. As expected, hyaluronic acids of larger molecular weights or those at higher concentrations displayed higher viscosity values. Within a range of the concentrations experimented (0.1 - 5.0 mg/mL), 10 kDa-hyaluronic acid showed the viscosity values from 0.73 to 0.88 cps (Fig. 1a), 100 kDa-hyaluronic acid from 0.74 to 2.38 cps (Fig. 1b), 1 MDa-hyaluronic acid from 0.97 to 28.1 cps (Fig. 1c), and 2 MDa-hyaluronic acid from 1.36 to 28.1 cps (Fig. 1d) at a shear rate of 90 $s^{-1}$. Hyaluronic acids of large molecular weights displayed increased viscoelastic properties, especially at a range of low shear rates.

The 10 kDa-hyaluronic acid showed lower viscosity values than human whole saliva at all six concentrations used in these experiments (Fig. 2a). The 100 kDa-hyaluronic acid at the concentration of 5 mg/mL displayed similar viscosity values

to those of SWS at lower shear rates (11.3, 22.5, and 45.0 $s^{-1}$), while that at the concentration of 2 mg/mL showed the viscosity values similar to SWS at 225 $s^{-1}$ and UWS at 450 $s^{-1}$ (Fig. 2b).

The 1 MDa-hyaluronic acid at the concentration from 0.2 mg/mL to 1.0 mg/mL showed viscosity values similar to human whole saliva. At the concentration of 1.0 mg/mL, 1 MDa-hyaluronic acid exhibited the similar viscosity values to SWS at the shear rates of 11.3 and 22.5 $s^{-1}$, at the concentration of 0.5 mg/mL similar to SWS at the shear rate of 225 $s^{-1}$ and similar to UWS at 450 $s^{-1}$, and at the concentration of 0.2 mg/mL similar to SWS at 450 $s^{-1}$ (Fig. 2c).

The 2 MDa-hyaluronic acid at the concentration of 0.5 mg/mL or higher, displayed the viscosity values higher than human whole saliva at all six shear rates experimented. The 2 MDa-hyaluronic acid at the concentration of 0.2 mg/mL displayed the similar viscosity value to those for SWS at 45.0 $s^{-1}$ and UWS at 225 $s^{-1}$, while that at the concentration of 0.1 mg/mL displayed the values similar to SWS at 225 $s^{-1}$ and 450 $s^{-1}$ (Fig. 2d).

**2. Effects of hyaluronic acid on enzymatic activity of lysozyme**

The effects of hyaluronic acid on the enzymatic activities of lysozyme were more significant on salivary lysozyme than HEWL, especially in high-molecular-weight ones, in both solution and surface assays.

All hyaluronic acids at 0.5 mg/mL did not affect the enzymatic activities of HEWL in solution, but 2 MDa-hyaluronic acid inhibited the enzymatic activity of salivary lysozyme in solution phase significantly ($P$ = 0.012). The 10 kDa-, 100 kDa-, and 1 MDa-hyaluronic acids did not inhibit the enzymatic activities of salivary lysozyme (Table 1).

While the enzymatic activities of both HEWL and salivary lysozyme on the hyaluronic acid-adsorbed hydroxyapatite surfaces (surface assay I) were not

affected at the molecular weights of 10 kDa, 100 kDa, and 1 MDa, the immobilized 2 MDa-hyaluronic acid inhibited the enzymatic activities of HEWL ($P$ = 0.017) and salivary lysozyme ($P$ = 0.012) significantly (Table 2).

When the pre-incubated mixture of hyaluronic acid and HEWL was adsorbed on the hydroxyapatite surfaces (surface assay II), the enzymatic activities of HEWL were not affected by 10 kDa- and 100 kDa-hyaluronic acids, but hyaluronic acids of 1 MDa ($P$ = 0.017) and 2 MDa ($P$ = 0.012) inhibited the enzymatic activities of HEWL significantly, especially in 2 MDa-hyaluronic acid decreasing more than 20%. In the case of salivary lysozyme, 10 kDa-hyaluronic acid did not affect the enzymatic activity of HEWL, but hyaluronic acids of 100 kDa ($P$ = 0.017), 1 MDa ($P$ = 0.012), and 2 MDa ($P$ = 0.012) inhibited the enzymatic activities of HEWL significantly.

## 3. Effects of hyaluronic acid on enzymatic activity of peroxidase

In the peroxidase assay, the results showed some differences from those of lysozyme assay. While it was similar to the lysozyme assay that the effects of hyaluronic acid on salivary peroxidase were more significant than those on the purified enzyme (bLPO), these effects were only observed on surface assay II. The hyaluronic acids of all molecular weights experimented did not affect the enzymatic activities of bLPO significantly in solution and on surface assay I, neither did the salivary peroxidase, even in large molecular weights such as 2 MDa (Table 3).

The pre-incubated mixture of bLPO and 2 MDa-hyaluronic acid inhibited the enzymatic activity of bLPO significantly ($P$ = 0.012) on hydroxyapatite surface (surface assay II), and pre-incubated salivary peroxidase with the hyaluronic acids of 1 MDa ($P$ = 0.012) or 2 MDa ($P$ = 0.017) decreased the salivary peroxidase activities significantly (Table 4).

### 4. Effects of hyaluronic acid on enzymatic activity of GO-PO

All the hyaluronic acids of four different molecular weights at 0.5 mg/mL did not affect the enzymatic activities of GO-PO significantly at three different glucose concentrations (0.02, 0.04, and 0.06 mg/mL) (Table 5).

## V. DISCUSSION

With regard to the development of effective saliva substitutes, most studies have focused on subjective satisfaction of patients with dry mouth or xerostomia (Duxbury et al., 1989; Levine, 1993; Momm et al., 2005; Oh et al., 2008; Olsson and Axéll, 1991; Visch et al., 1986; Vissink et al., 1983, 1987). However, there have been few objective studies that investigated the rheological and biological properties of candidate substances for saliva substitutes. While saliva substitutes using CMC are still the most popular products, rheological and biological properties of CMC are not much similar to those of human whole saliva (Vissink et al., 1984). Previous studies using mucins of animal origin, mucilages of plant origin, and hyaluronic acid have showed that these substances have viscoelastic properties similar to human saliva, and have reported the results of their interactions with potential antimicrobial supplements (Kang et al., 2011; Kho et al., 2014; Park et al., 2007, 2010; Vissink et al., 1984). Among these candidate substances, hyaluronic acid has an advantage as being one of the natural components present in human saliva. Hyaluronic acid of 1,630 kDa at the concentration of 0.5 mg/mL in SSB displayed similar viscosity values to those of SWS (Park et al., 2010). In the present study for further investigating the influences of molecular weight of hyaluronic acid, hyaluronic acids of four different molecular weights (10 kDa, 100 kDa, 1 MDa, and 2 MDa) at six different concentrations (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL) were

used to examine the viscosity values. The results showed that hyaluronic acids of low-molecular-weight except 10 kDa presented the viscosity values similar to human whole saliva at higher concentrations, while high-molecular-weight hyaluronic acids showed the similar viscosity values to those of human saliva at lower concentrations. The 100 kDa-hyaluronic acid at concentration of 5 mg/mL, 1 MDa-one at 0.5 mg/mL, and the 2 MDa-one at 0.2 mg/mL showed viscosity values similar to those of SWS at a range of shear rates (from 60 to 160 $s^{-1}$) which could reflect oral functions such as speaking or swallowing. These results supported the results of previous study that used 1,630 kDa-hyaluronic acid only (Park et al., 2010).

