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Sample collection and handling considerations for peptidomics studies in whole saliva; implications for biomarker discovery

Ebbing P. de Jong¹, Susan K. van Riper², Joseph S. Koopmeiners³, John V. Carlis², and Timothy J. Griffin^{1,*}

¹Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota; 321 Church St. SE, 6-155 Jackson Hall; Minneapolis, MN 55455.

²Biomedical Informatics and Computational Biology, University of Minnesota; 321 Church St. SE, 6-155 Jackson Hall; Minneapolis, MN 55455.

³Department of Biostatistics, University of Minnesota; 321 Church St. SE, 6-155 Jackson Hall; Minneapolis, MN 55455.

Abstract

BACKGROUND—Proteomics studies in saliva have demonstrated its potential as a diagnostic biofluid, however the salivary peptidome is less studied. Here we study the effects of several sample collection and handling factors on salivary peptide abundance levels.

METHODS—Salivary peptides were isolated using an ultrafiltration device and analyzed by tandem mass spectrometry. A panel of 41 peptides common after various treatments were quantified and normalized. We evaluated the effects of freezing rate of the samples, nutritional status of the donors (fed vs fasted), and room-temperature sample degradation on peptide abundance levels. Repeatability of our sample processing method and our instrumental analysis method were investigated.

RESULTS—Increased sample freezing rate produced higher levels of peptides. Donor nutritional status had no influence on the levels of measured peptides. No significant difference was detected in donors' saliva following 5, 10 and 15 min of room-temperature degradation. Sample processing and instrumental variability were relatively small, with median CVs of 9.6 and 6.6.

CONCLUSIONS—Peptide abundance levels in saliva are rather forgiving towards variations in sample handling and donor nutritional status. Differences in freezing methods may affect peptide abundance, so consistency in freezing samples is preferred. Our results are valuable for standardizing sample collection and handling methods for peptidomic-based biomarker studies in saliva.

Keywords

Peptidomics; saliva; sample handling; stability; normalization LC-MS/MS

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*to whom correspondence should be addressed, tgriffin@umn.edu, Tel. (612) 624-5249 Fax (612) 624-0432.

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Appendix A. Supplementary data

Supplementary data (tables 1-7 and related figures) associated with this article can be found, in the online version, doi:xxx

1. Introduction

Recent studies have shown the potential of saliva as a biofluid for clinical diagnostics [1]. Saliva has been used to measure steroid hormones, antibody levels [2], drugs, both illicit [3] and prescribed [4], DNA [5], and protein biomarkers [6,7]. Less studied, however, is the salivary peptidome [8–10]: the collection of naturally-occurring peptides being secreted or resulting from cleavage of larger proteins. Studies on salivary peptides have revealed information about peptides used in immune defense [8, 9], and on the origins, cleavage patterns, and post-translational processing and/or covalent modification of salivary proteins [11–13]. As a diagnostic tool, salivary peptides have also been used to identify peptides present in autism spectrum disorder [14].

Knowledge of sample handling requirements is crucial for the use of saliva in diagnostic testing. Salivary sample stability has been studied in regards to steroids [15,16], melatonin [17], salivary immunoglobulins [18–20], and other proteins and peptides [21–23]. Messina et al. found asymmetric dimethylation of arginine on multiple salivary peptides when samples were stored at -20°C , but not when stored at 4 or -80°C [21]. Jiang et al. found that levels of salivary β -actin and cystatin C were stabilized for at least 5 days at room temperature by the use of a proprietary reagent from a commercial RNA isolation kit [24]. In the works of Schipper et al., the role of several sample handling parameters on peptide abundances in whole saliva was studied by SELDI-TOF-MS [22,23]. This analysis showed the appearance and disappearance of some features in the SELDI spectra, which were assumed to be derived from salivary peptides, under different handling conditions, suggesting some effects of these conditions on the whole saliva peptidome. A similar study by Esser et al. also examined the difference in SELDI-TOF-MS features in response to various sample handling factors [25]. However, due to the inherent limitations of SELDI-MS (i.e., the absence of MS/MS spectra for sequence determination) the putative peptides affected by sample handling were not identified. Thus the full effect of sample handling factors on salivary peptide samples remains unclear. Here, we show the effects of various sample handling factors on the salivary peptidome, measuring abundance levels of a large panel of representative peptides via LC-MS/MS analysis. Five factors were selected for testing: sample freezing rate, donor nutritional status (fasting or fed state), sample degradation during room-temperature incubation, sample preparation repeatability, and LC-MS/MS instrument repeatability.

