Factors contributing to stability and instability in alpha-amylase activity in diluted saliva samples over time

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A B S T R A C T
For the measurement of salivary alpha-amylase (sAA) activity, saliva samples first have to be diluted. There is some evidence for instability, that is, a decline of sAA activity in diluted samples. It is not clear which factors during dilution may contribute to this phenomenon and how quickly this decline of sAA activity occurs.

Several experiments were conducted to investigate whether and how the material of the container (polystyrene (PS), polypropylene (PP), glass; experiment 1) and the diluent (saline (NaCl) solution, phosphate buffer saline (PBS), ultra-pure water; experiment 2) may affect sAA stability in diluted samples over a broad time window of up to 5 h. To study the velocity of the phenomenon in a fine-grained temporal resolution, sAA activity during the dilution process was studied (experiment 3).

The results suggest that the (in)stability of sAA activity in diluted samples is determined by the interaction of material, diluent, and time. The sAA activity was relatively stable if saliva samples were diluted with a NaCl solution or PBS in glass tubes. However, sAA activity in diluted samples decreased in plastic containers (PS, PP), or if ultra-pure water was used as the diluent. There was a clear time effect on this decline. However, the decline appears to require some time to evolve and may not occur immediately during the dilution process. To conclude, the dilution of saliva samples should preferably be conducted with NaCl solution or PBS in glass containers. If glass containers are not available, PS and PP containers can be used if the dilution is processed quickly (within 25 min) and the measurement is initiated immediately upon dilution.

1. Introduction

Over the last decades, the scientific community has become increasingly interested in salivary alpha-amylase, as research has demonstrated its importance as a significant enzyme for digestion, metabolism (see review, Butterworth et al., 2011; Elder et al., 2018), and oral health (e.g., Scannapieco et al., 1993), and its value as a biomarker of stress (e.g., Nater and Rohleder, 2009) as well as physical and mental health (e.g., Ali and Nater, 2020; Schumacher et al., 2013).

Salivary alpha-amylase (sAA) is a digestive enzyme, predominantly known for its crucial role in carbohydrate digestion and its contribution to lubrication, taste sensation, and antibacterial activity in the oral cavity (e.g., Fabian et al., 2015; Humphrey and Williamson, 2001; Scannapieco et al., 1993). It is synthesized and released by acinar cells of salivary glands, innervated by the two main branches of the autonomic nervous system, namely the parasympathetic (stimulates salivary flow rate, resulting in fluid-rich saliva) and sympathetic branch of the nervous system (stimulates the protein synthesis and secretion, resulting in protein-rich saliva) (Proctor, 2016). Thus, sAA represents a promising biomarker of the autonomic nervous system (Granger et al., 2006; Nater and Rohleder, 2009).

Despite these promising applications, researchers have observed that there is substantial variation in sAA, rendering its use as a valid and reliable biomarker somewhat challenging (e.g., Strahler et al., 2017). A better understanding of the sources of this variation is of crucial importance. Known sources of variation are a distinct diurnal rhythm, with a morning decline occurring about 30 min after awakening (‘awakening response’), followed by a continuous increase over the course of the day, with a relatively stable plateau or a slight decline towards the evening (Nater et al., 2007), response to environmental changes (e.g., stress exposure), copy number polymorphism of the salivary amylase gene (AMY1) (e.g., Santos et al., 2012; Yang et al., 2015) as well as influences by age, sex hormones, lifestyle (e.g., diet, physical activity, smoking, alcohol consumption, sleep), health status and medication usage (see reviews, Rohleder and Nater, 2009; Strahler et al., 2017). All of these factors are important sources of variation that

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need to be controlled for or considered when analyzing and interpreting sAA data.

Beyond the intra- and inter-individual variability in sAA activity, methodological factors related to the analysis method may additionally contribute to the variation in sAA activity. The most commonly used method of measuring alpha-amylase activity is kinetic colorimetric tests according to the IFCC recommendations (International Federation of Clinical Chemistry and Laboratory Medicine), which utilize the enzymatic ability of alpha-amylase to break down polysaccharides, such as starch, into disaccharide maltose. As alpha-amylase activity in saliva is higher compared to that of the pancreatic origin (usually measured in urine), saliva samples need to be diluted before applying kinetic colorimetric tests, allowing measurement within the detectable range of the assay. As a diluent of choice, various commercial manufacturers recommend either isotonic saline (NaCl) solution (e.g., DiaSys Diagnostic Systems; LT-Sys; Roche Diagnostics) or diluents containing phosphate-buffered saline (PBS) (e.g., IBL International; Salimetrics).

