Influences of Saliva Substitutes on Salivary Enzymatic Activity

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Many of the protective functions of saliva can be attributed to the biological, physical, structural, and rheological characteristics of salivary glycoproteins. Therefore, the development of ideal saliva substitutes requires understanding of the rheological as well as biological properties of human saliva. In the present study, we investigated the changes of salivary enzymatic activities by saliva substitutes and compared viscosity of saliva substitutes with human saliva. Five kinds of saliva substitutes such as Moi-Stir, Stoppers4, MouthKote, Saliva Orthana, and SNU were used. Lysozyme activity was determined by the turbidimetric method. Peroxidase activity was determined with an NbsSCN assay. α-Amylase activity was determined using a chromogenic substrate, 2-chloro-p-nitrophenol linked with maltotriose. The pH values of saliva substitutes were measured and their viscosity values were measured with a cone-and-plate digital viscometer at six different shear rates. Various types of saliva substitutes affected the activities of salivary enzymes in different ways. Stoppers4 enhanced the enzymatic activities of hen egg-white lysozyme, bovine lactoperoxidase (bLP), and α-amylase. Saliva Orthana and SNU inhibited bLP activity and enhanced α-amylase activity. MouthKote inhibited α-amylase activity. Moi-Stir inhibited the enzymatic activities of bLP and α-amylase. The pH values were very different according to the types of saliva substitutes. Stoppers4, MouthKote, and Saliva Orthana showed lower values of viscosity at low shear rates and higher values of viscosity at high shear rates compared with unstimulated and stimulated whole saliva. Moi-Stir and SNU displayed much higher values of viscosity than those of natural whole saliva. Collectively, our results indicate that each saliva substitute has its own biological and rheological characteristics. Each saliva substitute affects the enzymatic activity of salivary enzyme and finally oral health in different ways.

Key words: Saliva substitute, Lysozyme, Peroxidase, Amylase, Saliva

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I. INTRODUCTION

The importance of saliva becomes readily apparent in individuals whose capacity for saliva production is diminished. Patients with xerostomia may have complaints that include difficulties in speaking, eating, and swallowing. Some patients may also complain of oral malodor, a chronic burning sensation, taste disturbance, and intolerance to spicy food. Furthermore, decreased salivary production can lead to oral mucosal infection with *Candida*, and increased risk of dental caries and increased severity of periodontal diseases, which further worsen nutritional problems. Patients with dentures may have retention problems, soreness, and ulcers.[1-5] Consequently, inadequate saliva production can significantly diminish quality of life.[6-8]

To relieve problems related to xerostomia, most patients use saliva stimulants and/or saliva substitutes. When some residual salivary flow remains, saliva stimulants such as pharmaceutical sialogogues, sugarless candies, and chewing gum can be used to restore saliva production.[4,9,10] If stimulation of salivary secretion by means of saliva stimulants is ineffective, symptomatic treatment with saliva substitutes may be helpful.[4,11-15] Current saliva substitutes are generally divided into two categories: carboxymethylcellulose (CMC)-based saliva substitutes and animal mucin-based saliva substitutes.[16-18] Although these saliva substitutes may decrease some symptoms of oral dryness in xerostomic patients, the alleviating effects of today's commercially-available substitutes are short-lived and, therefore, of limited benefit to patients.[11,19] Despite this, several studies have reported that mucin-based saliva substitutes are more effective than their CMC-based counterparts.[20-22] Regarding the efficacy of artificial saliva, there have been previous reports which showed significant reduction of the dry mouth-related complaints in the patients suffering from severe xerostomia.[14,23,24]

Previous clinical studies on the effectiveness of saliva substitutes have largely depended on subjective reports of xerostomic patients.[19-21] Few objective data exist regarding the rheological (viscosity) and film-forming (wettability) properties essential to proper function of any saliva substitute. Although an ideal saliva substitute mimics the rheological and biochemical properties of natural human saliva,[16] the addition of antimicrobials to a solution otherwise having similar rheological properties to human saliva may be an even better solution, and this approach is presently feasible.[25] Since molecules in saliva substitutes and host-derived antimicrobial salivary molecules exist simultaneously in whole saliva of patients with salivary hypofunction, interactions between these molecules may occur. Indeed, such interactions are reported to induce an increase or decrease in enzymatic activities of antimicrobial molecules such as lysozyme and peroxidase.[26,27]

In the present study, we have investigated the effects of various types of commercial saliva substitutes on lysozyme, peroxidase and α-amylase activity. The viscosity of the saliva substitutes was also measured.