2. Materials and methods

2.1 Saliva sample collection

All samples were collected according to a protocol approved by the University of Minnesota Institutional Review Board. Subjects included in the study were all non-smokers free of confounding conditions: periodontal disease, auto-immune disease, a prior history of diseases of the oral mucosa, or current use of potentially confounding medications. Donors refrained from eating or drinking for at least one hour prior to donation. After a water rinse, donors allowed saliva to collect in their mouths before gently expectorating into a sterile 15 or 50 ml conical tube.

2.2 Isolation of peptides, clean-up and quantitation

Clarified saliva was prepared from fresh or thawed whole saliva samples by centrifuging at $3000 \times g$ at 4°C for 15 min, followed by $16100 \times g$ at 4°C for 1 min. The supernatant was mixed in a 10:1 ratio with denaturing buffer consisting of 4% SDS, 100 mM dithiothreitol and 100 mmol/l Tris, pH 7.4. The samples were boiled for 5 min, cooled to room temperature, then added to a centrifugal filter (Amicon Ultra, 0.5 ml, 10 kDa, Millipore).

Two hundred microliters of buffered urea (8 mol/l urea with 100 mmol/l tris pH 8.5) was added to the sample, and this mixture was centrifuged at $14000 \times g$ at room temperature for 40 min. An additional 200 μ l of buffered urea was added and the sample was centrifuged at $14000 \times g$ at room temperature for 40 min. The filters were discarded and the collected peptides were alkylated, by addition of iodoacetamide in buffered urea to 50 mmol/l, in the dark for 20 min. MCX cleanup was performed by diluting the samples to 3 ml with 2% formic acid and H₂O to pH ≤ 3 . The MCX columns (Oasis 3cc, 60mg, Waters Corp.) were equilibrated with 2 ml of 1:1 methanol:water followed by addition of the entire sample, washing with 3 ml of 0.1% formic acid, 2 ml of methanol, and elution with 1 ml of 95% methanol, 5% ammonium hydroxide. The eluted peptides were dried in a speed-vac, redissolved in water, and quantified by a modified BCA assay (Thermo Scientific) [26], using trypsin-digested saliva as a standard. Three micrograms of peptides were further purified and concentrated using the STAGE-tip protocol [27].

2.3 LC-MS/MS

Peptide samples were analyzed by online LC-ESI-MS on an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Waltham, MA) equipped with an Eksigent (Eksigent Technologies, Dublin, CA) 1DLC nanoflow system and a MicroAS autosampler. The column used was an in-house pulled capillary tip of 100 μ m inner diameter, packed to 13cm with Magic C18AQ 5- μ m, 200 Å pore particles (Michrom Bioresources, Inc., Auburn, CA). Samples were redissolved in an aqueous solution containing 2% ACN with 0.1% formic acid, and separated by a 2–40% ACN gradient in 0.1% formic acid over 60 min at 250 nl/min. Full scan mass spectra were acquired in the Orbitrap at 60000 resolution at m/z 400, followed by tandem mass spectrometry (MS/MS) in the LTQ of the five most intense ions from the full scan. Further details of the mass spectrometer were as previously reported [28].

2.4 Peptide identification and automated normalized quantitation

Tandem mass spectra were searched using Sequest v27.0 against an NCBI human database v200806 (70711 entries) with concatenated reversed sequences. The search parameters included variable oxidation of methionine, fixed acetamidylation and no enzyme specificity. The search results were filtered to include only peptides identified at 95% confidence with a charge 2+, 3+ and 4+, and with a 9 ppm precursor mass accuracy. This produced a peptide false discovery rate of $< 0.75\%$ for all experiments. We selected 41 peptides filtered through Scaffold (Proteome Software, Inc., Portland, OR) with 95% peptide identification probability in each degradation time course experiment donor sample and quantified the peptides using software developed in house. Peptide quantification included several steps. First, Thermo RAW data were converted to mzXML format using the program ReAdW (tools.proteomecenter.org) and MS¹ peaks were extracted from the mzXML files using a baseline noise threshold filter (intensity > 5000). Second, an MS¹ scan level charge assigned monoisotopic feature intensity ($CAMFI_{SL}$) was computed by summing isotopically charge state distributed peak intensities (within .005 Da tolerance) and normalizing to the total $CAMFI_{SL}$ extracted from its MS¹ scan. Third, a run level charge assigned monoisotopic feature intensity ($CAMFI_{RL}$) was computed by summing $CAMFI_{SL}$ for each feature across multiple MS¹ scans using retention time filter (± 90 seconds of the feature's most intense $CAMFI_{SL}$). Finally, peptide intensities were computed by summing their corresponding 2+ and 3+ charge $CAMFI_{RL}$. To ensure specificity of the XIC peak, we verified that the peptide of interest was the only sequence identified during the peak, within the specified precursor m/z .