Previous anecdotal observations from our and other laboratories suggest that sAA activity might be somewhat instable in highly diluted saliva samples, as indicated by a steady decline of sAA activity over time. This potential instability of sAA in dilutions may result in an increased risk of measurement inaccuracy and underestimation of the sAA activity, and thus further contribute to sAA variation. Therefore, the investigation of the underlying mechanisms and the trajectory of this phenomenon may help to identify strategies to increase stability or reduce instability of sAA in highly diluted saliva samples. This notion gives rise to the following two research questions.

1. (What) factors contribute to the (in)stability of sAA activity over time?
2. What is the trajectory (i.e., regarding shape and timing) of this instability of sAA in diluted saliva samples?

Materials differ in their tendency to adsorb peptides and proteins on their surfaces (e.g., Goebel-Stengel et al., 2011), and may thus also impair enzyme stability and activity (Nakanishi et al., 2001). Glass is presumed to have low or negligible binding tendencies (and is therefore considered as ‘gold standard’). However, this notion has been shown to be only partially true, depending on the substance, solvent, and pre-treatment of the glass surface (e.g., Goebel-Stengel et al., 2011; Sueter and DeLuca, 1983). Plastic containers are commonly used in the laboratory. More specifically, polystyrene (PS) is predominantly used for optical measurements such as immunoassays, due to its high transparency and binding ability (e.g., coating a plate with antibodies). Polypropylene (PP), on the other hand, is suitable for long-term storage due to its resistance to chemicals, low-binding characteristics, and thermal stability (resistant to extremely low and high temperatures). Accordingly, we hypothesize that the decline of sAA activity over time is less pronounced in glass, followed by PP and PS (experiment 1).

The characteristics of the diluent may affect enzyme activity. Laboratory experiments using human saliva suggest an optimum pH of 7, meaning that the highest enzyme activity is found at neutral pH (Rudeekulthamrong and Kaulpiboon, 2012; Tatab and Ottijo, 2015). In line with this finding, human saliva is found to be neutral to slightly acidic (normal pH: 6–7), maintained by a bicarbonate pH-buffering system (Humphrey and Williamson, 2001; Proctor, 2016). Furthermore, in-vitro experiments in human saliva have shown that ions may have activating (e.g., Cl−, Br−, Li+, Na+, K+, NH4+, Mg2+, Ca2+, Ba2+) or inhibiting effects (e.g., Ag+, Hg2+) (Clifford, 1986; Rudeekulthamrong and Kaulpiboon, 2012; Tatab and Ottijo, 2015) on sAA activity. Saliva contains a variety of electrolytes which have beneficial effects on salivary functioning such as sAA activity (e.g., Na+, K+, Ca2+, Mg2+, HCO3, PO43) (Humphrey and Williamson, 2001). Considering pH and the presence of ions, we compare three different diluents with neutral pH (6–7): isotonic saline solution (NaCl, 0.9%), phosphate-buffered saline (PBS), and ultra-pure distilled water (H2O). Given the neutral pH of all diluents of interest, we expect the diluents to exhibit comparable sAA activity patterns over time (experiment 2).

To tackle the question of how quickly this decline of sAA activity occurs, sAA activity is measured a) repeatedly in 15-minute intervals for up to the first hour and again five hours after dilution in order to cover a broad time period (experiments 1 and 2) and b) within the analysis plate (44 diluted samples) using a pooled saliva sample in order to cover the short time period of dilution (experiment 3). We hypothesize a steady decline of sAA activity with the passing of time.

2. Materials and methods

2.1. Collection of saliva samples

To cover a broad range of sAA activity due to intra- and inter-individual variability, saliva samples were collected from different individuals, each of whom collected four samples: upon awakening, 30 min after awakening, 2 pm (±1 h), and 8 pm (±1 h). All participants provided informed consent. In total, eleven healthy participants (6 females, 29.0 ± 5.6 years, 23–40 years; 1 smoker), recruited from among university staff at the University of Vienna, Austria and their acquaintances, provided saliva samples using the passive drool method. Participants were asked to collect at least 1 mL saliva in total. In brief, participants were instructed to collect saliva in their mouth for 1–2 min and subsequently transfer accumulated saliva into a SaliCap tube (ultrapure PP) via a straw (SaliCap Set; IBL, Hamburg, Germany), and were instructed to repeat the collection procedure if necessary. Participants were asked to refrain from alcohol consumption, physical exercise, and chewing gum for 2 h prior to saliva collection, and from food consumption, smoking, and teeth brushing for the last hour prior to saliva collection. For the two morning samples, participants were allowed to drink water only (no coffee, tea, or juice).