As salivary substitutes and human whole saliva might exist at the same time in the oral cavity, the interaction between molecules in human whole saliva and salivary substitutes should be considered to develop effective salivary substitutes. It has been reported that 1,630 kDa-hyaluronic acid did not affect the enzymatic activity of HEWL, salivary lysozyme, bLPO, and salivary peroxidase in solution phase (Park et al., 2010). In the present study using hyaluronic acids of different molecular weights at the concentration of 0.5 mg/mL, high-molecular-weight hyaluronic acids inhibited the lysozyme or peroxidase activities more than low-molecular ones. Large-sized hyaluronic acids could interfere with diffusion of enzymes, which provide less opportunities to exert enzymatic activities. It has been reported that 1,630 kDa-hyaluronic acid inhibited candidacidal activities of lysozyme and peroxidase more at high concentration than at low concentration (Kang et al., 2011), suggesting that limitation of diffusion by concentrated hyaluronic acids could be a possible mechanism. The ionic interactions between hyaluronic acid and lysozyme and between hyaluronic acid and peroxidase have been reported (Green et al., 1990; Moss et al., 1997; Van Damme et al., 1991, 1994). The strength of ionic interaction could be affected by molecular weight of hyaluronic acid. However, enzymatic activity was not affected by these interactions

especially in the solution assays using purified ones in the present study. It has been reported that lysozyme activity of leucocytic lysosome was inhibited by hyaluronic acid even at low-molecular-weight, while peroxidase activity was not affected (Avila and Convit, 1975). The Ionic complex formation between hyaluronic acid and myeloperoxidase has been reported, although the complex did not affect the activity of myeloperoxidase significantly at low concentrations (Green et al., 1990). Therefore, the results of interactions between hyaluronic acid and antimicrobials enzymes could depend on the types of enzyme and experimental conditions.

The results of present study showed that salivary lysozyme and peroxidase are more significantly inhibited by hyaluronic acids than purified enzymes both in solution and on hydroxyapatite surface except in solution assay of peroxidase. It could be explained that the influences of other molecules in saliva and formation of complex molecules could enhance the inhibitory effects of hyaluronic acids. It has been reported that mucinous component in saliva could interfere the diffusion of water molecules, which might explain more significant inhibitory effects of hyaluronic acids on salivary enzymes than purified enzymes (Lamy et al., 1990). Comparing solution assays with surface ones, it was observed that enzymatic activities were more affected by hyaluronic acid on hydroxyapatite surface than in solution. It could be assumed that the molecular conformations of hyaluronic acid or enzymes might be changed on the hydroxyapatite surface and these changes could have induced the different results between surface and solution assays. As surface assays, we performed the experiments in two different ways (surface assay I and surface assay II) to determine the influence of the reaction sequence, and the results were quite different. When pre-incubated hyaluronic acids with lysozyme or peroxidase were adsorbed to hydroxyapatite beads (surface assay II), the inhibitory effects were more significant than adsorption of hyaluronic acids to the beads before adsorption of enzymes (surface assay I). This might be due to increased adsorption of the ionic-complex molecules between hyaluronic acid and lysozyme or

peroxidase formed during pre-incubation to hydroxyapatite surface.

The enzymatic activity of GO-PO was not inhibited by hyaluronic acids with all molecular weights experimented. It means that hyaluronic acids did not affect the activity of GO-PO even at high-molecular-weight in solution phase. Although enzymatic activity is not directly proportional to candidacidal activity, this finding is consistent with previous study which presented that hyaluronic acid inhibited candidacidal activities of glucose oxidase-mediated lactoperoxidase system less than those of lysozyme or peroxidase, suggesting that glucose oxidase-mediated lactoperoxidase system might be more advantageous as saliva substitutes for dry mouth patients in respect of antimicrobial properties (Cho et al., 2013).

Based on the results of the present study, it should be discussed whether low-molecular-weight at high concentrations or high-molecular-weight at low concentrations would be more beneficial for oral health. To solve the question, additional studies on both biological and rheological properties should be needed. In this study, we investigated the viscosity of hyaluronic acids in respect of interfacial rheology and effects on enzymatic activities. Further studies of other rheological properties including extensional variables such as surface tension and biocompatibility considering intraoral comfort should be considered.

There were some limitations to extrapolate these in vitro results into the in vivo system. In the present study, the interactions between hyaluronic acids and antimicrobials were too simplified to evaluate the real interactions in human oral cavity, because there are various molecules which could also affect rheological and biological properties of hyaluronic acids and induce additional molecular interactions in the oral cavity.

In conclusion, the present study investigated the effects of molecular weights of hyaluronic acid both on rheological (viscosity) and biological property (effects on enzymatic activity). High-molecular-weight hyaluronic acids at low concentration and low-molecular-weight at high concentration showed similar viscosity values to

human whole saliva at 60 - 160 $s^{-1}$. Inhibitory effects of hyaluronic acids on lysozyme and peroxidase activities were more significant in high-molecular-weight ones, on-surface than in-solution, and on saliva than purified enzymes.

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Table 1. Effects of hyaluronic acid with different molecular weights on enzymatic activities of HEWL and whole salivary lysozyme in solution

| Source of lysozyme | MW of hyaluronic acid | Lysozyme activity (Units/mL) | | | *P* value |
|---|---|---|---|---|---|
| | | Sample only | With hyaluronic acid | Ratio (%) | |
| HEWL (n=8) | 10 kDa | 2553.0 ± 121.6 | 2509.0 ± 108.9 | 98.4 ± 4.3 | 0.263 |
| | 100 kDa | 2595.6 ± 69.3 | 2592.9 ± 82.9 | 100.0 ± 4.3 | 0.889 |
| | 1 MDa | 2477.0 ± 67.3 | 2451.0 ± 123.5 | 98.9 ± 4.5 | 0.575 |
| | 2 MDa | 2575.0 ± 156.9 | 2559.0 ± 122.0 | 99.5 ± 3.3 | 0.575 |
| Human saliva (n=8) | 10 kDa | 620.0 ± 103.9 | 629.3 ± 106.6 | 101.5 ± 3.4 | 0.161 |
| | 100 kDa | 629.5 ± 108.7 | 626.7 ± 90.8 | 100.1 ± 4.1 | 0.674 |
| | 1 MDa | 613.5 ± 88.7 | 605.7 ± 82.0 | 98.9 ± 2.1 | 0.161 |
| | 2 MDa | 651.0 ± 87.6 | 601.5 ± 81.5 | 92.4 ± 2.3 | 0.012* |

HEWL, hen egg-white lysozyme
MW, molecular weight
Ratio (%), enzymatic activities of HEWL or human saliva with hyaluronic acids compared to those without hyaluronic acids
*P* values were obtained from the Wilcoxon signed rank test. *$P < 0.05$

