ORIGINAL

Effect of saliva collection method on the concentration of protein components in saliva

Fumiko Michishige[1], Kyoko Kanno[1], Sumiko Yoshinaga[1], Daisuke Hinode[2], Yozo Takehisa[3], and Susumu Yasuoka[3]

[1]*Department of Fundamental Nursing, School of Health Sciences, The University of Tokushima, Tokushima, Japan ;* [2]*Department of Preventive Dentistry, Institute of Health Biosciences , The University of Tokushima Graduate School, Tokushima,Japan ; and* [3]*Hakuai Kinen Hospital, Tokushima, Japan*

Abstract : In order to clarify how we collect saliva for analyzing salivary protein in aged subjects who can not eat well, we compared the effects of suction, spitting and the swab saliva collection method on the yield of protein components in saliva samples from normal volunteers. The saliva collected by suction, spitting and the swab method were designated as, Saliva I, II and III, respectively.

The saliva volume collected by Saliva I was about 2-fold greater than that by of Saliva II and III. This is mainly due to the fact that saliva secretion was stimulated by the suction itself. The content of total protein, S-IgA, trypsin-like activity and human airway trypsin-like protease (HAT) were almost the same in Saliva I and II, and significantly lower in Saliva III than in Saliva I and II. Kallikrein activity was almost the same in Saliva I, II and III. The concentration of each total protein, S-IgA, kallikrein activity, trypsin activity and HAT in Saliva I were significantly positively correlated with that in Saliva II.

These results indicate that we can obtain information of change of salivary protein by analyzing saliva collected by suction method, although this method caused the stimulation of saliva to some extent. J. Med. Invest. 53 : 140-146, February, 2006

Keywords **:** *saliva, S-IgA, kallikrein, trypsin-like enzyme, saliva collection*

INTRODUCTION

It is well known that the measurement of the flow rate of saliva (1-4) and the contents of various proteins in saliva (5-12) are useful for clarifying the physiological functions of saliva and also useful for estimating the pathophysiological state of oral diseases and systemic diseases (13-17).

Saliva is involved in the defense mechanism of oral cavity by various mechanisms (18). Of the salivary components, many proteins are related to local host defense. We are aiming to develop an effective oral care method for preventing the occurrence of aspiration pneumonia and oral infection in aged subjects who can not eat well due to dysphagia, dementia and consciousness disturbance, without lowering the biological anti-infectious activity of saliva. In Japan, a considerable part of these patients are receiving nutrients and fluids via a naso-gastric tube or percutaneous gastrostomy tube. We considered that it is important to clarify the characteristics of change of the salivary protein profile in these patients, in order to perform oral care suitable for each subjects.

Spitting, suction and swab (adsorbent) method are

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Address correspondence and reprint requests to Fumiko Michishige, Department of Fundamentel Nursing, School of Health Sciences, The University of Tokushima, Kuramoto-cho, Tokushima 770-8509, Japan and Fax : +81-88-633-9038.

reported as methods for collecting whole saliva (19). Navazesh summarized that of these methods, the spitting method is recommended for both unstimulated and stimulated whole saliva collection, but the suction and swab method introduced some degree of stimulation, and thus are not recommended for unstimulated whole saliva collection. However, most of our subjects could not spit saliva well. Therefore, we needed to collect saliva from these subjects by the suction method or swab method.

Human airway trypsin-like protease (HAT) was found in the human sputum (20). Recent reports suggested that HAT is involved in the local defence of airway (21-23). Recently, we found that saliva contains HAT (unpublished).

