

⟨Brief Note⟩

Validation of human salivary proteins affected by normal diet

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Summary Eating is important for maintaining physical and mental health and necessarily involves the production of saliva, which is a requirement for taste. Changes in the composition of saliva that relate to the satisfaction produced by eating may serve as objective indicators for the development of medical food products. Therefore, we conducted a study on the possibility of using the stimulus of eating food to change the components of secreted saliva. Under moderately hungry conditions when they were going to eat their regular lunch meal, 12 female nursing students in their twenties were asked to eat a popular type of food that is generally considered to be delicious, and saliva was collected at three time points: (i) prior to the arrival of the food, (ii) during visual and olfactory stimulation by the food, and (iii) at six minutes after commencement of eating. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis, alterations in several salivary proteins were detected after the stimulation. Five salivary proteins, including alpha-amylase 1 precursor, immunoglobulin light chain, unknown protein, cystatin S precursor, and S100A8, were identified by matrix-assisted laser desorption time-of-flight mass spectrometry and are suggested as possible markers for the assessment of diet.

Key words: Medical food products, Saliva, SDS-PAGE, Salivary proteins, Mass spectrometry

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1. Introduction

Saliva has attracted attention as a secretion that can be noninvasively sampled from humans. It is made up mostly of water but is also known to contain minerals and various types of proteins^{1,2}. Additionally, the proteins contained in saliva have been reported to vary depending on whether the saliva secretion is stimulated by sweetness, umami, bitterness, or acidity³.

We have previously studied the relationship between nationality and differences in the perception of taste⁴. In that study, Japanese and Sri Lankan students ingested the same foods and were asked questions about their taste perceptions of sweetness, saltiness, sourness, bitterness, astringency, and deliciousness. Results of the subjective evaluation varied according to the composition of food and nation of origin. Indeed, individuals show different preferences for food and taste and levels of satisfaction from eating the same food. In order to maintain a healthy body, a diet with tastes that are suitable for each individual is important. However, little is known about the evaluation of food products that can be expected to have such effects. Therefore, we conducted a study on the association of food stimulation with the changes in the protein composition of saliva. Identification of salivary protein markers that are affected by food stimulation may contribute to the development of medical food products in the future.

2. Materials and Methods

Food products

In the present study, the test food was “Yu-rinchii bento”, which is popular and commercially available in Japanese supermarkets and comprises chicken seasoned with sweet soy sauce and rice. This lunch was selected because in the previous study, all Japanese subjects evaluated the bento as delicious⁴.

Saliva collection

Under moderately hungry conditions when they were going to eat their regular lunch meal, 12 female nursing students in their twenties were asked to eat the bento. Samples of their saliva were collected at three time points: (i) before the meal, (ii) during visual and olfactory stimulation by the food, and (iii) at six minutes after the commencement of eating (Fig. 1). Each person’s oral cavity was rinsed with water prior to sample collection, followed by placement of an oral swab (Salimetrics Oral Swab; Salimetrics, Carlsbad, CA, USA) under the tongue for one minute. Then the swab was immediately placed in a storage tube and centrifuged at $1500 \times g$ for 15 minutes to collect the saliva. The weight of each saliva sample was measured with an electronic balance.

This study was approved by the Institutional Review Board for Clinical Research of Niigata University (#2016-2657). All participants provided written consent after receiving an explanation of the purpose, methods, potential risks, and benefits of the study.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

A sample buffer solution (2×) containing 2-mercaptoethanol (Cosmo Bio, Tokyo, Japan) was added to 15 μL of the collected saliva; this mixture was then incubated at 95 °C for five minutes. The samples were separated using 4 to 20% of precast protein gel (Mini-PROTEAN TGX gel; Bio-Rad, Hercules, CA, USA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer (0.025 mol/L Tris, 0.192 mol/L glycine, and 0.1% SDS) at a constant voltage of 200 V for 30 min

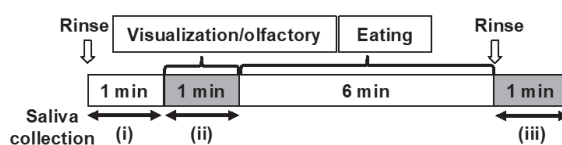


Fig. 1 Experimental scheme: (i) before food intake, (ii) during visualization and olfactory stimuli, and (iii) after commencement of eating. Rinse = oral rinse with water.

at room temperature. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 (Merck, Darmstadt, Germany). Each band was subjected to comparative analysis using ImageJ software⁵.