Participants stored saliva samples in their refrigerator or freezer at home and brought them to the laboratory on the following day, where samples were stored at −20 °C. Samples were thawed, vortexed (20 s), and centrifuged (1930g, 1 min). Clear supernatant was transferred into a new clean SaliCap, vortexed thoroughly for 20 s, and up to nine 100 μL aliquots were prepared, which were immediately stored at −30 °C until analysis. For each experiment, a separate aliquot was used in order to control for repeated freeze-and-thaw cycles.

2.2. Sample dilution procedure and kinetic colorimetric test for the determination of salivary alpha-amylase activity

Kinetic colorimetric tests are based on enzyme kinetics. In brief, alpha-amylase in the sample cleaves the substrate 4,6-ethylidene-(G7)-p-nitrophenyl-(G1)-alpha-o-maltodextrinase (EPS-G7) into various fragments. In a second step, these fragments are further hydrolyzed by another enzyme called alpha-glucosidase, resulting in glucose and by-product p-nitrophenol. The increase in absorbance due to p-nitrophenol (measured at 405 nm) reflects the direct proportional amount of the total of alpha-amylase activity in the sample (e.g., Nater et al., 2015).

On the day of the experiment, aliquots of the saliva samples were thawed and brought to room temperature. To assure that no enzyme gradient was built, SaliCaps were mixed for 10 s using a vortex mixer (Biosan, Riga, Latvia). Subsequently, to obtain a clear supernatant, SaliCaps were centrifuged at 1930g for 11 min. For economic reasons, the dilution procedure was performed in two steps; each saliva sample was diluted 1:400 with isotonic saline solution (NaCl, 0.9 %) (Medipharm, Kufstein, Austria) in a standard 96-well PS microplate (Sarstedt, Nümbrecht, Germany). After each dilution step, the dilutions in the microplate were mixed for 2 min at 120 g with the help of an orbital plate shaker (Heidolph, Schwabach, Germany).

Salivary alpha-amylase activity was measured using a kinetic colorimetric test, with reagents obtained from DiaSys Diagnostic Systems.
Systems (Holzheim, Germany). Calibrator (TruCal U) and assay controls (TruLab N, TruLab P) were prepared in advance according to the instructions provided by the manufacturer, aliquoted and immediately stored at −30 ºC. Following the manufacturer’s instructions, substrate reagent was prepared by mixing four parts R1 (containing alpha-glucosidase) and one part R2 (containing EPS-G7). Before analysis, reagents were brought to room temperature. Next, 20 μL of the diluted saliva samples, blank (NaCl, 0.9 %), calibrator, and two assay controls were transferred into the analysis plate (96-well PS microplate). Subsequently, 100 μL of substrate reagent were pipetted into each well by a multipette (Eppendorf, Hamburg, Germany). Without delay, the analysis plate was shaken within a pre-heated (37 ºC) absorbance reader (Biotek Synergy HTX, BioTek Instruments, Winooski, VT, USA) for 2 min, before the measurement at the wavelength of 405 nm was automatically initiated. The measurement comprises nine cycles, with one minute between each cycle; however, for the estimation of sAA activity, only two cycles were considered (cycle 1 and 5). Using Gen5 Data Analysis Software (BioTek Instruments, Winooski, VT, USA), absorbance values were blank-corrected first, with NaCl wells serving as blank; and blank-corrected absorbance increases were calculated by subtracting the first measurement from the fifth measurement. The alpha-amylase activity (in U/mL) of the saliva samples was estimated by applying the following formula, considering the calibrator and dilution factor: blank-corrected absorbance increase of the sample * dilution factor (0.4) * reference calibrator value (provided by manufacturer) / blank-corrected absorbance increase of the calibrator.

2.3. Experiment 1: material

To investigate whether and how the adsorption effect may contribute to the decline of sAA activity in diluted saliva samples, different materials which varied in terms of their binding tendencies were tested. Aliquots of 44 saliva samples were diluted 1:400 with NaCl solution in PS microplates, following the protocol described above (see 2.2). After dilution, duplicates of diluted samples as well as blank, calibrator, and assay controls were transferred into the analysis plate (PS microplate) and measured immediately. We repeated the measurement of the samples taken from the same dilution after 15 min (t1), 30 min (t2), 45 min (t3), 1 h (t4), and 5 h (t5) relative to the first measurement (t0). The same experiment was repeated using a PP deep-well plate (Biologix, Jinan, Shandong, China) and borosilicate glass tubes (Hirschmann, Eberstadt, Germany) attached to a deep-well plate for the dilution of the saliva samples.

