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Rheological Properties of Human Saliva and Salivary Mucins

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Abstract : It is unclear which factors in saliva contribute to its rheological properties such as viscosity and spinnbarkeit. In this study, we investigated relationships between the rheological properties (viscosity and spinnbarkeit) and two salivary mucin (MUC5B and MUC7) levels in saliva. Unstimulated (UWS) and chewing-stimulated (CWS) whole saliva were collected from healthy young adults, and the viscosities and spinnbarkeits were measured. The image densities of MUC5B and MUC7 bands in saliva were measured by Stains-All staining after electrophoresis. Both the viscosity and spinnbarkeit of UWS were significantly higher than those of CWS. The viscosities were correlated positively with the spinnbarkeit in UWS, but not in CWS. Image-density analysis of mucins demonstrated that the viscosities increased with the levels of MUC5B and the spinnbarkeit with the levels of MUC7. These results suggest that MUC5B contributes to viscosity and MUC7 to spinnbarkeit.

Introduction

Various studies have reported some relationships between two rheological properties of saliva, viscosity and spinnbarkeit, and oral diseases, such as dental caries and periodontitis. Elevated salivary viscosity is associated with an increase in dental caries¹⁾, and subjects with a higher spinnbarkeit exhibit a significantly greater extent and severity of periodontitis²⁾. The increases of viscosity of artificial saliva produced by mixing purified mucins from the gastric mucosa of pigs improve the condition of oral mucosa in dry-mouth patients^{3,4)}. An increase in the spinnbarkeit is frequently observed in xerostomia⁵⁾. These circum-

stances suggest that it is important to clarify in detail the rheological properties of saliva in order to more fully understand the mechanisms of oral diseases, and thereby assist in the treatment and prevention of oral diseases.

Viscosity means the quality or state of being viscous, while spinnbarkeit indicates the capability to draw a thread of a fluid. The correlation between viscosity and spinnbarkeit is complex. It has been reported that there is a positive correlation between the viscosity and spinnbarkeit in both unstimulated whole saliva (UWS) and nasal mucus^{6,7)}, while there is a report of a negative correlation in the sputum of patients with chronic bronchitis⁸⁾. These strongly suggest that the viscosity and spinnbarkeit are not always determined by the same parameters.

Saliva is composed of various components such as water, inorganic molecules, sugars, lipids, and protein.

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Among these, glycoproteins are thought to be the most closely involved in the rheological properties of saliva. Mucins are glycoproteins with a variety of subtypes, for example, MUC1-4, MUC5B, MUC5AC, and MUC6-8⁹). Human salivary glands secrete two types of mucin: higher molecular weight MUC5B (above 1 MDa) and lower molecular weight MUC7 (200–250 kDa)¹⁰). These two salivary mucins were previously termed MG1 and MG2, respectively. Nieuw Amerongen *et al.* have reported that salivary mucins comprise up to 26% of the total protein content of saliva¹¹). The viscosity of chewing-stimulated whole saliva (CWS) has been reported to be lower than that of UWS¹²), which indicates that the mucin level is higher in the latter^{13,14}). However, as far as we know, no report has demonstrated a direct correlation between the rheological properties and the levels of the two types of mucin in human saliva.

In the present study, we investigated the relationships between the rheological properties (viscosity and spinnbarkeit) and two salivary mucin (MUC5B and MUC7) levels in the saliva of young healthy adults. Since it has been reported that there are differences in the viscosity and levels of salivary mucins between UWS and CWS^{12–14}), both UWS and CWS were measured and their rheological properties were compared. Furthermore, since the flow rate and protein concentration have already been reported as salivary viscosity-related factors by Shannon¹⁵), the two salivary parameters were also measured to confirm the relationships. Additionally, as the protein concentrations were different between UWS and CWS, and the difference was due to the amylase levels in saliva, amylase activity was also measured.

Materials and Methods

The subjects were 51 healthy young adults (26 males and 25 females), chosen at random from under and post-graduate students at Kyushu Dental College. The mean age \pm SD of the subjects was 25.2 ± 3.1 years (20 to 32 years). The subjects were not taking drugs and did not have any diseases that might affect salivary secretion. The procedures were explained in full to all subjects and signed consent forms were

obtained prior to the start of the experiments. The study design was approved by the Institutional Review Board of Kyushu Dental College, Japan, in accordance with the Helsinki Declaration.