Matrix-assisted laser desorption time-of-flight mass spectrometry analysis

Each CBB-stained band was removed from the gel and analyzed at Genomine (Pohang, Republic of Korea). Peptide mass fingerprinting by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex LRF 20; Bruker, Massachusetts, USA) was performed at Genomine, according to the manufacturer's protocol. The mass spectrometry data was registered in the JPOST repository⁶ (accession number: JPST000688, PXD015944).

3. Results

The measured weights of the saliva samples for the 12 subjects (A–L) are summarized in Table 1.

The proteins contained in 10 μ L of saliva were separated by SDS-PAGE and comparative analysis of the protein fluctuations was performed (Fig. 2). In this experiment, the saliva collected before food stimulation was used as the control. The densities of the bands in the saliva were compared between the control samples and the samples after stimulation. In association with the visual and olfactory stimuli, the band with a molecular weight of about 50 kDa had increased intensity in the samples from subjects B, H, J, and L; and decreased intensity in the sample from subject K. However, the intensities of bands 1 to 6 increased significantly after food stimulation. The intensities increased in band 1 in four individuals (C, F, G, and J); in band 2 in eight individuals (A, B, C, D, E, H, J, and K); in band 3 in four individuals (C, E, H, and J); in band 4 in 11 individuals (A–K); in band 5 in 10 individuals (A–F and H–K); and in band 6 in 10 individuals (B–K). For subject K, the intensity of band 1 decreased further after eating, compared with the effects of the visual and olfactory stimuli. No result was obtained for subject L.

Table 1 Weights (g) of the saliva samples

Sample name	(i)	(ii)	(iii)
A	0.388	0.336	0.773 \uparrow
B	0.513	0.976 \uparrow	0.888 \uparrow
C	0.349	0.571 \uparrow	0.603 \uparrow
D	0.281	0.244	0.476 \uparrow
E	0.562	0.888 \uparrow	0.885 \uparrow
F	1.630	1.337	1.597
G	1.288	0.979	1.078
H	1.346	1.121	0.949
I	0.705	0.542	0.490
J	0.502	0.251 \downarrow	0.186 \downarrow
K	0.491	0.425	0.170 \downarrow
L	0.088	0.096	0.000 \downarrow
Average (standard deviation)	0.679 (\pm 0.459)	0.647 (\pm 0.383)	0.675 (\pm 0.430)

Weight of saliva (g) at three time points: (i) prior to the arrival of the food, (ii) during visual and olfactory stimulation by the food, and (iii) at six minutes after the commencement of eating. \uparrow : 1.5 times heavier than (i), \downarrow : 0.5 times lighter than (i)

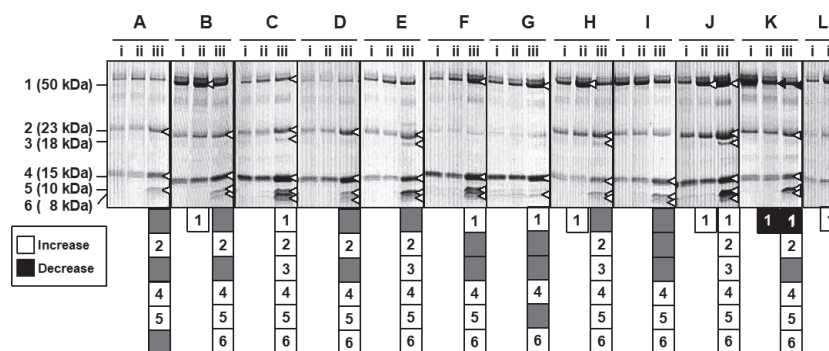


Fig. 2 Separation of salivary proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis. (i) before food intake, (ii) during visualization and olfactory stimuli, and (iii) after commencement of eating. Using (i) control samples for comparison, increases in band intensity ratio exceeding 1.5 (white arrows and white boxes) and decreases in band intensity ratio of <0.5 (black arrows and black boxes) were measured by ImageJ software analysis. The bands changed by stimulation were detected at positions 1 to 6 (1: 50 kDa, 2: 23 kDa, 3: 18 kDa, 4: 15 kDa, 5: 10 kDa, and 6: 8 kDa, respectively). In sample C (iii), all six bands were detected and were used for the subsequent mass spectrometry analysis. Sample L (iii) was excluded because data were not collected.