From these backgrounds, in the present work we compared the concentrations of total protein and specific protein components (S -IgA, kallikrein, trypsin-like protease and HAT) in the saliva samples obtained by the three saliva collection methods in healthy subjects, in order to clarify the difference of the effect of these saliva collection methods on the yield of proteins contained in saliva, and thus to clarify which is a suitable method as a saliva collection method to analyze the characteristics of change of the salivary protein profile in the patients with various diseases.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant HAT (rHAT) and two different rabbit polyclonal antibodies against rHAT were supplied by the Teijin Institute for Bio-Medical Research (Tokyo, Japan). Streptavidin-horse radish peroxidase conjugate was purchased from Biosource (Camarillo, CA). Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO, USA). Boc-Phe-Ser-Arg- 4-methyl-coumaryl-7-amide (MCA) and Pro-Phe-Arg-MCA were obtained from the Peptide Institute (Osaka, Japan). Peroxidase-conjugated rabbit anti-human secretory component antibody, goat affinity purified antibody to human IgA (α chain) and purified human secretory IgA from colostrums were obtained from Dako (Glostrup, Denmark), Cappel TM (Durham NC) and ICN Pharmaceuticals (Aurora,OH), respectively.

Subjects

Saliva samples were obtained from 10 nonsmoker female volunteers aged 20-22 years old.

Before collecting the saliva, written informed consent was obtained from all subjects after a full explanation of the procedures involved. The experimental procedure was approved by the Ethics Committee of Tokushima University Hospital.

Collection of samples

Collection of whole saliva samples were performed under resting conditions in a quiet room during the afternoon, between 14:00 and 15:00, at least 1 h after eating. Saliva samples were collected from each subject by following three different methods.

Method I, suction method : Saliva was collected by suction using a saliva aspiration set, which consisted of an aspiration catheter, a trap (Type P, 10Fr, Termo, Tokyo) and a low-pressure continuous aspirator (SeaStar Corporation, Kyoto). Saliva was collected via the catheter, the tip of which was inserted into the floor of mouth, by gentle continuous suction for 5 min. Saliva flow rate (ml/min) was estimated by measuring the volume of saliva collected.

Method II, the spitting method or direct expectoration method : Subjects were asked to collect saliva in their mouths and to spit it into a sterile plastic dish with a diameter of 6 cm for 5 min, according to the method of Nagler and Hershkovich (12).

Method III, Swab method using the Salivette® collection kits (Sarstedt, Numbrechet, Germany) (4, 7, 10): A neutral, non-covered cotton roll was placed under the tongue of each subject for exactly 5 min, the subject being asked not to swallow saliva in the period. Then the roll was returned to a Salivette, and the Salivette was centrifuged at 5000 g for 5 min at 4°C. The resulting aliquots were pipetted into analytical test tubes.

We obtained saliva samples by the above three methods from each subject, in order of Method I, II and III, in an interval of 15 min between each method.

Saliva samples were kept in a deep freezer at -80°C until use.

Measurement of total protein

The protein concentration was measured according to the method of Lowry *et al.* (24) with BSA as a standard.

Assay of protease activity

Trypsin-like activity was measured according to the method of Yasuoka *et al.* (20). Briefly, the assay mixture (1.5ml), containing 50 mM Tris-HCl (pH8.6), Boc-Phe-Ser-Arg-MCA (100μM, BSA at 100μg/ml) and 100μl of the test sample, was incubated at 37°C for 60 min, and the reaction was stopped by the addition of 1 ml of 30% acetic acid. The fluorescence intensity of the released aminomethyl-coumarin (AMC)

was measured with a fluorescence spectrophotometer (F-3010 Hitachi Co, Japan) at 440 nm with excitation at 380 nm. The amount of AMC released was calculated from a standard curve. One unit of enzyme was defined as the amount that produced 1 μmole of AMC per min.

Kallikrein activity was also measured by the fluorometry described above with Pro-Phe-Arg-MCA as a substrate. Saliva samples were mixed with 9 volumes of physiological saline and homogenized using an IKA homogenizer (IKA-Werke, Ultra-Turrax T8) for 1 min in ice-bath. Trypsin-like and kallikrein activity were measured using 100μl and 20μl, respectively, of the whole homogenate as an enzyme solution.