UWS and CWS were collected for 5 min by the spitting method at 14:00–18:00 on different days, described in our previous studies^{16–18}). To collect CWS, a 1-g piece of paraffin gum (HORIBA, Japan) was used. All subjects were instructed to swallow at time zero and spit out all saliva into the sample tube at the end of the collection. CWS was collected 5–10 min after the start of chewing, because it takes a few min to completely soften paraffin by chewing.

The viscosity and spinnbarkeit of saliva were measured just after the collection of saliva. The viscosity was measured with a round-vibration-type viscometer (Viscomate VM-10 A, CBC Materials Co., Ltd., Japan), the probe of which oscillates at 500 Hz, using a sample volume of 1 mL in a purpose-made plastic cup. The viscosity of distilled water was 0.93 mPa·s. In 11 of 51 UWS samples, because the volumes collected were extremely small and/or often markedly foamy, the viscosities could not be measured. The spinnbarkeit was measured with the Neva Meter (IMI-021, Ishikawa Ironworks Co., Ltd., Japan) as reported previously⁶). The device detects the point at which the electrical resistance of liquids produced by the application of a constant voltage reaches infinity when the liquid thread is severed. The stretching rate was set as 5 mm/s, and the sample volume was 50 μ L. For spinnbarkeit values, we selected the wet mode, because saliva always wets two contact surfaces of mucous membranes in the oral cavity. Measurements were automatically taken five times, and the spinnbarkeit was determined by calculating the averages of three values after excluding the highest and lowest values. Furthermore, other salivary parameters, for example, pH, Na⁺, and K⁺ concentrations, were measured just after collection. However, because such parameters did not show any correlation with rheological properties, these results were excluded from the present report. Purified mucin from the bovine submandibular gland (MP Biomedicals, Inc., France) was diluted with saline (0.1 to 1%) and the viscosity and spinnbarkeit were measured in

the same way as for human saliva. The measurements were performed three times.

The saliva samples were stored at -80°C for the following biochemical analyses, as described in our previous studies^{16,17}. After thawing and mild sonication, total protein concentrations were measured by Bradford's method, using bovine serum albumin as a standard. Amylase activities were measured using the Neo-Amylase Test (Daiichi Pure Chemicals Co., Japan).

To study the relationships of the two types of mucin with the viscosity and spinnbarkeit, the samples of UWS and CWS were classified into six groups on the basis of the viscosity and spinnbarkeit values. Forty of 51 UWS samples were analyzed for viscosity, all 51 were analyzed for spinnbarkeit, and all 51 UWS and CWS samples were analyzed for viscosity and spinnbarkeit. Samples in each group were mixed and centrifuged. The supernatants ($10\ \mu\text{L}$) were mixed 1 : 1 with SDS-PAGE sample loading buffer and 10% 2-mercaptoethanol and heated at 37°C for 30 min. They were layered onto polyacrylamide gels (Ready gels J, 10% separating gel and 5% stacking gel, Bio-Rad Co., Inc.) and run at 40 mA per gel for 1.5 h. The gels were fixed with 25% isopropanol solution, then stained with Stains-All (Sigma) solution (30 mM Tris/HCl (pH 8.8) containing 0.025% (w/v) Stains-All, 7.5% formamide, 25% isopropanol) overnight, and de-stained with H_2O ¹⁹. From the known molecular sizes of MUC5B (above 1 MDa) and MUC7 (200–250 kDa)¹⁰, the two mucins were identified. MUC5B bands were located near loading sites in the gel because of the very large molecular size. To standardize the staining densities of mucin bands between different gels, a UWS sample was used as a control in all electrophoreses. Image densities of MUC5B and MUC7 in each gel were calculated by analysis software, Scion Image (Scion Corporation). The averaged image densities of MUC5B and MUC7 of UWS in 51 subjects were defined as 100%, and the MUC5B and MUC7 levels of each sample were expressed as a percent ratio.

The significance of differences between UWS and CWS was assessed using Student's *t*-test and Pearson's correlation coefficient (*r*). Relationships

yielding *p*-values less than 0.05 were considered significant. All values are expressed as the mean \pm SD, and *n* represents the number of subjects.

Results

Table 1 shows salivary parameters of UWS and CWS in the subjects. For UWS, the number of samples was limited to 40 for viscosity measurement. The viscosities and spinnbarkeits of UWS were significantly higher than those of CWS. The flow rates of UWS were significantly lower than those of CWS. The protein concentrations of UWS and CWS did not differ significantly, but amylase activities of UWS were significantly lower than those of CWS. These results suggest that although there is no difference in the total protein concentration between UWS and CWS, there is some variation in their protein components.