Table 2. Effects of hyaluronic acid with different molecular weights on enzymatic activities of HEWL and whole salivary lysozyme on hydroxyapatite surface

| Source of lysozyme | Type of assay | MW of hyaluronic acid | Lysozyme activity (Units) | | | *P* value |
|---|---|---|---|---|---|---|
| | | | Sample only | With hyaluronic acid | Ratio (%) | |
| HEWL (n=8) | Surface assay I | 10 kDa | 52.1 ± 2.9 | 51.7 ± 3.0 | 99.3 ± 1.9 | 0.327 |
| | | 100 kDa | 52.2 ± 4.2 | 51.7 ± 3.7 | 99.2 ± 1.7 | 0.208 |
| | | 1 MDa | 54.6 ± 2.9 | 54.3 ± 2.9 | 99.5 ± 1.1 | 0.161 |
| | | 2 MDa | 54.3 ± 3.6 | 53.7 ± 3.3 | 99.0 ± 1.2 | 0.017* |
| | Surface assay II | 10 kDa | 52.4 ± 2.4 | 52.6 ± 2.4 | 100.3 ± 2.3 | 0.726 |
| | | 100 kDa | 52.8 ± 2.8 | 52.1 ± 2.7 | 98.7 ± 2.9 | 0.263 |
| | | 1 MDa | 54.4 ± 6.7 | 49.9 ± 6.4 | 92.0 ± 7.4 | 0.017* |
| | | 2 MDa | 57.2 ± 5.6 | 43.6 ± 4.9 | 76.4 ± 7.6 | 0.012* |
| Human Saliva (n=8) | Surface assay I | 10 kDa | 35.1 ± 5.9 | 34.9 ± 5.6 | 99.7 ± 2.7 | 0.779 |
| | | 100 kDa | 34.9 ± 3.9 | 34.3 ± 4.3 | 98.3 ± 4.0 | 0.263 |
| | | 1 MDa | 32.7 ± 4.1 | 31.9 ± 5.0 | 97.1 ± 3.8 | 0.069 |
| | | 2 MDa | 32.6 ± 2.7 | 30.3 ± 3.3 | 93.1 ± 5.8 | 0.012* |
| | Surface assay II | 10 kDa | 43.8 ± 2.7 | 43.7 ± 1.1 | 100.0 ± 6.9 | 0.674 |
| | | 100 kDa | 44.1 ± 4.6 | 41.3 ± 5.4 | 93.6 ± 5.3 | 0.017* |
| | | 1 MDa | 36.1 ± 7.8 | 33.1 ± 7.0 | 91.6 ± 2.2 | 0.012* |
| | | 2 MDa | 38.4 ± 6.5 | 29.5 ± 6.0 | 76.6 ± 7.3 | 0.012* |

HEWL, hen egg-white lysozyme
MW, molecular weight
Ratio (%), enzymatic activities of HEWL or human saliva with hyaluronic acids compared to those without hyaluronic acids
Surface assay I, hyaluronic acids were adsorbed to hydroxyapatite beads first, and HEWL or human saliva was incubated on the hyaluronic acid-adsorbed beads next.
Surface assay II, hyaluronic acids were pre-incubated with HEWL or human saliva, and the mixtures were adsorbed to hydroxyapatite beads.
*P* values were obtained from the Wilcoxon signed rank test. *$P < 0.05$

Table 3. Effects of hyaluronic acid with different molecular weights on enzymatic activities of bLPO and whole salivary peroxidase in solution

| Source of peroxidase | MW of hyaluronic acid | Peroxidase activity (Units/mL) | | | *P* value |
|---|---|---|---|---|---|
| | | Sample only | With hyaluronic acid | Ratio (%) | |
| bLPO (n=8) | 10 kDa | 7.14 ± 1.00 | 6.95 ± 0.80 | 97.8 ± 5.7 | 0.327 |
| | 100 kDa | 8.31 ± 0.96 | 8.27 ± 1.04 | 99.6 ± 5.8 | 0.779 |
| | 1 MDa | 8.17 ± 0.71 | 8.06 ± 0.86 | 99.0 ± 11.8 | 0.889 |
| | 2 MDa | 8.04 ± 0.88 | 7.84 ± 1.01 | 97.6 ± 7.0 | 0.674 |
| Human saliva (n=8) | 10 kDa | 0.430 ± 0.040 | 0.429 ± 0.045 | 99.7 ± 2.6 | 0.779 |
| | 100 kDa | 0.439 ± 0.028 | 0.434 ± 0.030 | 99.0 ± 3.0 | 0.310 |
| | 1 MDa | 0.457 ± 0.053 | 0.461 ± 0.050 | 101.1 ± 2.9 | 0.362 |
| | 2 MDa | 0.459 ± 0.030 | 0.459 ± 0.032 | 100.1 ± 1.9 | 0.888 |

bLPO, bovine lactoperoxidase
MW, molecular weight
Ratio (%), enzymatic activities of bLPO or human saliva with hyaluronic acids compared to those without hyaluronic acids
*P* values were obtained from the Wilcoxon signed rank test. **P* < 0.05

Table 4. Effects of hyaluronic acid with different molecular weights on enzymatic activities of bLPO and whole salivary peroxidase on hydroxyapatite surface

| Source of peroxidase | Type of assay | MW of hyaluronic acid | Peroxidase activity (mUnits) | | | *P* value |
|---|---|---|---|---|---|---|
| | | | Sample only | With hyaluronic acid | Ratio (%) | |
| bLPO (n=8) | Surface assay I | 10 kDa | 5.87 ± 0.11 | 5.90 ± 0.08 | 100.7 ± 2.0 | 0.528 |
| | | 100 kDa | 5.53 ± 0.21 | 5.40 ± 0.18 | 97.7 ± 3.5 | 0.069 |
| | | 1 MDa | 6.14 ± 0.40 | 6.09 ± 0.37 | 99.2 ± 1.6 | 0.236 |
| | | 2 MDa | 5.78 ± 0.25 | 5.63 ± 0.19 | 97.5 ± 3.5 | 0.092 |
| | Surface assay II | 10 kDa | 4.97 ± 0.31 | 4.97 ± 0.29 | 100.0 ± 2.0 | 0.944 |
| | | 100 kDa | 5.88 ± 0.27 | 5.90 ± 0.33 | 100.3 ± 2.6 | 0.726 |
| | | 1 MDa | 5.22 ± 0.22 | 5.14 ± 0.27 | 98.5 ± 3.4 | 0.182 |
| | | 2 MDa | 6.25 ± 0.24 | 5.87 ± 0.26 | 94.0 ± 2.8 | 0.012* |
| Human Saliva (n=8) | Surface assay I | 10 kDa | 7.49 ± 0.24 | 7.42 ± 0.22 | 99.2 ± 2.2 | 0.401 |
| | | 100 kDa | 7.50 ± 0.54 | 7.50 ± 0.56 | 100.0 ± 2.1 | 0.889 |
| | | 1 MDa | 7.30 ± 0.57 | 7.27 ± 0.53 | 99.6 ± 1.9 | 0.483 |
| | | 2 MDa | 7.14 ± 0.25 | 7.00 ± 0.29 | 98.1 ± 4.1 | 0.093 |
| | Surface assay II | 10 kDa | 6.40 ± 0.60 | 6.39 ± 0.64 | 99.8 ± 3.7 | 0.889 |
| | | 100 kDa | 6.84 ± 0.41 | 6.69 ± 0.50 | 97.8 ± 2.7 | 0.068 |
| | | 1 MDa | 6.96 ± 0.60 | 6.60 ± 0.69 | 94.7 ± 3.2 | 0.012* |
| | | 2 MDa | 6.81 ± 0.58 | 6.40 ± 0.48 | 94.2 ± 4.8 | 0.017* |

bLPO, bovine lactoperoxidase
MW, molecular weight
Ratio (%), enzymatic activities of bLPO or human saliva with hyaluronic acids compared to those without hyaluronic acids
Surface assay I, hyaluronic acids were adsorbed to hydroxyapatite beads first, and bLPO or human saliva was absorbed to the hyaluronic acid-adsorbed beads next.
Surface assay II, hyaluronic acids were pre-incubated with bLPO or human saliva, and the mixtures were adsorbed to hydroxyapatite beads.
*P* values were obtained from the Wilcoxon signed rank test. *$P < 0.05$