II. MATERIALS AND METHODS

1. Saliva substitutes

Five kinds of saliva substitutes were used in the present study. Moi-Stir (Pharma Science Inc., Montreal, Canada), Stoppers4 (Woodridge Labs, Inc., Van Nuys, CA, USA), MouthKote (Parnell Pharmaceuticals, San Rafael, CA, USA), Saliva Orthana (AS Pharma, UK) were commercially available and purchased. The other one (SNU) was saliva substitute used in the Seoul National University Dental Hospital.

2. Measurement of lysozyme activity

Lysozyme activity was determined by the turbidimetric method.[28] Samples were placed in a lyophilized cell suspension of *Micrococcus lysodei kticus* ATCC 4698, starting OD_{450} = 0.65 - 0.70, so

$$\text{Lysozyme activity (units/mL)} = \frac{(\Delta A_{450nm}/\text{min Test} - \Delta A_{450nm}/\text{min Blank})\ (df)}{(0.001)\ (0.1)}$$

df: dilution factor
0.001: change in absorbance at A_{450nm} as per the unit definition
0.1: volume (in mL) of enzyme used

that the lysozyme present in solution could degrade the bacterial substrate. After incubation, the optical density of each samples' liquid phase was determined. The lysozyme activity was calculated from the below equation. The lysozyme activity was expressed as Units/mL.

3. Influence of saliva substitutes on lysozyme activity

Hen egg-white lysozyme (HEWL, Sigma-Aldrich, St Louis, MO, USA) dissolved in simulated salivary buffer (SSB, 0.021 M Na_2HPO_4/NaH_2PO_4, pH 7.0, containing 36 mM NaCl and 0.96 mM $CaCl_2$)[29] served as a lysozyme source. The effects of saliva substitutes on lysozyme activity were examined by incubating 250 μL of each saliva substitute with 250 μL of HEWL (10 μg/mL) for 10 min at RT. The incubated mixture was placed in a suspension of *M. lysodeikticus* and an incubated mixture of buffer with HEWL was used as a control. Either an incubated mixture of each saliva substitute with buffer, or an incubated buffer alone was used as a blank.

4. Measurement of peroxidase activity

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 $OSCN^-$ ions generated during the oxidation of SCN^- by bLPO.[30] Peroxidase activity was expressed as mUnits/mL.

5. Influence of saliva substitutes on peroxidase activity

Bovine lactoperoxidase (bLP, Sigma-Aldrich, St Louis, MO, USA) dissolved in SSB served as a peroxidase source. The effects of saliva substitutes on peroxidase activity were examined by incubating 250 μL of each saliva substitute with 250 μL of bLP (25.0 μg/mL) for 10 min at RT. To 300 μL of reaction mixture for NbsSCN assay, 15 μL of KSCN (final concentration of 4.2 mM SCN^-) and 15 μL of sample solution were added, and reaction was initiated by the addition of 15 L of H_2O_2 (final concentrations were 50 μM). An incubated mixture of buffer with bLP was used as a control. For the blank reaction, an incubated mixture of each saliva substitute with buffer, or an incubated buffer alone was used.

6. Measurement of α-amylase activity

α-Amylase activity was determined using salivary α-amylase assay kit (Salimetrics, State College, PA, USA) which utilizes a chromogenic substrate, 2-chloro-p-nitrophenol linked with maltotriose. The enzymatic action of α-amylase on this substrate yields 2-chloro-p-nitrophenol, which can be spectrometrically measured at 405 nm. α-Amylase activity was expressed as Units/mL.

7. Influence of saliva substitutes on α-amylase activity

Human salivary α-amylase (Sigma-Aldrich, St Louis, MO, USA) dissolved in SSB served as an amylase source. The effects of saliva substitutes on α-amylase activity were examined by incubating 100 μL of each saliva substitute with 100 μL of α-amylase (50 units/mL) for 10 min at RT. An incubated mixture of buffer with α-amylase was used as a control. No α-amylase activity was detected in SSB or any saliva substitutes used in the experiments.

8. pH measurement of saliva substitutes

The pH values of saliva substitutes were measured using a pH meter (pH meter 440, Corning Inc., Lowell, MA, USA).

9. Measurement of viscosity

Viscosity measurement was conducted 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℃, and 0.5 mL volume of fluid was used in each test. The viscosity of each sample was measured three times.

10. Statistics

The Wilcoxon signed rank test was used to analyze the effects of salivary substitutes compared with their controls. P-values less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Influence of saliva substitutes on lysozyme activity

The influences of various kinds of saliva substitutes on lysozyme activity were shown in Fig. 1. Stoppers4 enhanced the enzymatic activities of HEWL (P < 0.01). Moi-Stir, MouthKote, Saliva Orthana, and SNU did not affect the enzymatic activities of HEWL.