Table 2 shows the correlation coefficients between salivary parameters. The viscosity was correlated positively with the spinnbarkeit in UWS, but not in CWS. Furthermore, the viscosities were positively correlated with the total protein concentrations in both UWS and CWS. Additionally, viscosity was correlated negatively with the flow rates in CWS, but showed no correlation with the flow rate in UWS. Spinnbarkeits of both UWS and CWS did not correlate with the total protein concentrations and flow rates. Thus, the correlations of the two rheological properties with the other salivary parameters tested were not identical. These results indicate that the parameters of viscosity and spinnbarkeit in saliva are influenced by different factors. Then, we investigated the relationships between the rheological properties of saliva and the two types of salivary mucin.

The relationships between the volumes of mucin subtypes (MUC5B and MUC7) and viscosity or spinnbarkeit are shown in Fig. 1. The upper panels in Fig. 1 show the image densities of MUC5B and MUC7 bands. When the averaged image densities of MUC5B and MUC7 bands of UWS in the 51 subjects were regarded as 100%, those of UWS in 40 subjects, within which the viscosity could be measured, were slightly decreased to 92% and 93%, respectively.

Table 1 Salivary parameters of unstimulated and stimulated whole saliva

	n = 51		n = 40 ^{a)}	
	UWS	CWS	UWS	CWS
Viscosity (mPa·s)		1.06 ± 0.12	1.09 ± 0.11	1.05 ± 0.08*
Spinnbarkeit (mm)	2.31 ± 0.41	1.72 ± 0.26**	2.2 ± 0.39	1.73 ± 0.23**
Flow rate (mL/min)	0.43 ± 0.23	1.71 ± 0.87**	0.49 ± 0.22	1.90 ± 0.85**
Protein (mg/mL)	0.71 ± 0.35	0.66 ± 0.28	0.70 ± 0.30	0.63 ± 0.23
Amylase (IU/mL)	331 ± 221	520 ± 249**	325 ± 199	538 ± 241**

Values for all salivary parameters are expressed as the mean and standard deviation.

UWS : Unstimulated whole saliva for 0—5 min.

CWS : Chewing-stimulated whole saliva for 5—10 min.

^{a)} Viscosities of UWS were not measured in 11 of 51 subjects.

*p < 0.05 ; **p < 0.001 as compared with UWS.

Table 2 Correlation of each salivary parameter

	Viscosity		Spinnbarkeit	
	UWS (n = 40)	CWS (n = 51)	UWS (n = 51)	CWS (n = 51)
Spinnbarkeit	0.47*	0.25		
Flow rate	0.22	-0.48**	0.05	-0.03
Protein	0.50**	0.54**	0.12	0.04
Amylase	0.31	0.15	-0.05	-0.12

Table shows Pearson's correlation coefficients.

*p < 0.01, **p < 0.001.

CWS samples were also grouped using the two parameters of viscosity and spinnbarkeit. Two averaged image densities of the two kinds of mucin in CWS showed the same values (MUC5B : 84%, and MUC7 : 28% in grouping with viscosity and spinnbarkeit), indicating the reliability of analysis. The results suggest that MUC5B and MUC7 of CWS are at lower levels than in UWS. The lower panels of Fig. 1 show their relative percentage against averaged values of viscosity and spinnbarkeit. The levels of MUC5B increased linearly with viscosity in both UWS and CWS (Fig. 1A), but those of MUC7 did not (Fig. 1B). Although the levels of MUC5B seemed to increase with spinnbarkeit, the elevated rates in UWS and CWS were not consistent (Fig. 1C). On the other hand, the level of MUC7 increased with spinnbarkeit, and the elevated rates in UWS and CWS were almost the same (Fig. 1D). These results suggest that the

levels of MUC5B and MUC7 in human saliva correlate with viscosity and spinnbarkeit. We also investigated the relationships between the mucin levels and total protein concentrations. Both in UWS and CWS, the level of MUC5B increased in correlation with the total protein concentrations, but the level of MUC7 did not (data not shown).