Table 5. Effects of hyaluronic acid with different molecular weights on enzymatic activity of glucose oxidase-mediated peroxidase in solution

| MW of hyaluronic acid (n=6) | Concentration of glucose (mg/mL) | GO-PO activity (OD) | | | *P* value |
|---|---|---|---|---|---|
| | | Sample only | With hyaluronic acid | Ratio (%) | |
| 10 kDa | 0.02 | 0.364 ± 0.007 | 0.362 ± 0.006 | 99.2 ± 0.7 | 0.068 |
| | 0.04 | 0.703 ± 0.010 | 0.700 ± 0.009 | 99.6 ± 0.7 | 0.207 |
| | 0.06 | 1.011 ± 0.016 | 1.006 ± 0.013 | 99.5 ± 0.6 | 0.075 |
| 100 kDa | 0.02 | 0.364 ± 0.007 | 0.361 ± 0.007 | 99.0 ± 1.0 | 0.078 |
| | 0.04 | 0.703 ± 0.010 | 0.697 ± 0.009 | 99.1 ± 0.8 | 0.068 |
| | 0.06 | 1.011 ± 0.016 | 1.004 ± 0.017 | 99.5 ± 0.9 | 0.207 |
| 1 MDa | 0.02 | 0.364 ± 0.007 | 0.362 ± 0.007 | 99.3 ± 1.1 | 0.207 |
| | 0.04 | 0.703 ± 0.010 | 0.701 ± 0.010 | 99.8 ± 1.3 | 0.673 |
| | 0.06 | 1.011 ± 0.016 | 1.010 ± 0.019 | 99.9 ± 1.3 | 0.833 |
| 2 MDa | 0.02 | 0.364 ± 0.007 | 0.362 ± 0.007 | 99.3 ± 1.3 | 0.223 |
| | 0.04 | 0.703 ± 0.010 | 0.702 ± 0.012 | 99.9 ± 1.9 | 0.833 |
| | 0.06 | 1.011 ± 0.016 | 1.006 ± 0.018 | 99.5 ± 1.0 | 0.345 |

GO-PO, glucose oxidase-mediated peroxidase
MW, molecular weight
Ratio (%), enzymatic activities of GO-PO with hyaluronic acids compared to those without hyaluronic acids
*P* values were obtained from the Wilcoxon signed rank test. *$P < 0.05$

## Figure Legends

Fig. 1. Viscosity values of hyaluronic acids at different concentrations. Viscosity measurements were performed at 6 different shear rates (11.3, 22.5, 45.0, 90.0, 225, and 450 $s^{-1}$). (a) 10 kDa-hyaluronic acid, (b) 100 kDa-hyaluronic acid, (c) 1 MDa-hyaluronic acid, (d) 2 MDa-hyaluronic acid

Fig. 2. Viscosity values of hyaluronic acids compared with human whole saliva at different concentrations. Viscosity measurements were performed at 6 different shear rates (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL). The viscosity values for 1 MDa-hyaluronic acid at 2.0 and 5.0 mg/mL and 2 MDa-hyaluronic acid at 1.0, 2.0, and 5.0 mg/mL were not shown for more detailed comparison. (a) 10 kDa-hyaluronic acid, (b) 100 kDa-hyaluronic acid, (c) 1 MDa-hyaluronic acid, (d) 2 MDa-hyaluronic acid

UWS, unstimulated whole saliva; SWS, stimulated whole saliva

*The viscosity values of UWS and SWS was cited from previous data (Park et al., 2010).

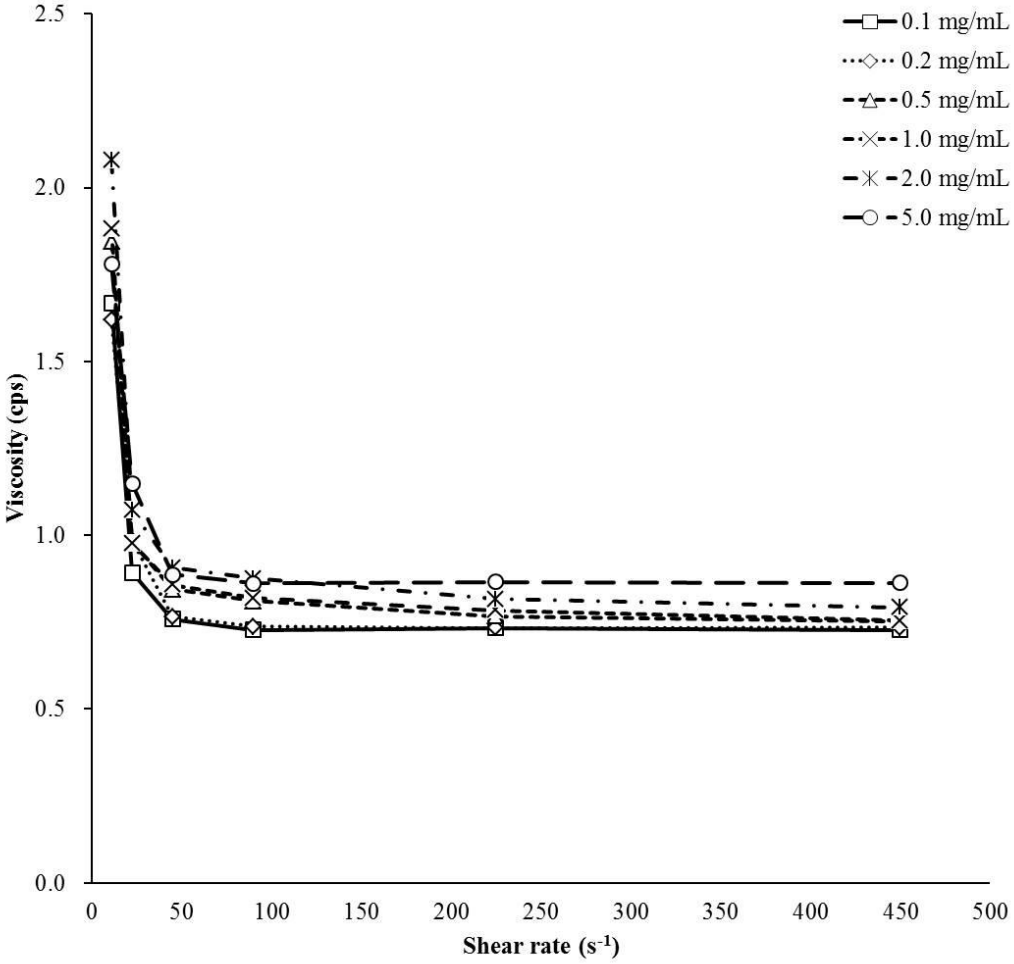
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
5.0 mg/mL
Viscosity (cps)
Shear rate (s-1)
0.0
0.5
1.0
1.5
2.0
2.5
0
50
100
150
200
250
300
350
400
450
500