2. Influence of saliva substitutes on peroxidase activity

The influences of various kinds of saliva substitutes on peroxidase activity were shown in Fig. 2. Stoppers4 enhanced the enzymatic activities of bLP (P < 0.01). Saliva Orthana, Moi-Stir and SNU inhibited the enzymatic activities of bLP (P < 0.01). MouthKote did not affect the enzymatic activities of bLP.

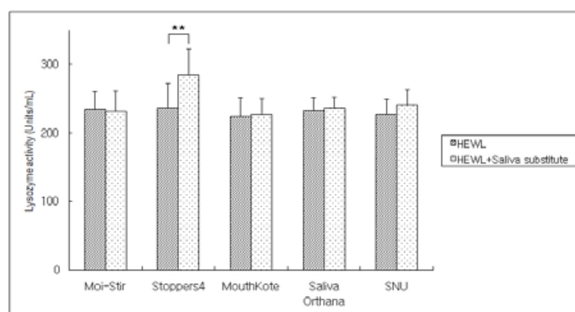

Fig. 1. Influence of saliva substitutes on lysozyme activity. The assay was performed 10 times.
HEWL, hen egg-white lysozyme
** P < 0.01

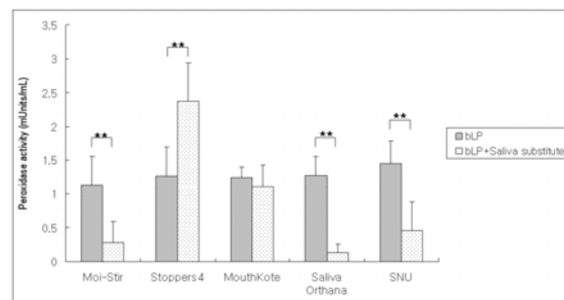

Fig. 2. Influence of saliva substitutes on peroxidase activity. The assay was performed 10 times.
bLP, bovine lactoperoxidase
** P < 0.01

3. Influence of saliva substitutes on α-amylase activity

The influences of various kinds of saliva substitutes on α-amylase activity were shown in Fig. 3. The enzymatic activities of α-amylase were enhanced by Stoppers4 (P < 0.01), Saliva Orthana (P < 0.01), and SNU (P < 0.05). Moi-Stir and MouthKote inhibited the enzymatic activities of α-amylase (P < 0.01).

Table 1. The pH values of commercial saliva substitutes

Saliva substitute	pH
Moi-Stir	7.0
Stoppers4	4.0
MouthKote	3.0
Saliva Orthana	5.8
SNU	6.7

4. Measurement of pH

The pH values of saliva substitutes were shown in Table 1. MouthKote and Stoppers4 were pH 3.0 and 4.0, respectively. Saliva Orthana and SNU were weak acidic. Moi-Stir was neutral.

5. Measurement of viscosity

Stoppers4, MouthKote, and Saliva Orthana showed lower values of viscosity at low shear rates and higher values of viscosity at high shear rates compared with unstimulated (UWS) and stimulated whole saliva (SWS) (Fig. 4-1). Moi-Stir and SNU displayed much higher values of viscosity than those of natural saliva (Fig. 4-2).

Ⅳ. DISCUSSION

Many of the protective functions of saliva can be attributed to the biological, physical, structural, and rheological characteristics of salivary glycoproteins.[31] Therefore, the development of ideal saliva substitutes requires understanding of the rheological as well as biological properties of human saliva. In the present study, we investigated the changes of salivary enzymatic activities by saliva substitutes and compared viscosity of saliva substitutes with human saliva at different shear rates.

Of the antimicrobial molecules identified in saliva, lysozyme and peroxidase are prominent antibacterial components widely distributed in various biological fluids including saliva, tears, milk, and cervical secretions.[32,33] Lysozyme provides its antimicrobial activity through a muramidase-dependent mode and

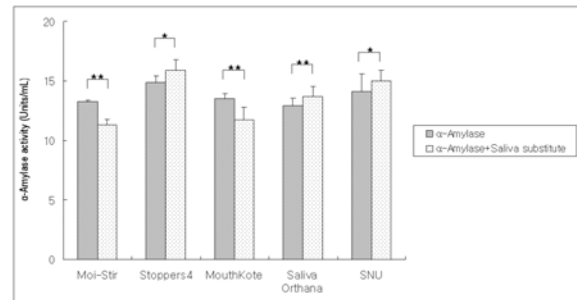

Fig. 3. Influence of saliva substitutes on α-amylase activity. The assay was performed 10 times.
* P < 0.05, ** P < 0.01