To demonstrate the hypothesis that MUC5B and MUC7 are related to viscosity and spinnbarkeit, we finally investigated how purified mucin from the bovine submandibular gland affects viscosity and spinnbarkeit. The viscosity of purified mucin increased with the concentration in a dose-dependent manner (Fig. 2A). On the other hand, the spinnbarkeit showed no appreciable change with mucin concentration (Fig. 2A). The components of purified mucin were investigated with Stains-All staining and compared with those of a UWS sample. The purified

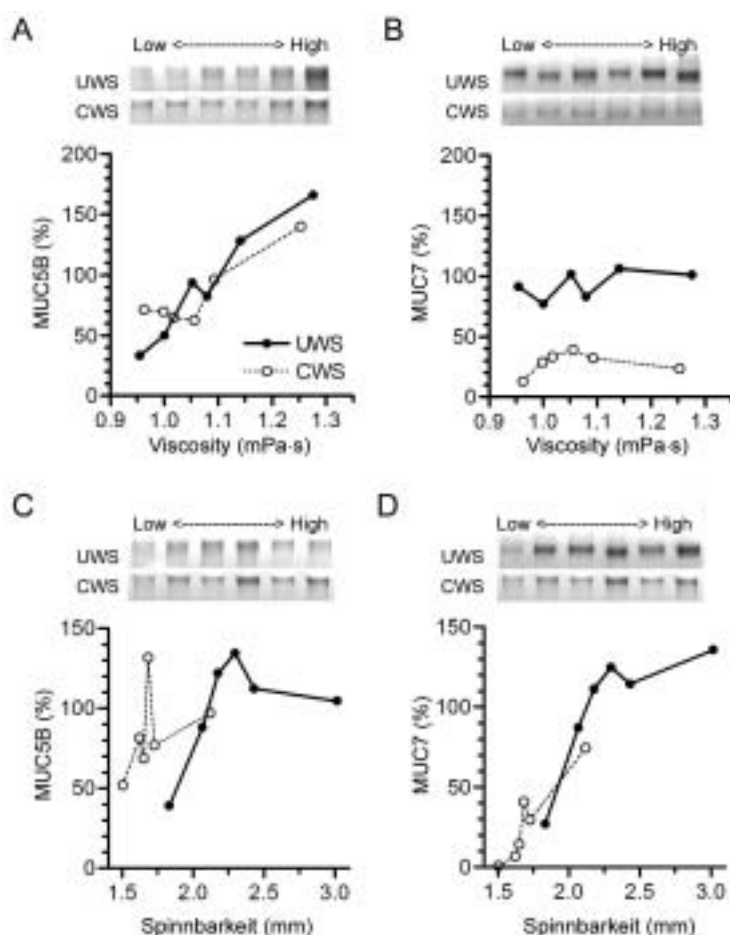


Fig. 1 MUC5B and MUC7 levels in samples of UWS and CWS

The samples were grouped into six groups ($n=5-10$ in each group, $10 \mu\text{L}$ of each sample was mixed) on the basis of similar values for rheological properties. The upper panels show the electrophoretic patterns of MUC5B (A and C) and MUC7 (B and D) in the samples of the six groups by Stains-All staining. The lower panel shows a plot of the image densities of MUC5B (A and C) and MUC7 (B and D) in UWS (closed circles) and CWS (open circles), against the average values of the viscosities (A and B) and spinnbarkeits (C and D) of saliva samples in each group.

mucin contained only MUC5B and not MUC7 (Fig. 2B).

Discussion

The present study demonstrated that viscosity and spinnbarkeit in UWS were higher than those in CWS (Table 1), and there was a positive correlation between the rheological properties in UWS (Table 2). The latter is consistent with observations by another laboratory in our college⁶. Similar observations have

been reported in the nasal mucus⁷. On the other hand, a negative correlation between viscosity and spinnbarkeit has also been identified in the sputum⁸. Even in the present study, there was no observed correlation between viscosity and spinnbarkeit in CWS. These results indicate that viscosity and spinnbarkeit are different rheological properties. The present study also demonstrated that viscosity and spinnbarkeit rose with increasing levels of MUC5B and MUC7, respectively (Fig. 1). From these, it is suggested that MUC5B may contribute to viscosity, and