2.4. Experiment 2: diluent

The second experiment aimed to study the effect of pH-neutral diluents, varying in their composition, on the stability of sAA activity. Again, aliquots of the same 44 saliva samples as used in experiment 1 were diluted in glass tubes according to the procedure described in experiment 1. We compared three commonly used diluents: NaCl solution (Medipharm, Kufstein, Austria), PBS, pH 7.2 1x (NaCl, Na2HPO4, KH2PO4) (HiMedia Laboratories, Mumbai, India) and H2O utilizing a water purification system (Adrona, Riga, Latvia). Solutions were pH-neutral to slightly acidic (6–7), confirmed by pH-indicator strips (Scharlab, Barcelona, Spain).

2.5. Experiment 3: pool sample

The third experiment set out to investigate whether and how quickly the decline of sAA activity may occur during the dilution process. The manually performed dilution process of 44 samples (fitting on the 96-well analysis plate) until the start of the measurement took approximately 25 min in total. This approach allows for a comparison of sAA activity between 44 dilutions of the same saliva sample which differ in their positions on the dilution plate and thus in time since dilution. If the instability of sAA activity occurs immediately upon dilution, sAA activity of the last diluted sample is assumed to be higher than that of the first diluted saliva sample. For this experiment, eleven saliva samples were pooled and diluted with NaCl solution in a PS microplate. In total, 44 diluted samples of the pooled saliva sample were transferred into the analysis plate (PS microplate) and subsequently measured (see also Fig. 2C). The same experiment was repeated for the diluted with NaCl solution in a PP deep-well plate, for which six saliva samples were pooled.

2.6. Statistics

Using G*Power (Faul et al., 2007) for the priori sample size estimation (expected medium effect size of .25 in power of .90, three conditions, six time points), at least 39 samples were required. We decided to run the experiments using 44 samples corresponding with the maximum samples that can be run in duplicates on a microtiter plate. First, all measurement values which were not detectable (sAA < 3 U/mL) were assigned the value ‘1’ because the subsequent ln-transformation of ‘0’ is not applicable and ln-transformation of ‘1’ results in the value ‘0’. Second, sAA data were ln-transformed to obtain a normal distribution of the data. Using ln-transformed data, repeated measures ANOVAs (with Greenhouse-Geisser correction) were computed for experiments 1 and 2 in order to investigate the effect of time and condition (material and diluent, respectively) on sAA activity. To determine the relative amount of sAA activity with reference to the initial measurement (t0), percent recovery was calculated by applying the following formula: sAA at tx / sAA at t0 * 100, with tx referring to the respective measurement time point (1-15). Degradation rates were computed to quantify the relative loss of sAA activity after 1 h and 5 h relative to the initial measurement time point (formulas: sAA at t0 − sAA at t4) / sAA at t0 * 100 and (sAA at t0 − sAA at t5) / sAA at t0 * 100, respectively. For experiment 3, Kendall’s Tau τ were computed to investigate whether ln-transformed sAA and the position of diluted sample on the analysis plate were correlated. For figures and computation of percent recovery and degradation rates, raw data were used. All analyses were conducted using IBM SPSS 26.0 (Chicago, IL).

3. Results

3.1. Experiment 1: material

The first experiment aimed to investigate whether the material of plates (PS, PP) or tubes (glass) used for dilution with NaCl solution may affect the sAA activity over time. Overall, there was a significant main effect of time (F(1.04, 44.50) = 25.55, p < .001; η² = .373), indicating changes of sAA activity over time. The significant main effect of material (F(1.75, 75.40) = 24.36, p < .001; η² = .362) and significant material x time interaction effect (F(1.13, 48.36) = 20.15, p < .001; η² = .319) further suggested that the choice of material may affect the sAA activity and its stability over time (Fig. 1A), which is also reflected by percent recovery (see Table 1). The materials differed significantly in the total loss of sAA activity within the first hour (glass < PS < PP; F(1.47, 63.17) = 63.93, p < .001; η² = .598), indicated by degradation rates of 31.5 ± 19.6 %, 13.9 ± 15.5 %, and 2.4 ± 3.5 % for PS, PP, and glass, respectively. Degradation rates after 5 h further increased to 42.1 ± 32.1 %, 15.9 ± 22.9 %, and 6.1 ± 5.9 % for PS, PP, and glass, respectively (glass < PS < PP; F(1.64, 70.66) = 50.49, p < .001; η² = .540). Notably, sAA activity was not detectable in seven of the 44 samples 5 h after dilution conducted in PS plates.