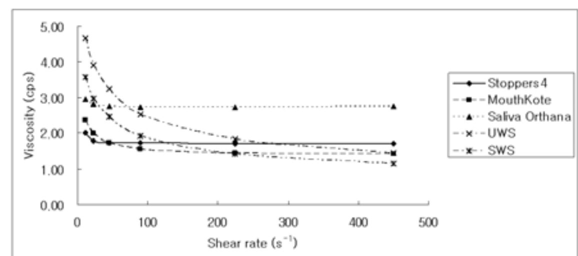

Fig. 4-1. Viscosity values of human saliva and saliva substitutes. The viscosity of saliva substitutes was measured 4 times. The viscosity values of human saliva were adopted from Park *et al.* (2007).[25]
UWS, unstimulated whole saliva; SWS, stimulated whole saliva

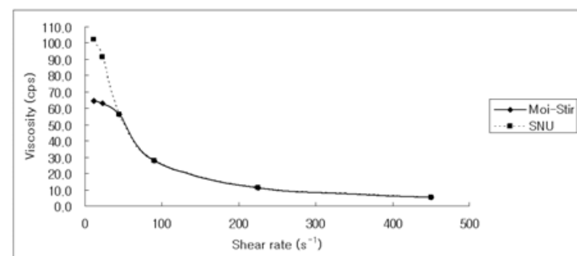

Fig. 4-2. Viscosity values of saliva substitutes. The viscosity of saliva substitutes was measured 4 times.

cationic-dependent or structure-related bactericidal mechanisms.[34,35] Two species of peroxidase are present in human saliva. Salivary peroxidase is secreted by the salivary glands, whereas myeloperoxidase emerges from leucocytes reaching the oral cavity. Peroxidase provides antimicrobial activity and protection of oral tissues from oxygen toxicity through oxidation of SCN^- and consumption of H_2O_2.[36,37] In patients with dry mouth, HEWL or bLP, either alone or in combination with other molecules, has been incorporated in oral health care products to restore the antimicrobial capacity of saliva.[38] It is not simple to interpret the effects of saliva substitutes on α-amylase activity. It is because α-amylase may have both protective and detrimental properties.[13] α-amylase can interact with various viridans streptococci to facilitate their clearance from the oral cavity.[39] However, α-amylases adsorbed to the tooth surface can promote adherence of these bacteria and also digest dietary starch to maltose that can be used by the bacteria to produce acid.[40]

In the present study, various types of saliva substitutes affected the activities of salivary enzymes in different ways. Stoppers4 enhanced the enzymatic activities of HEWL, bLP and α-amylase. Saliva Orthana and SNU inhibited bLP activity and enhanced α-amylase activity. MouthKote inhibited α-amylase activity. Moi-Stir inhibited the enzymatic activities of bLP and α-amylase. Therefore regarding the effects on antimicrobial activities, Stoppers4 was better saliva substitute. Stoppers4 contains hydroxyethylcellulose, lysozyme, lactoferrin, and glucose oxidase which may act as antimicrobial supplements in saliva of the patients with dry mouth. However, MouthKote and Stoppers4 were acidic in nature which may increases dental caries in patients with dry mouth. The changes of salivary enzymatic activity by saliva substitutes suggested the existence of a complex molecule between molecules in saliva substitutes and salivary enzymes. Previous studies have shown that mucins form heterotypic complexes with other salivary molecules, such as secretory IgA, lysozyme, cystatins, α-amylase, PRPs, statherins, histatins, lactoferrin, and agglutinin.[41,42] In fact, porcine gastric mucin, one of candidate molecule for saliva substitutes, affected peroxidase activity as well as lysozyme activity.[26,27].

The efficacy of saliva as a lubricant is at least partially dependent on its viscosity and how this changes with shear rates.[43] According to our results, Moi-Stir and SNU displayed much higher viscosity than human whole saliva. Stoppers4, MouthKote, and Saliva Orthana showed lower values of viscosity at low shear rates and higher values of viscosity at high shear rates compared with human whole saliva. It is known that an excessively sticky salivary substitute gives rise to unpleasantness and difficulty in masticatory function.[16,44] Considering the clinical preference of mucin-based saliva substitutes over traditional CMC-based formulations, which have comparatively higher viscosity values,[10,16,18] high viscosity is not always desirable in terms of the function of the salivary substitute.