**Fig. 1. (a)**

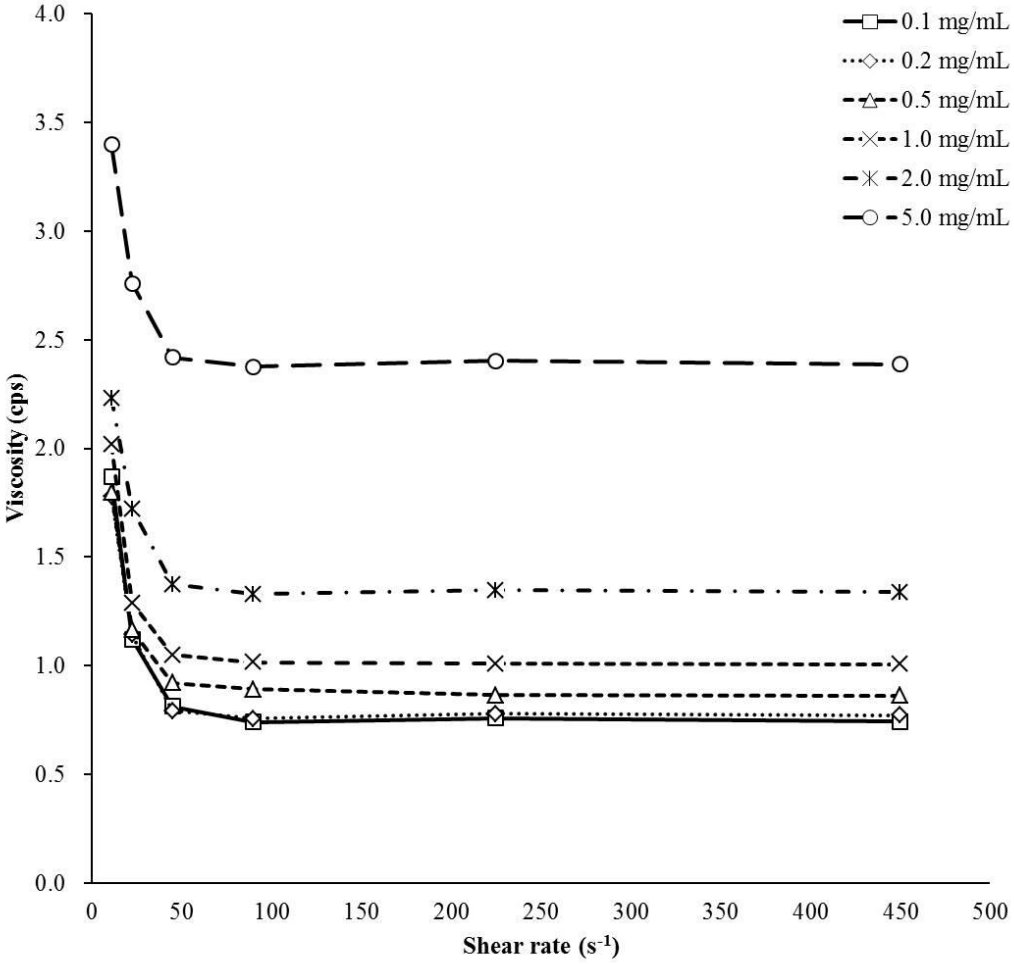
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
5.0 mg/mL
Viscosity (cps)
Shear rate (s-1)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
350
400
450
500

**Fig. 1. (b)**

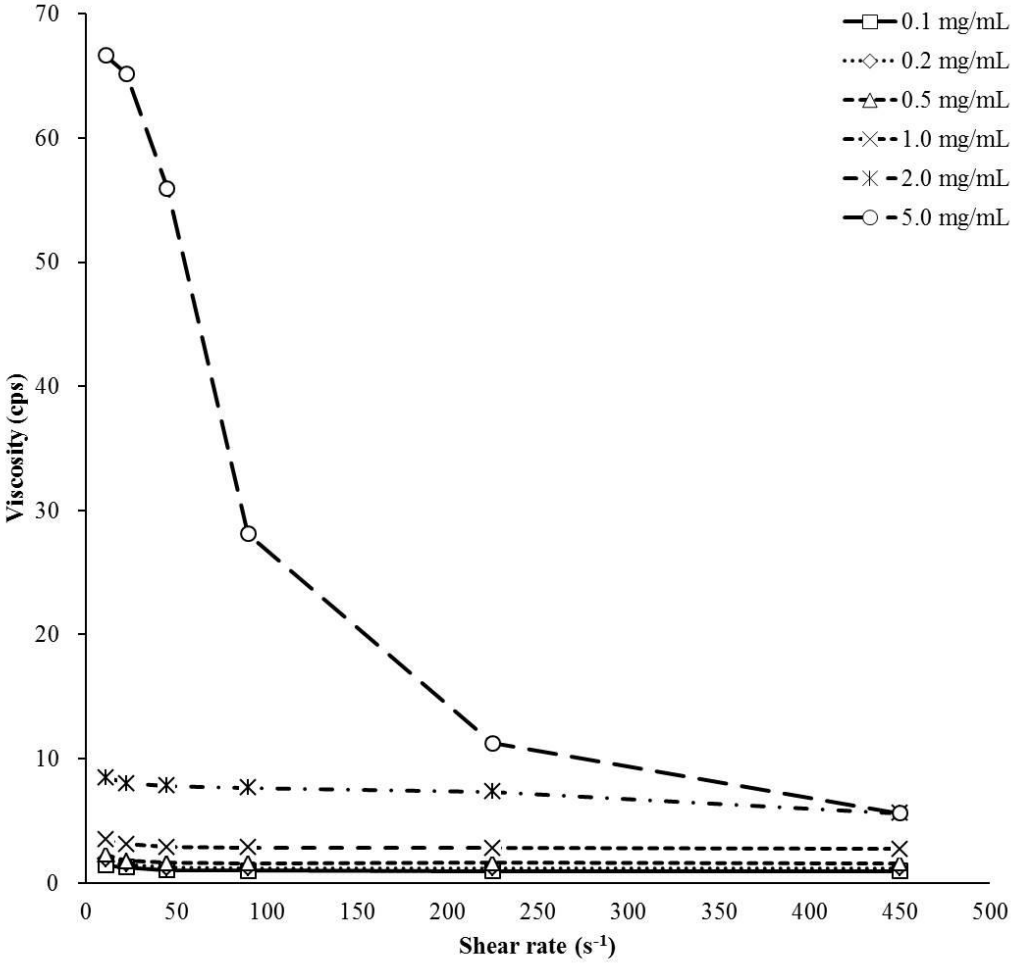
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
5.0 mg/mL
Viscosity (cps)
Shear rate (s-1)
0
10
20
30
40
50
60
70
0
50
100
150
200
250
300
350
400
450
500