The accuracy of our automated peptide normalization and quantification software program was manually validated via the Xcalibur peak area calculation of selected peptides. Additionally the accuracy of the program was confirmed via analysis of a serial dilution of

saliva peptides, wherein our program improved the average linear correlation coefficient from $r^2 = 0.45$ (un-normalized) to $r^2 = 0.92$ (normalized). This data is shown in Supplemental Table 1.

2.5 Freezing rate experiments

Whole saliva samples (n=8), collected at 9 am, were split into 2 aliquots. The first aliquot was immediately placed at -80°C , and the second placed on wet ice for approximately an hour before being stored at -20°C . Samples were frozen at the specified temperature overnight and processed the following day.

2.6 Patient nutritional status experiments

Saliva samples were collected at 9am from donors following an overnight fast, and again from the same donors (n=7) at 2 pm following *ad libitum* feeding, except during the hour prior to sample collection.

2.7 Room-temperature degradation experiments

Saliva from seven donors was collected at 9 am, and aliquots placed at -80°C at the following time points: 0, 5, 10 and 15 min, and 24 h. The aliquots collected at 24 h were frozen for at least 2 h, after which all samples were thawed and processed.

2.8 Repeatability experiments

Freshly collected saliva from a single donor was processed in triplicate. In another study, an autosampler vial was filled with sufficient sample from a single experiment for 3 replicate injections.

2.9 Statistics and analysis

Freezing rate and patient nutritional status data were analyzed by taking the difference in a peptide's signal for a given donor under the two treatment conditions (e.g. -80 vs. -20) and dividing by the standard deviation of the differences for that peptide. This provides standardized differences, which allow us to look for consistent trends across peptides by analyzing all peptides on the same scale. Standardized differences were analyzed using a linear mixed model with random effects for subject and peptide to account for possible within-subject or within-peptide correlation [29]. A p-value was produced by testing whether or not the intercept was equal to zero. An intercept significantly different from zero indicates that there is a consistent difference in signal between the two treatment conditions. An identical analysis was completed for the room-temperature degradation data with a peptide's signal at 5, 10, 15 min and 24 h compared to baseline ($t = 0$ timepoint).

3. Results

3.1. Database searching and selection of peptides panel

Processing of 100 μl of clarified saliva (supernatant) typically yielded 25 – 50 μg of peptides, as determined by the BCA assay. Database searches of the tandem mass spectra typically identified 20–40 proteins (data not shown), dominated by proline-rich proteins and their isoforms, submaxillary gland androgen regulated protein 3A, statherins and histatins. It is noteworthy that many high-abundance proteins seen in whole saliva studies such as amylase, mucin and albumin, are nearly absent from the peptide fraction isolated here. Peptide identification was performed using no enzyme specificity, to allow detection of all types of enzymatically cleaved peptide products. Peptides present in all $t=0$ samples of the degradation time course study produced a panel of 41 peptides for quantification in all

subsequent experiments. A list of these peptides sequences is given in Supplementary Table 2.

3.2. Freezing rate

The effect of freezing rate was studied by comparing samples placed on wet ice and then at -20°C against samples immediately frozen at -80°C . Figure 1 displays the standardized differences between the two freezing rates, and shows a general trend of increased abundance of the quantified peptides for samples frozen at -80°C ($p = 0.002$). Normalized peptide intensities are shown in Supplementary Table 3.

3.3. Donor nutritional status

Figure 2 displays the standardized differences showing the effect of donors' nutritional status on the measured amount of peptides in saliva ($p = 0.848$). For all the peptides examined, some donors expressed more peptide in the fasted state, while others displayed the opposite trend. Importantly, there is no *consistent* trend for either increased or decreased peptide levels in either state, for any of the peptides. Normalized peptide intensities are shown in Supplementary Table 4.