Table 2 Proteins that were altered in the stimulated saliva

No.	Accession number	Protein name	Protein sequence coverage	Monoisotopic mass
1	NP_004029.2	Alpha-amylase 1 precursor	53%	57731
2	ACF34428.1	Immunoglobulin light chain, partial	52%	23628
3	BAG34753.1	Unnamed protein product	52%	18867
4	NP_001890.1	Cystatin S precursor	69%	16204
5	NP_001306125.1	S100-A8 isoform a	69%	13523
6	NP_002955.2	S100-A8 isoform d	67%	10828

No. indicates the band stained with CBB. The isoforms of S100A8 are transcribed from the same gene.

In order to identify the proteins in each band, MALDI-TOF MS analysis was performed on each protein band detected after the eating stimulus. Bands 1 to 6 were ultimately identified as alpha-amylase 1; immunoglobulin light chain (kappa), partial; unknown protein (BAG34753.1); cystatin S; S100A8 isoform a; and S100A8 isoform d, respectively (Table 2).

4. Discussion

In this study, we found that alpha-amylase 1 was the only protein for which the concentration in saliva changed when the subject was exposed to visual, olfactory, and food stimuli. Alpha-amylase is involved in the hydrolysis of polysaccharides^{7,8}. Surprisingly, the intensity of band for the enzyme alpha-amylase, which has an important function in

saliva, altered following visual, and olfactory stimuli. Thus, alpha-amylase whose intensity of band differ not only food but also visual and olfactory stimulation, can be important candidates of salivary protein markers for dietary assessment.

The immunoglobulin light chain protein^{9,10}, which is part of an antibody involved in the humoral immune reaction and binds to the immunoglobulin heavy chain, showed an increase only under the stimulus of eating. Since the heavy and light chains are paired by disulfide bonds and these are cleaved by SDS-PAGE, they should be detected separately as different bands (light chain: 25 kDa, heavy chain: 50 kDa). The reason that the intensity of only the light chain bands was found to alter is not known, and this remains a possible new target for salivary protein markers.

The band associated with cystatin S increased in intensity due to food stimuli in all samples except sample L, which was excluded. Cystatin is a cysteine protease inhibitor that blocks the action of endogenous, bacterial, and parasitic protozoal proteases¹¹. The inhibitor has been reported to be induced by bitterness³. The increase in cystatin in this study may be related to bitterness, but further verification is necessary to confirm the relationship.

Although S100A8 was barely detected before the meal, following visual or olfactory stimuli either isoform a or isoform d of S100A8 was detected in all samples except sample L. If this can be used as a protein marker, it will be a very useful one. S100A8 is a component of calprotectin (an S100A8/9 complex) and is reported to bind calcium or zinc and to exhibit antibacterial action as a zinc chelate in saliva¹². S100A8 has been reported to be induced by umami, bitterness, or sourness³. Interestingly, the intensities of the two S100A8 isoforms were confirmed separately in this study even though the isoforms are transcribed from the same gene. This may imply that the secretion of proteins is regulated differently for different individuals when sensing food. Hence, S100A8 may be a very strong candidate for the food stimulation index. In the future, it will be necessary to ascertain how these variations are related to food stimulation.

On the other hand, there are already many reports indicating that the saliva flow rate is increased by the smell of food^{7,13} or by chewing^{14,15}. Therefore, it was predicted that the weight of saliva would increase, but this showed three patterns: increase, decrease, and no change. In order to estimate the saliva flow rate from the saliva weight in this study, the saliva collection method must be considered. There are two saliva collection methods: (1) the passive drool technique, and (2) the absorbent device technique. The latter was used in this study, and we chose a swab method that can be easily collected, avoiding stress from the act of spitting out saliva before eating. This method can easily collect saliva from local glands such as the sublingual glands, and the collected saliva can be used to measure proteins and hormones. However, Salimetrics Co. Ltd mentions that stimulation with swabs may change the salivary flow. Therefore, in order to accurately analyze the saliva flow rate and the amount of secreted protein estimated from the flow rate, it might be better to use the passive drool method.