**Fig. 1. (c)**

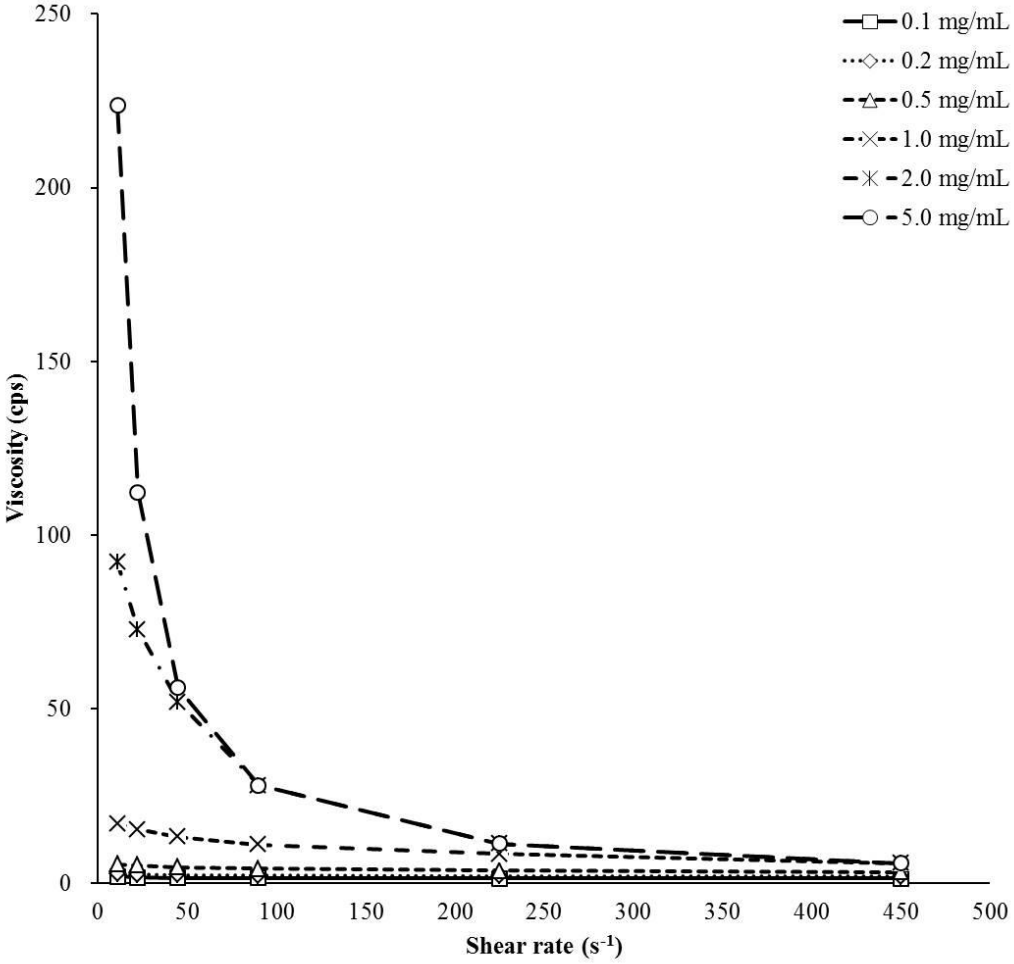
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
5.0 mg/mL
Viscosity (cps)
Shear rate (s-1)
250
200
150
100
50
0
0
50
100
150
200
250
300
350
400
450
500

**Fig. 1. (d)**

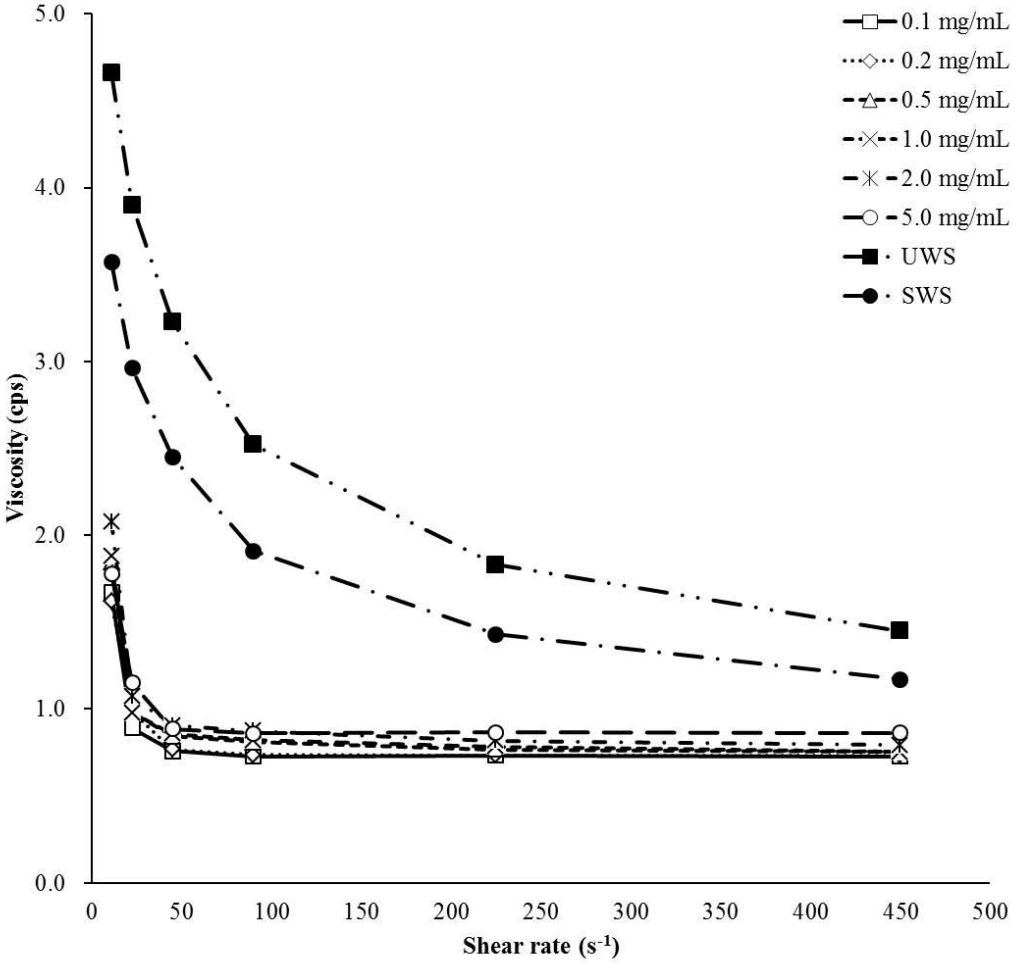
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
5.0 mg/mL
UWS
SWS
Viscosity (cps)
Shear rate (s-1)
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300
350
400
450
500