3.2. Experiment 2: diluent

The second experiment addressed the diluent used for dilution of the saliva sample, comparing NaCl solution (data used from the first experiment), PBS, and H2O using glass tubes. There was a significant
the loss of sAA activity at one and five hours after dilution (1 h: 11.5 ± 3.28%; 5 h: 8.26 ± 2.59%) as compared to the immediate measurement, t1 = 15 min, t2 = 30 min, t3 = 45 min, t4 = 1 h, t5 = 5 h.

**Table 1**

Stability of sAA activity over time, indicated by percent recovery relative to initial measurement.

| Diluent | Material | t0 - t1 (n = 44) M ± SD in % (Min – Max) | t0 - t2 (n = 44) M ± SD in % (Min – Max) | t0 - t3 (n = 44) M ± SD in % (Min – Max) | t0 - t4 (n = 44) M ± SD in % (Min – Max) | t0 - t5(n = 44) M ± SD in % (Min – Max) |
|---------|----------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| NaCl    | PS       | 83.19 ± 5.88 (73.51–98.06)             | 76.58 ± 11.04                          | 69.96 ± 14.20                          | 68.46 ± 19.62                          | 57.89 ± 32.12                          |
| NaCl    | PP       | 93.07 ± 9.20 (72.95–107.56)            | 86.33 ± 12.06                          | 87.76 ± 14.61                          | 86.07 ± 15.49                          | 84.12 ± 22.87                          |
| NaCl    | Glass    | 98.29 ± 2.06 (93.97–102.97)            | 99.33 ± 2.63                           | 97.56 ± 3.05                           | 95.98 ± 3.51                           | 93.94 ± 5.89                           |
| PBS     | Glass    | 96.57 ± 2.60 (85.79–102.09)            | 92.90 ± 3.93                           | 96.50 ± 2.74                           | 95.98 ± 2.59                           | 88.54 ± 5.30                           |
| H2O     | Glass    | 71.28 ± 22.78 (8.47–100.60)            | 48.10 ± 29.20 (3.21–99.61)             | 34.29 ± 29.62 (2.41–94.16)             | 27.48 ± 32.44 (1.38–106.51)            | 17.44 ± 28.91 (0.71–99.38)            |

Descriptions: M = mean, SD = standard deviation, H2O = ultra-pure water, NaCl = isotonic saline solution (0.9 %), PBS = phosphate-buffered saline, PS = polystyrene, PP = polypropylene, t0 = immediate measurement, t1 = 15 min, t2 = 30 min, t3 = 45 min, t4 = 1 h, t5 = 5 h.

**Fig. 1.** Illustration of mean sAA activity in diluted saliva samples over the time, comparing materials of the container (A) and diluent (B).

**Fig. 1.** Illustration of mean sAA activity in diluted saliva samples over the time, comparing materials of the container (A) and diluent (B).

The effect of time (F(2.11, 90.62) = 95.02, p < .001; η² = .688), diluent (F(1.23, 53.08) = 94.62, p < .001; η² = .688), and solvent x time interaction (F(2.16, 92.76) = 82.38, p < .001; η² = .657), driven by the H2O condition (Table I Fig. 1B). Diluents differed significantly with regard to the loss of sAA activity at one and five hours after dilution (1 h: F(1.02, 43.65) = 197.07, p < .001; η² = .821; 5 h: F(1.06, 45.64) = 317.19, p < .001; η² = .881), with the highest degradation rates in H2O (1 h: 72.5 ± 32.4 %; 5 h: 82.6 ± 28.9 %), followed by PBS (1 h: 4.0 ± 2.6 %; 5 h: 11.5 ± 5.3 %) and NaCl solution (1 h: 2.4 ± 3.5 %; 5 h: 6.1 ± 5.9 %). Notably, sAA activity was not detectable in 1, 8, 9, 16, and 29 samples 15 min, 30 min, 45 min, 1 h, and 5 h after dilution using H2O, respectively (Fig. 1).