Enzyme-linked immunosorbent assay (ELISA) for HAT

Coating of a 96-well plate for ELISA (Microlon, high binding,Greiner bio-one, Frickenhausen, Germany) with primary antibody was carried out at 4°C overnight, using a rabbit polyclonal anti - rHAT antibody. Blocking of the plate with 1 % BSA was then carried out at room temperature for 3 hours. The plate, which contained 50μl each of test sample and secondary biotinylated rabbit polyclonal anti-rHAT antibody solution per well, was left to stand at 4°C overnight. Then, the plate that contained 100μl of a streptavidin-horse radish peroxidase conjugate solution per well, was incubated at room temperature for 1 hour. Finally, the plate that contains 100μl of 0.023% tetramethylbenzidine dihydrochloride dihydrate(TMBZ•HCl)/2.5mM H_2O_2, was incubated at room temperature for 30 min. The reaction was terminated with 100μl of 1 N H_2SO_4 per well, and the absorption at 450 nm was measured using a microplate reader (Model 550, Bio-Rad).

The test samples were diluted 100-400 fold with PBS/2 % Triton X-100/0.6 % SDS.

Measurement of S-IgA

The S-IgA content was measured by a sandwich ELISA with a minor modification of the method of Takizawa *et al.* (25).In this ELISA, a goat anti-human IgA (α-chain) antibody and peroxidase-conjugated rabbit anti-human secretory component antibody were used as the primary antibody and secondary antibody, respectively. Human S-IgA from human colostrums was used as a standard.

Statistical analysis

Data represent the mean ±SD. The significance of differences between flow rates and protein concentrations in the saliva samples collected by each saliva collection method was tested by the Fisher's protected least significant difference. Statistical significance was established at the $p<0.05$ level. The significance of correlation between protein concentrations or protein contents in the saliva samples by each collection method was tested using Pearson's correlation coefficient. Statistical analysis was done by Stat View 5.0 (SAS Institute, Inc., USA).

RESULTS

Volume of saliva

The mean saliva volume (ml/min) collected by the suction method (0.79±0.28) was significantly (about 2-fold) larger ($p<0.01$) than that by the spitting method (0.40±0.19) or the swab method (0.38±0.18). The mean saliva volume collected by the spitting method was almost the same as that in the cotton roll method.

Contents of total protein, S-IgA, kallikrein activity, trypsin-like activity and HAT in the saliva samples collected by different methods (Table 1)

The saliva samples collected by the suction, spitting and swab method, were named Saliva I, Saliva II and Saliva III, respectively.

In the Saliva I, the mean values of total protein concentration (μg/ml), S -IgA concentration (μg/ml), kallikrein activity (mU/ml), trypsin -like activity (mU/ml) and HAT concentration (ng/ml) are 2505±956, 89.7±33.1, 58.5±30.3, 12.6±7.0 and 47.5±35.2, respectively.

As shown in Table 1, each mean value of the total protein concentration, S-IgA concentration, trypsin-like activity and HAT concentration were not significantly different between Saliva I and II, but were significantly lower in the Saliva III compared with Saliva I or Saliva II.

The HAT concentration in the individual saliva samples collected by three different methods are shown in Fig.1.

In Saliva I, the HAT concentration was significantly positively correlated with the trypsin-like activity ($r=0.94$, $p=<0.0001$). The HAT concentration was correlated with the trypsin-like activity in Saliva II ($r=0.57$, $p=0.084$) and Saliva III ($r=0.61$, $p=0.059$) (Fig. 2).

In contrast with the other proteins tested, the mean value of the kallikrein activity was not significantly different among the saliva samples I, II and III (Table 1).

The mean ratios of each protein component content in Saliva III to that in Saliva I were 0.45 in HAT concentration, 0.36 in trypsin-like activity, 0.70 in S-IgA, 0.76 in total protein, and 1.08 in the kallikrein activity,

Table 1. Contents of total protein, S-IgA, kallikrein activity, trypsin-like activity and HAT in the saliva samples collected by suction, spitting and swab methods

	unit	Suction method (Saliva Ⅰ)	Spitting Method (Saliva Ⅱ)	Swab method (Saliva Ⅲ)	P		
					Ⅰ,Ⅱ A	Ⅰ,Ⅲ B	Ⅱ,Ⅲ C
Total protein	μg/ml	2505±956	2464±422	1868±557	0.895	0.047*	0.619
S-IgA	μg/ml	89.7±33.1	84.6±22.9	59.0±20.3	0.661	0.013*	0.037*
Kallikrein activity	mU/ml	58.5±30.3	69.6±38.2	60.8±36.1	0.496	0.884	0.591
Trypsin-like activity	mU/ml	12.6±7.0	11.0±5.0	4.1±2.1	0.503	0.001**	0.005**
HAT	ng/ml	47.5±35.2	33.1±14.8	16.7±7.9	0.163	0.004**	0.114