3.4. Sample degradation

The effect of room-temperature sample degradation was studied by allowing whole saliva samples to remain at room temperature, with aliquots being removed and immediately frozen (-80°C) after 0, 5, 10, 15 min, and 24 h (Figure 3; p equal to 0.065, 0.824, 0.602, 0.024 for 5, 10, 15 min and 24 h, respectively). Peptide abundance levels fluctuated slightly with time after donation, however especially for short time periods after collection (up to 15 min), abundance levels were unchanged. A significant decrease in overall peptide abundance at 24 h was observed, however some peptides increased in abundance. This is consistent with other studies [22,24,25] suggesting increased protein degradation with longer incubation times. Normalized peptide intensities are shown in Supplementary Table 5.

3.5. Sample processing repeatability

Sample processing repeatability was assessed by preparing three aliquots of the same sample in parallel using our method (ultrafiltration followed by LC-MS/MS). The median CV for the normalized peptide intensities was 9.6% (range 1.1 to 97.0%; Supplementary Table 6).

3.6. Instrumental repeatability

Finally, instrument repeatability was estimated by three consecutive analyses of a sample from the same autosampler vial. The median CV for the normalized peptide intensities was 6.6% (range 0.7 to 44.7%; Supplementary Table 7).

4. Discussion

In this study, we present an in-depth examination of salivary peptide abundance levels and the possible effects due to sample collection and handling factors. Previous work by others using SELDI-TOF-MS has measured changes in mass spectral peaks, putatively derived from peptides, due to centrifugation speed, the presence of protease inhibitors, storage temperature (-20 vs -80°C) and storage time [22]. A significant limitation in these studies was the lack of identification of the analytes corresponding to the peaks detected by SELDI-TOF-MS. In order to provide greater insight into the question of saliva peptide stability, we targeted salivary peptides identified via MS/MS analysis and sequence database searching. Our study examined 41 abundant peptides in whole saliva which are found in nearly all samples. Although these peptides are all derived from proteolysis from larger starting

polypeptides, the mechanisms and proteases of these cleavage events are diverse and in many cases unknown [11,13,30].

A faster freezing rate was found to increase the abundance levels of peptides in this study. Our data does not allow us to draw conclusions regarding the mechanisms responsible for this observation, however possibilities include differences in (i) the rate of cleavage of the protein to the observed peptide, (ii) the rate of cleavage of the observed peptide to a smaller, unobserved peptide, (iii) peptide hydrolysis, or (iv) peptides binding irreversibly to the walls of the tubes. We suspect the latter explanation is the main contributor, but an in-depth study would be required to confirm this. Other studies have suggested some effects on salivary peptides due to freezing rates, although the focus of these studies were different from ours. Asymmetric dimethylation of arginine has been reported when freezing at -20°C , but not at -80°C [21], suggesting a possible “cold shock” response that leads to peptide modification. Although our study did not focus on post-translationally modified peptides, our results are consistent with this previous study in that we also found freezing to have an effect on salivary peptides. Another study looked at the effects of storage duration (1 or 6 months) at -20°C compared to -80°C [22]. The authors did observe, via SELDI-TOF-MS, spectral changes dependent on storage duration at the different temperatures relative to a control sample. Unlike these previous studies, our findings address the important question of whether or not initial freezing conditions affect salivary peptide abundance levels. Our results suggest that attention should be paid to ensuring consistent procedures in freezing samples.

Patient nutritional status showed little effect on the peptides examined in this study. We chose to evaluate the effects of nutritional status of donors on salivary peptides, given the well-known changes in saliva protein composition and enzyme activities induced by food intake [31, 32]. We reasoned that knowledge of the possible effects of feeding versus fasting would be valuable for peptidomic-based biomarker discovery studies in saliva, as well as subsequent studies developing clinical tests utilizing these peptide biomarkers. In a clinical setting, time elapsed since eating and patient compliance with orders to fast are not always easy to control. Our findings point to minimal effects on salivary peptide abundance levels due to feeding or fasting. We point out however that the fed state samples were collected at least one hour after eating. Thus effects on salivary peptide abundances within shorter times after eating cannot be ruled out.

Room temperature storage on saliva samples showed no significant influence on the peptides studied here within the first 15 min (Fig. 3). After 24 hours of incubation however, a statistically significant decrease in peptides abundances was observed. This may not be surprising given past studies which suggested decreases in salivary protein abundances when left at room temperature for extended periods of time. Jiang et al. found that the use of RNAlater (Qiagen) stabilized the abundance levels of β -actin and cystatin C in saliva for at least five days, whereas complete loss of these proteins was observed with or without the use of protease inhibitors [24]. Somewhat contradicting these results, Morris et al. reported that IgG levels in saliva are stable for up to 7 days at 10 or 20°C in the absence of any protease inhibitors [19]. Esser et al. [25] and Schipper et al. [22] have studied changes in low molecular weight salivary components detectable by SELDI-TOF-MS over three or four hours with and without protease inhibitors. Both papers find that the addition of protease inhibitors reduced the number of changes in SELDI-TOF-MS profiles, but neither paper confirms that the peaks showing changes upon incubation are proteinaceous material. Our results show that at least for relatively short amounts of time prior to freezing (≤ 15 min), salivary peptide abundances remain stable without the need for protease inhibitors or other additives.