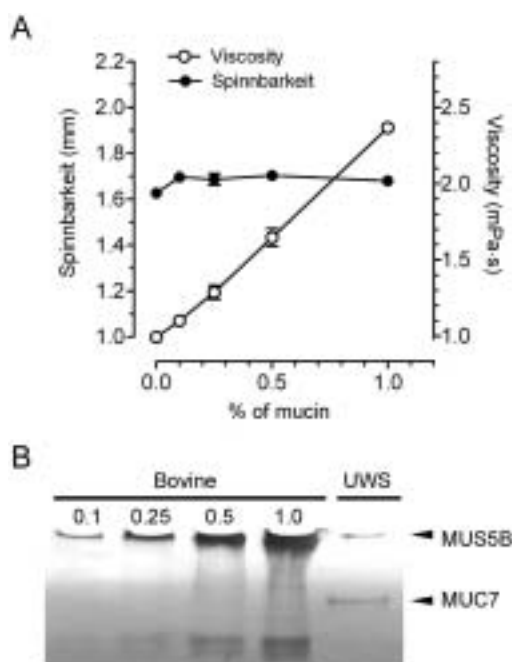


Fig. 2 Effects of purified mucin on rheological properties

(A) The relationships of viscosity (open circles) and spinnbarkeit (closed circles) with purified mucin from the bovine submandibular gland. Values represent means \pm SD of three experiments. (B) The electrophoretic patterns of mucin-containing solutions and UWS samples by Stains-All staining.

MUC7 to spinnbarkeit in human saliva. This hypothesis was further supported by the present experiment using purified bovine submandibular mucin (Fig. 2).

As above-mentioned, the present study did not identify any correlation between viscosity and spinnbarkeit in CWS, while there was a strong correlation between them in UWS (Table 2). Although we have no clear explanation for this at present, one reason may be the change in the ratio between MUC5B and MUC7 between stimulated and unstimulated conditions. The flow rate increased approximately four times from 0.43 mL/min in UWS to 1.71 mL/min in CWS, as shown in Table 1. This leads to a reduction of the levels of MUC7 in CWS to about one quarter of those in UWS (from 100% to 28%, as shown in Results). It is likely that such a marked reduction in

MUC7 levels in CWS is due to dilution caused by chewing-stimulated salivary production. It has been reported that MUC7 levels in submandibular/sublingual saliva remain unchanged on gustatory stimulation by fruit-flavoured candies²⁰. From these results, it is suggested that MUC7 is secreted at a constant rate in both chewing-stimulated and unstimulated conditions.

In the present study, the viscosity of UWS from 11 subjects, who showed slow salivary flow rates, sometimes with foamed saliva, were not measured. As a result, no correlation between the viscosity and flow rate was detected in UWS in 40 subjects, while a negative correlation was detected in CWS in 51 subjects (Table 2). As mentioned in Results, the averaged levels of MUC5B and MUC7 in the UWS of 40 subjects were lower than those in 51 subjects. This implies that saliva samples from the 11 subjects had relatively higher mucin levels, and, therefore, a higher viscosity, as expected.

The total protein concentrations in both UWS and CWS showed positive correlations with the viscosity (Table 2). Similarly, Shannon reported that the viscosities of UWS correlated positively with the total protein levels¹⁵. On the other hand, the present study also demonstrated that amylase activity did not show any correlation with viscosity. Further, there was no significant difference in the total protein concentration between UWS and CWS, and the amylase activity of CWS was significantly higher than that of UWS (Table 2). These results indicate that the relative concentrations of other proteins, except amylase, are more decreased in CWS than UWS. In fact, the level of MUC5B was decreased to 84% in CWS, compared to that in UWS (as shown in Results). Hence, the positive correlations between the total protein concentration and viscosity are probably secondary effects due to MUC5B in both UWS and CWS. In addition, the absence of a correlation between the total protein concentration and spinnbarkeit may also be reflected by the lack of a correlation between the total protein concentration and level of MUC7. Further, it should be noted that the level of MUC7 may reach a maximum at a spinnbarkeit of more than 2.25 (Fig. 1D). Thus, the increase in the spinnbarkeit

beyond 2.25 may depend on some other factors.

In summary, MUC5B contributes to viscosity and MUC7 to spinnbarkeit in human saliva. Increasing the viscosity by adding purified mucins to artificial saliva will improve the symptoms of dry-mouth patients^{3,4}. For oral health, MUC5B and MUC7 are not only related to rheological, but also biochemical properties. It has been reported that MUC5B exhibits a higher affinity for hydroxyapatite²¹) and that the MUC7-sIgA complex binds to mucosal pathogens²²). Therefore, measurements of both the viscosity and spinnbarkeit of saliva may be effective methods to assess mucin-related oral defenses.

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