Values are means ±SD * p<0.05 ** p<0.01
The saliva samlples collected by suction, spitting and swab method, were designated as saliva Ⅰ, Ⅱ and Ⅲ, respectively.
A : Saliva Ⅰ versus Saliva Ⅱ, B : Saliva Ⅰ versus Saliva Ⅲ, C : Saliva Ⅱ versus Saliva Ⅲ

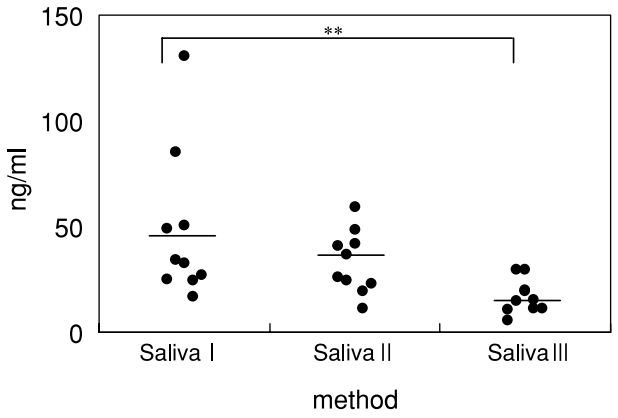
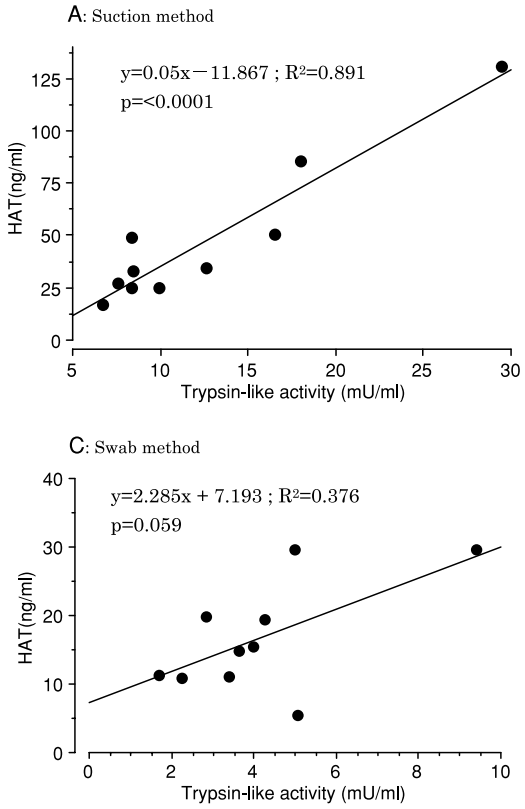


Fig. 1. HAT concentrations of the saliva samples collected by three different methods
The saliva samples collected by the suction, spitting and swab method, were designated Saliva Ⅰ, Ⅱ and Ⅲ, respectively. Horizontal lines show mean values. **p<0.01



as shown in Fig 3. The ratios of of each protein component content in the Saliva Ⅲ to that in Saliva Ⅱ were similar to the Ⅲ/Ⅰ ratios.

Relationship between contents of each protein component in the Saliva Ⅰ and Ⅱ

As shown in Table 2, each correlation coefficient (r) between each content of total protein, S-IgA, kallikrein activity, trypsin activity and HAT in Saliva Ⅰ and Ⅱ, were 0.71, 0.72, 0.79, 0.63 and 0.79, respectively, and thus each content of total protein, S-IgA, kallikrein activity, trypsin activity and HAT in Saliva Ⅰ were significantly positively correlated with that in Saliva Ⅱ.