Our results on room-temperature degradation has a number of consequences for salivary diagnostics targeting salivary peptides. First, although some peptides do show decreased abundance after 24 h, many remain stable. This suggests that some salivary peptides may be amenable to diagnostic testing via overnight shipping to a central laboratory, without the need for freezing, as has been investigated for other salivary diagnostic tests [16,19,24,33]. Second, our results also have implications for point-of-care diagnostic devices for use in the clinic [34–36] that may be developed for targeting salivary peptides. An advantage of these point-of-care devices is the very short time lapse between sample collection and analysis. Our results confirm that a relatively short time lapse (≤ 15 min) between collection and analysis should have no effect on salivary peptide abundance levels.

We chose a method combining ultrafiltration for salivary peptide isolation followed by LC-MS/MS on an LTQ-Orbitrap and quantification by extraction of normalized peptide peak features. Measurement based on summed peak intensities was possible due to the high relative abundance of the peptides selected for analysis. The variability of measured peptide levels, either due to sample processing or instrumental imprecision in our MS-based assay, is adequately low for detecting changes in salivary peptide abundances. The variability measured in our study after normalizing (median CV reduction of 53% in instrument variability) is in-line with other prominent studies quantitating peptides from LC-MS data, including global normalization methods (20–30% median SD reduction) and linear regression followed by analysis order normalization (43% median SD reduction) [37]. The normalization procedure helps to control for variables such as imprecision in sample loading by the autosampler and electrospray instability.

Despite the important new knowledge on sample processing and collection effects for salivary peptides provided by our study, some questions remain. First, although we have selected a large set of naturally occurring salivary peptides, our results do not guarantee that the factors investigated here will have similar effects on all other salivary peptides. Initial studies on the entire complement of salivary peptides, performed by semi-quantitative analysis via spectral counting, suggested minimal differences in peptide abundances due to room temperature degradation and donor nutritional status. Nonetheless, studies targeting specific peptides of interest are the only way to conclusively investigate the potential effects of sample collection and handling factors. Second, the sample donors in this study were all non-smokers, free from oral diseases. Clinical samples from patients with oral disease might not behave the same as those examined here, and further studies are warranted to determine whether the conclusions drawn here can be extended to clinical samples. Lastly, the sample size in this study was sufficient to detect large abundance changes in peptides due to the factors investigated. However, a larger sample size is required to detect more subtle changes that may be important.

In conclusion, we have performed the most in-depth study of sample collection and handling effects on salivary peptide abundance levels to-date. Our results indicate that the salivary peptidome is relatively resistant to two factors: fasting versus fed status of donors, and sample degradation due to room temperature incubation. Since higher levels of peptides are seen at an increased rate of freezing, we recommend consistency in the method of freezing samples. These results can be used to guide future studies for peptide biomarker discovery in saliva, and potential development of clinical assays targeting validated peptides derived from these studies.

Highlights

- We explore the effect of several pre-analytical factors on the salivary peptidome

- Low molecular weight proteins and peptides were isolated and analyzed by LC-MS²
- Faster sample freezing increased the measured peptide intensity
- Donor nutritional status had no effect on peptide levels
- 15 min room temperature sample incubation did not change measured peptide levels

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

SELDI	surface-enhanced laser desorption ionization
TOF	time of flight
BCA	bicinchroic acid
MCX	mixed-mode cation exchange
XIC	extracted ion chromatogram

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Figure 1.

Box plots of the standardized (i.e. divided by their standard deviation) differences between peptides measured from samples frozen at -20 and -80 °C (frozen at -80 – frozen at -20), $p = 0.002$.

Figure 2.
Box plots of the standardized differences between peptides measured from samples under fasting and fed conditions (fed – fasted), $p = 0.848$.

Figure 3.
Box plots of the standardized differences between peptides measured 5 minutes, 10 minutes, 15 minutes and 24 hours compared to baseline, p equal to 0.065, 0.824, 0.602, 0.024, respectively.