Collectively, our results suggest that different saliva substitutes affect the enzymatic activities of salivary enzyme differently. Therefore, saliva substitutes may affect the function of antimicrobial components in natural saliva and saliva substitutes, and the composition of salivary pellicle on tooth surfaces. Although there are many issues to be considered before the results of the present *in vitro* study can be extrapolated to an *in vivo* situation, the results of the present study provide valuable information on the effects of saliva substitutes on antimicrobial activities in oral health care products and possibly in the oral cavity.

V. CONCLUSIONS

Many of the protective functions of saliva can be attributed to the biological, physical, structural, and rheological characteristics of salivary glycoproteins. Therefore, the development of ideal saliva substitutes requires understanding of the rheological as well as biological properties of human saliva. In

the present study, we investigated the changes of salivary enzymatic activities by saliva substitutes and compared viscosity of saliva substitutes with human saliva. Five kinds of saliva substitutes such as Moi-Stir, Stoppers4, MouthKote, Saliva Orthana, and SNU were used. Lysozyme activity was determined by the turbidimetric method. Peroxidase activity was determined with an NbsSCN assay. α-amylase activity was determined using a chromogenic substrate, 2-chloro-p-nitrophenol linked with maltotriose. The pH values of saliva substitutes were measured and their viscosity values were measured with a cone-and-plate digital viscometer at six different shear rates. Various types of saliva substitutes affected the activities of salivary enzymes in different ways. Stoppers4 enhanced the enzymatic activities of HEWL, bLP, and α-amylase. Saliva Orthana and SNU inhibited bLP activity and enhanced α-amylase activity. MouthKote inhibited α-amylase activity. Moi-Stir inhibited the enzymatic activities of bLP and α-amylase. The pH values were very different according to the types of saliva substitutes. Stoppers4, MouthKote, and Saliva Orthana showed lower values of viscosity at low shear rates and higher values of viscosity at high shear rates compared with unstimulated and stimulated whole saliva. Moi-Stir and SNU displayed much higher values of viscosity than those of natural whole saliva. Collectively, our results indicate that each saliva substitute has its own biological and rheological characteristics. Each saliva substitute affects the enzymatic activity of salivary enzyme and finally oral health in different ways.

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국문요약

타액대체제가 타액 효소 활성에 미치는 영향

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타액의 보호작용은 주로 타액 당단백질의 생물학적, 물리적, 구조적 성질 및 유동학적 성질과 관련이 있다. 그러므로 이상적인 타액 대체제의 개발을 위해서는 인체 타액의 생물학적 성질 뿐만 아니라 유동학적 특성을 이해하여야 한다. 본 연구의 목적은 타액 대체제가 인체 타액에 존재하는 효소의 활성에 미치는 영향을 파악하고 다양한 타액 대체제의 점도와 인체 타액의 점도를 비교하기 위해서 시행되었다. Moi-Stir, Stoppers4, MouthKote, Saliva Orthana 및 서울대학교치과병원 타액 대체제(SNU)를 사용하였으며, lysozyme 활성은 turbidimetric 법으로, peroxidase 활성은 NbsSCN 법으로, α-amylase 활성은 maltotriose와 결합된 2-chloro-p-nitrophenol를 사용하여 시행하였다. 타액 대체제의 pH를 측정하였으며 cone-and-plate 형태의 점도계를 이용하여 다양한 전단율에서 점도를 측정하였다. 본 연구에 사용된 다양한 타액 대체제는 타액 효소 활성에 각기 다른 영향을 미쳤다. Stoppers4는 hen egg-white lysozyme, bovine lactoperoxidase (bLP) 및 α-amylase 활성을 증가시켰고, Saliva Orthana와 SNU는 bLP 활성은 저해하였으며 α-amylase 활성은 증가시켰다. MouthKote는 α-amylase 활성을 저해하였으며, Moi-Stir는 bLP와 α-amylase 활성을 저해하였다. 타액 대체제의 pH는 타액 대체제의 종류에 따라 매우 달랐다. Stoppers4, MouthKote 및 Saliva Orthana는 낮은 전단율에서는 인체 타액보다 낮은 점도를 높은 전단율에서는 인체 타액보다 높은 점도를 나타내었다. Moi-Stir와 SNU는 인체 타액보다 매우 높은 점도를 나타내었다. 결론적으로 본 연구결과는 각각의 타액 대체제는 각기 다른 생물학적 기능과 유동학적 특성을 가지고 있음을 알 수 있다. 타액 대체제의 사용은 사용하는 타액 대체제의 종류에 따라 타액 효소 활성에 각기 다른 영향을 미치고 궁극적으로는 구강건강에 다른 영향을 미칠 수 있을 것이다.

주제어: 타액 대체제, Lysozyme, Peroxidase, Amylase, 타액