DISCUSSION

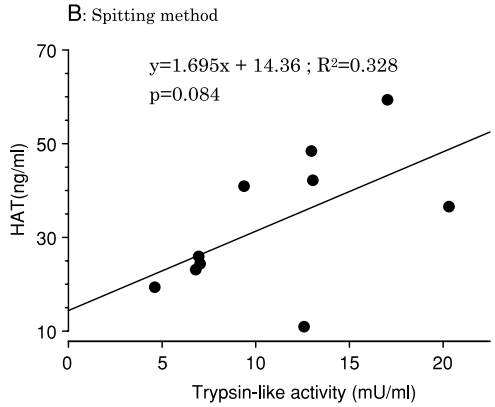


Fig. 2. Relationship between the trypsin-like activity and HAT concentration in the saliva samples collected by suction, spitting and the swab method
The trypsin-like activity was measured by a fluorometric method with Boc-Phe-Ser-Arg-MCA as a substrate, and the HAT content was measured with an ELISA. Circles ; individual data, n=10

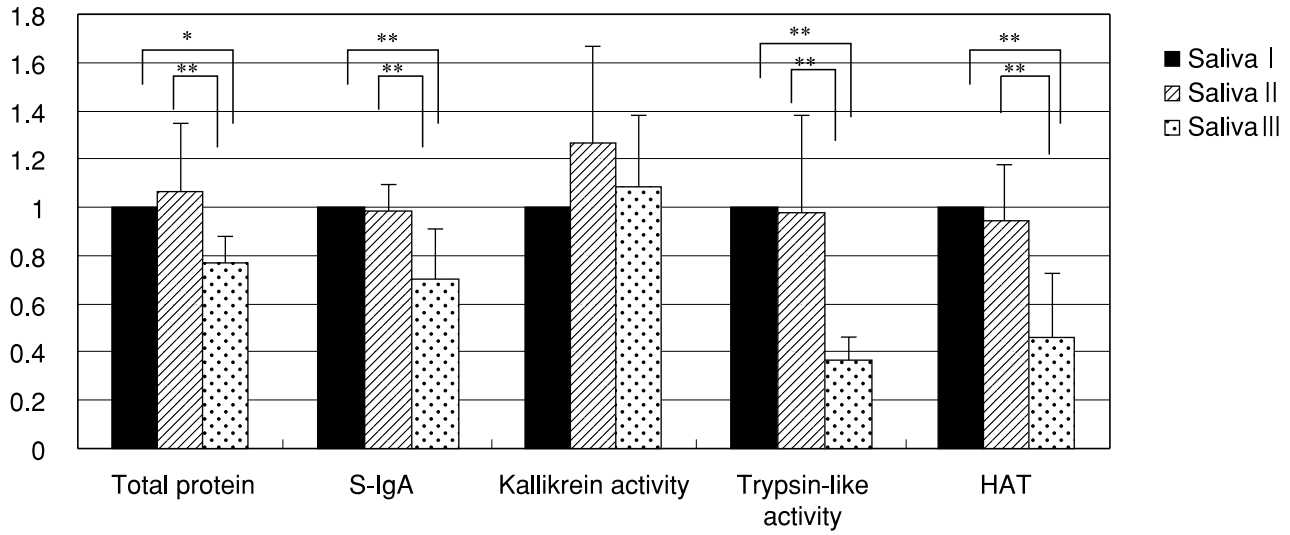


Fig. 3. The ratios of each protein content of Saliva II and III to that of Saliva I
Bars show mean values, and horizontal lines SD. * $p<0.05$ ** $p<0.01$

Table 2. Correlation of content of total protein, S-IgA, kallikrein activity, trypsin-like activity and HAT between Saliva I and Saliva II

Protein	Correlation coefficient	p
Total protein	0.71*	0.032
S-IgA	0.72*	0.015
Kallikrein activity	0.79**	0.003
Trypsin-like activity	0.63*	0.045
HAT	0.79**	0.004

Significance of correlation between two saliva samples was tested by Pearson's correlation coefficient. * $p<0.05$ ** $p<0.01$