**Fig. 2. (a)**

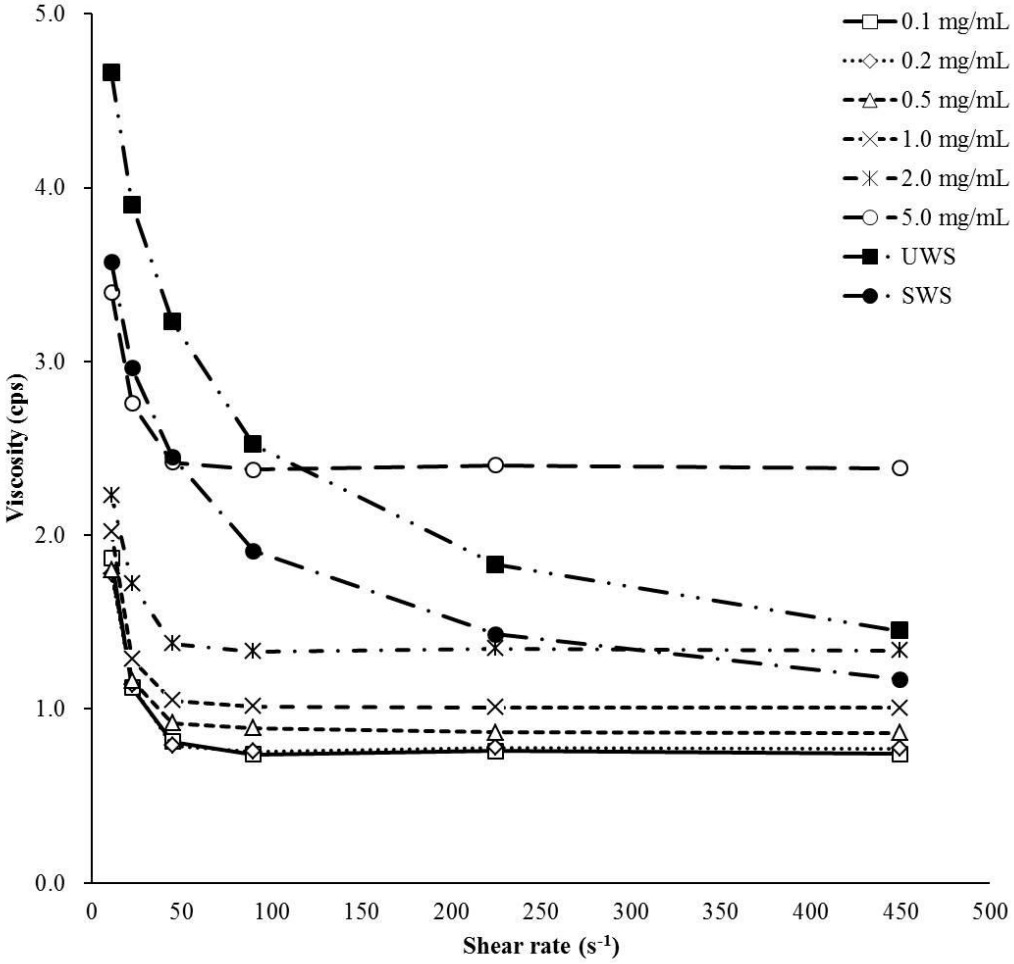
5.0
4.0
3.0
2.0
1.0
0.0
Viscosity (cps)
0
50
100
150
200
250
300
350
400
450
500
Shear rate (s-1)
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
5.0 mg/mL
UWS
SWS

**Fig. 2. (b)**

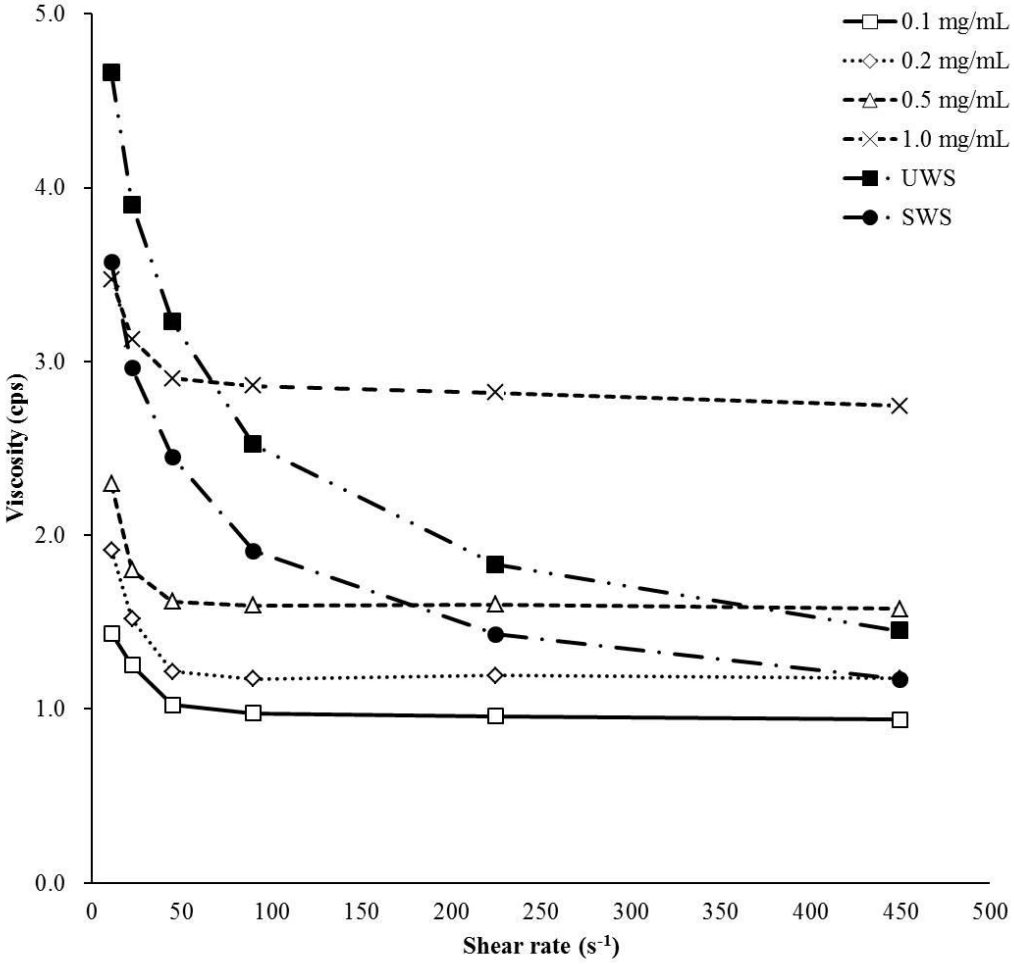
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
1.0 mg/mL
UWS
SWS
Viscosity (cps)
Shear rate (s-1)
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300
350
400
450
500