However, more accurate comparisons can be developed by considering experimental conditions, such as comparable food products, number of chews, normalization with standard protein, salivary flow rate, and total protein concentration. Although the number of samples was limited in this experiment, we were able to show that changes in the salivary components can be examined using simple analysis by SDS-PAGE. The causes of these protein changes from food satisfaction and the mechanism of salivation need to be studied in sensory science. If salivary protein changes can be used as more effective objective indicators of satisfaction with food, this may help develop medical food products.

Conflicts of interest

The authors have no conflicts of interest.

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References

1. Ghafouri B, Tagesson C, and Lindahl M: Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics*, 3: 1003-1015, 2003.
2. Ventura TM da S, Ribeiro NR, Dionizio AS, Sabino IT, and Buzalaf MAR: Standardization of a protocol for shotgun proteomic analysis of saliva. *J Appl Oral Sci*, 26: e20170561, 2018.
3. Neyraud E, Sayd T, Morzel M, and Dransfield E: Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. *J Proteome Res*, 5: 2474-2480, 2006.
4. Koyama Y, Premarathne SD, Oppilamany T, Ohnuma A, Okuda A, Iijima A, Onoma N, and Uchiyama M: Differences in subjective taste between Japanese and Sri Lankan students depending on food composition, nationality, and serum zinc. *Clin Nutr Exp*, 23: 60-68, 2019.
5. Schneider CA, Rasband WS, and Eliceiri KW: NIH image to ImageJ: 25 years of image analysis. *Nat Methods*, 9: 671-675, 2012.
6. Okuda S, Watanabe Y, Moriya Y, et al.: jPOSTrepo: an international standard data repository for proteomes. *Nucleic Acids Res*, 45: D1107-D1111, 2017.
7. Morquecho-Campos P, Bikker FJ, Nazmi K, de Graaf K, Laine ML, and Boesveldt S: Impact of food odors signaling specific taste qualities and macronutrient content on saliva secretion and composition. *Appetite*, 143: 104399, 2019.
8. Freitas D, Le Feunteun S, Panouillé M, and Souchon I: The important role of salivary α -amylase in the gastric digestion of wheat bread starch. *Food Funct*, 9: 200-208, 2018.
9. Basile U, Gulli F, Gragnani L, Napodano C, Pocino K, Rapaccini GL, Mussap M, and Zignego AL: Free light chains: Eclectic multipurpose biomarker. *J Immunol Methods*, 451: 11-19, 2017.
10. Heaney JJJ, Killer SC, Svendsen IS, Gleeson M, and Campbell JP: Intensified training increases salivary free light chains in trained cyclists: Indication that training volume increases oral inflammation. *Physiol Behav*, 188: 181-187, 2018.
11. Dickinson DP: Salivary (SD-type) cystatins: over one billion years in the making--but to what purpose? *Crit Rev Oral Biol Med*, 13: 485-508, 2002.
12. Vogl T, Gharibyan AL, and Morozova-Roche LA: Pro-Inflammatory S100A8 and S100A9 proteins: Self-Assembly into multifunctional native and amyloid complexes. *Int J Mol Sci*, 13: 2893-2917, 2012.
13. Ferriday D and Brunstrom JM: I just can't help myself: effects of food-cue exposure in overweight and lean individuals. *Int J Obes (Lond)*, 35: 142-149, 2011.
14. Polland KE, Higgins F, and Orchardson R: Salivary flow rate and pH during prolonged gum chewing in humans. *J Oral Rehabil*, 30: 861-865, 2003.
15. Karami-Nogourani M, Kowsari-Isfahan R, and Hosseini-Beheshti M: The effect of chewing gum's flavor on salivary flow rate and pH. *Dent Res J*, 8: S71-S75, 2011.