In the present study, we selected S-IgA, kallikrein, and trypsin-like protease as salivary proteins, in addition of total protein level. S-IgA is one of major proteins in the saliva, and major immunoglobulin related local humoral immunity of mucous membrane (26). Kallikrein is known to be present in relatively high levels in salivary glands (27-28), and its activity in the saliva are often measured to examine the phathophysiological states of oral and systemic diseases (14,15,17). There are few reports that analyze the trypsin-like activities in saliva. As described in the Introduction, HAT is a novel trypsin-like protease found in the human airways (20), and we found that saliva samples from normal volunteers contain HAT, and that the trypsin-like activity in the saliva samples is mainly due to HAT. Moreover, the immunohistochemical analysis showed that the HAT protein was found specifically in the ducts of human salivary glands, indicating that HAT in the saliva is derived from salivary glands (umpublished data).

In the analysis of the saliva samples of normal subjects, the saliva volume collected using the suction method was about 2-fold greater than that of the spitting method and swab method. This is mainly due to the fact that saliva secretion was stimulated by the suction itself. Navazesh and Christensen reported that the suction methods introduced some degree of stimulation of saliva production (29).

In the present study, the total protein contents and each protein component in the saliva samples were expressed as the concentration per ml. Using gel filtration and ELISA for HAT, we have found that the trypsin-like activity in the saliva samples of normal volunteers is mainly due to HAT (unpublished data). In the present study, the trypsin-like activity was positively correlated with the HAT concentration in all of Saliva I, II and III. This result supports the above-described results.

As shown Table 1, the content of each S-IgA, trypsin-like activity and HAT were almost the same in Saliva I and II, and significantly lower in Saliva III than in Saliva I and II. The degree of difference between Saliva I or II and Saliva III was different in each protein component, the difference being the most prominent in the trypsin-like activity and HAT concentration ; the contents of trypsin-like activity and HAT in Saliva III were about half of those in Saliva I and II, while the S-IgA concentration of Saliva III was about 70 % of those of Saliva I and II. Most likely, the lower content of certain proteins in Saliva III than in Saliva I and II is thought to be caused by the following two mechanisms in the swab method : 1) The cotton roll adsorbs some parts of these proteins when it is placed in the mouth, and the degree of adsorption of proteins by the cotton roll differs between each protein. 2) Some parts of these proteins in Saliva III were precipitated when the cotton

roll was centrifuged in the Salivette at 5000 g for 5 min at 4°C. Both the degree of adsorption of proteins by the cotton roll and that of the precipitation of proteins are thought to differ between each protein.

To contrast to the trypsin-like activity and HAT, the kallikrein activity was not significantly different among the saliva samples I, II and III. This is considered to be mainly due to the fact that the loss of salivary kallikrein by the above-described two mechanisms is very slight.

The present results of the analysis of the saliva samples of normal subjects indicated that the yield of salivary proteins is lower and more variable in the saliva samples collected by the swab method than in those by the spitting method and suction method, supporting the idea that the swab method was found to be the least reliable of the known saliva collection methods (19).

If all of the subjects whose saliva samples we are aiming to analyze, can spit saliva well, it is thought that it is better to collect their unstimulated saliva samples using the spitting method, because the stimulation of saliva secretion is more slight in the spitting method than in the suction method. As described in the Introduction, we are planning to clarify the characteristics of salivary protein change of the aged subjects who can not eat well due to various kinds of diseases. In most of these subjects, we can not collect saliva using the spitting method, because most of these subjects can not spit saliva well. Accordingly, we need to collect saliva from these subjects using the suction method or swab method. However, many of these subjects can not keep well the cotton swab, and the swab method is not reliable as described above. Ultimately, we have to collect saliva using the suction method from these subjects.

The concentration of each total protein, S -IgA, kallikrein activity, trypsin activity and HAT in Saliva I were significantly positively correlated to that in Saliva II. These results indicate that we can obtain information of the change of salivary protein by analyzing the saliva samples collected using the suction method, as we can by analyzing the saliva samples collected using the spitting method, although the former method caused some extent of stimulation of saliva during its collection.

Finally, these results indicated that the analysis of proteins in saliva samples collected by the suction method is useful to obtain information of changes of salivary protein profiles in oral and systemic diseases of the aged subjects who can not eat or spit saliva well.

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