**Fig. 2. (c)**

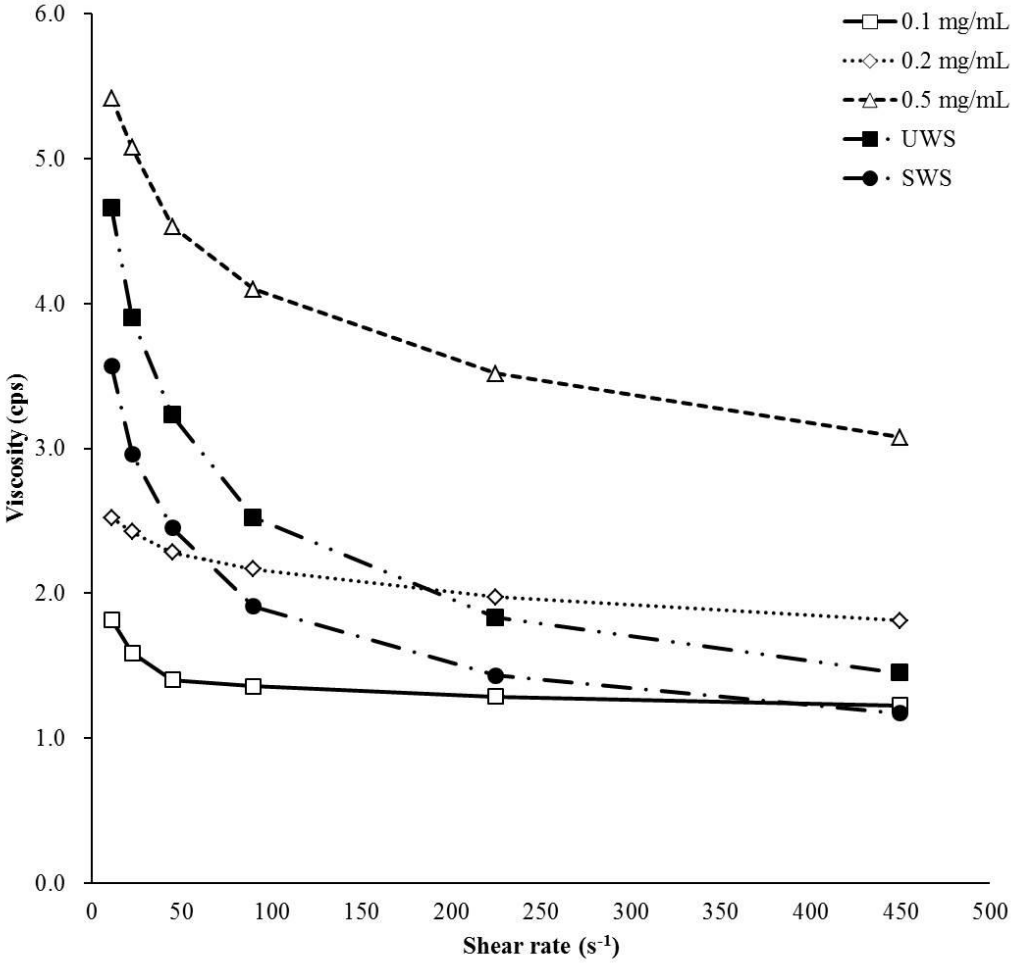
0.1 mg/mL
0.2 mg/mL
0.5 mg/mL
UWS
SWS
Viscosity (cps)
Shear rate (s-1)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
250
300
350
400
450
500

**Fig. 2. (d)**

국문초록

# Hyaluronic acid의 분자량에 따른 차이가 점도 및 Lysozyme과 Peroxidase 관련 효소 활성에 미치는 영향

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(지도교수 **고 홍 섭**)

Hyaluronic acid는 glycosaminoglycan의 일종으로 인체에도 존재하므로 생체 적합성이 뛰어나고 타액과 유사한 점탄성의 특성을 나타내므로 타액대체물의 후보물질로 제시된 바 있다. 본 연구의 목적은 hyaluronic acid의 분자량이 유동학적 및 생물학적 특성에 미치는 영향을 조사하기 위한 것으로, 다양한 분자량의 hyaluronic acid의 점도를 분석하고 분자량의 차이에 따라 hyaluronic acid가 lysozyme과 peroxidase 관련 효소의 활성에 미치는 영향을 평가하는 것이다.

실험에 사용된 hyaluronic acid는 네 가지 서로 다른 분자량의 상용화된 hyaluronic acid (10 kDa, 100 kDa, 1 MDa 및 2 MDa)를 사용하였고, 상용화된 효소로는 hen egg-white lysozyme (HEWL, 30 $\mu$g/mL), bovine lactoperoxidase (bLPO, 25 $\mu$g/mL)와 glucose oxidase-mediated peroxidase (GO-PO)를 사용하였으며, 연구대상자로부터 인체 타액을 채취하여 타액 내 lysozyme과 peroxidase에 대한

분석도 시행하였다. Hyaluronic acid의 점도 측정을 위해 cone-and-plate digital viscometer를 활용하여 6가지 농도 (0.1, 0.2, 0.5, 1.0, 2.0 및 5.0 mg/mL)에서 전단 속도 (shear rate)를 11.3 $s^{-1}$부터 450 $s^{-1}$까지 6단계로 증가시키면서 각각의 점도를 측정하였다. Lysozyme의 효소 활성은 형광물질이 부착된 *Micrococcus lysodeikticus*가 가수분해되는 정도를 측정하여 분석하였고, peroxidase의 효소 활성은 fluorogenic 2’,7’-dichlorofluorescein (LDCF)이 fluorescing 2’,7’-dichlorofluorescein (DCF)으로 산화되는 정도를 측정하였으며, GO-PO의 효소 활성은 산화된 o-dianisidine을 측정하여 분석하였다. Lysozyme과 peroxidase의 활성은 용액 상태와 hydroxyapatite 표면 상태에서 각각 측정하였고, GO-PO의 활성은 용액 상태에서만 측정하였다. 효소 활성에 대한 영향을 측정하기 위해 0.5 mg/mL 농도의 hyaluronic acid를 사용하였다.

서로 다른 분자량의 hyaluronic acid를 이용하여 점도를 측정한 결과, 연하 및 대화 시 발생하는 전단 속도인 60 $s^{-1}$부터 160 $s^{-1}$까지의 범위에서 100 kDa-hyaluronic acid는 5 mg/mL의 농도에서, 1 MDa-hyaluronic acid는 0.5 mg/mL의 농도에서, 2 MDa-hyaluronic acid는 0.2 mg/mL의 농도에서 인체 타액과 비슷한 점도를 보였다. 효소 활성에 대한 hyaluronic acid의 영향을 분석한 결과, 용액 상태에서는 2 MDa-hyaluronic acid가 유일하게 타액 내 lysozyme 활성을 저해하였고, 다른 분자량의 hyaluronic acid는 유의한 영향을 미치지 않았으며, 또한 상용화된 효소들은 유의한 영향을 받지 않았다. 표면 상태에서는 고분자량의 hyaluronic acid가 lysozyme과 peroxidase의 활성을 억제함이 관찰되었고, 그 정도는 상용화된 효소보다 타액 내 효소들에서 더 명확하게 관찰되었다. GO-PO의 활성은 본 실험에서 사용된 모든 분자

량의 hyaluronic acid가 유의한 영향을 미치지 않았다.

결론적으로, 고분자량의 hyaluronic acid는 저농도에서, 저분자량의 hyaluronic acid는 고농도에서 인체 타액과 유사한 점도를 보였고, hyaluronic acid의 효소 활성 억제 효과는 저분자량보다는 고분자량에서, 용액 상태보다는 표면 상태에서, 상용화된 효소보다는 타액 내 효소에서 더 명확하게 관찰되었다.

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주요어 : Hyaluronic acid, 분자량, 점도, Lysozyme, Peroxidase
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