**3.3. Experiment 3: pool sample**

To investigate the time sensitivity of the phenomenon at the early stage of the dilution process, sAA activity was determined in 44 separate dilutions of a pool sample and correlated with the position on the plate. The position on the plate was not significantly correlated with sAA activity, either for the pool sample diluted in the PS microplate (Kendall’s Tau τ = 0.15, p = .151) or for the pool sample diluted in the PP deep well plate (Kendall’s Tau τ = −.08, p = .442), suggesting negligible associations. Fig. 2 shows fluctuations of sAA activity across the plate, separately for PS and PP.

**4. Discussion**

The current study set out to investigate whether and how the material of the container and the diluent contribute to the decrease in sAA activity in diluted saliva samples. In summary, our results suggest a relative stability in samples diluted with NaCl solution or PBS in glass tubes. However, a steady decline of sAA activity was found if plastic containers (PP, PS) for dilutions with NaCl solutions, or if H2O as a diluent in glass tubes, were used. Our findings provide evidence for a time effect of this phenomenon, that is, sAA activity in highly diluted samples decreased steadily over time. However, this time-sensitive decline of sAA activity may take some time to reveal detectable detrimental effects on the sAA activity.

The material effect on sAA activity in diluted samples can be explained by adsorption. Adsorption describes the adherence of adheres, ions, or molecules of an absorbate on a surface due to hydrophobic and electrostatic interactions (Nakanishi et al., 2001; Song et al., 1996). It is assumed that the immobilization of enzymes upon adsorption is associated with conformational changes (i.e., momentous structural changes and instability of the enzyme) and functional changes (i.e., reduction and loss of catalytic activity) (Nakanishi et al., 2001; Ulbrich et al., 1991). More specifically, it is conceivable that conformational changes in the domain A and B of alpha-amylase (containing the active site, calcium-binding site, and chloride-binding site), which are essential for the structural integrity and catalytic activity (Payan, 2004; Ramasubbu et al., 1996), are closely linked to the reduction and loss of the enzymatic activity.

1 In a subsequent experiment, aliquots of twenty selected saliva samples (covering a wide range of sAA activity, based on previous measurements from experiments 1 and 2) were again analyzed in duplicates to closely investigate the diluent ultra-pure water from two different sources, namely filtered water utilizing a water purification system (Adrona, Riga, Latvia) or commercially available LG-MS Chromasolv water (Honeywell, Seelze, Germany). Repeated measures ANOVA revealed a significant time effect (F(1.58, 29.94) = 95.02, p < .001; η² = .546); while the main effect of water (F(1.19, 50.92) = 95.02, p = .906; η² = .001) and the time x water interaction (F(2.33, 44.28) = 95.02, p = .739; η² = .018) were not significant, confirming sAA instability in ultra-pure water dilution.

2 For the PP condition, there was an outlier. The exclusion of the outlier did not change the results (Kendall’s Tau τ = −.06, p = .721).
We can only speculate as to why sAA is more prone to bind to plastic surfaces (PP < PS) compared to glass surfaces. The adsorption is believed to be determined by an interplay of various factors related to the adsorbate, the adsorbing surface, and environmental conditions (e.g., Nakanishi et al., 2001; Song et al., 1996). The properties of the adsorbing material may facilitate the interaction with the enzyme, for example, physical properties, chemical properties and composition, hydrophobicity, and electrical properties (Goebel-Stengel et al., 2011; Nakanishi et al., 2001; Suelter and DeLuca, 1983). Furthermore, the enzyme’s properties may determine whether and to what degree the enzyme is apt to attach to certain surfaces, for example, the charge, size, stability of the structure (‘hard proteins’ with high internal stability, ‘soft proteins’ with low internal stability), amino acid composition, and steric conformation of the protein (Nakanishi et al., 2001). However, some researchers argue that it is almost impossible to predict how peptides and proteins act on surfaces, as previous research failed to identify clear patterns in terms of the properties of adsorbates and the adsorption affinity to specific surfaces (Goebel-Stengel et al., 2011). Finally, environmental factors were found to affect adsorption, such as the pretreatment of the surface or diluent (e.g., Goebel-Stengel et al., 2011).

In our study, the usage of glass per se was unable to prevent the reduction of sAA activity in diluted samples, supporting the hypothesis that adsorption is an interaction of the adsorbed protein, adsorbing surface, and diluent. In contrast to NaCl solution and PBS, samples which were diluted with H2O in glass tubes were found to show a steady decrease in sAA activity. This finding was in contrast to our initial assumption that the stability of sAA activity in diluted samples is comparable between pH-neutral diluents. One possible explanation for this finding may lie in the absence of ions in H2O, while both NaCl solution and PBS contain Na+ and Cl− ions. There is some evidence that the addition of salts can reduce adsorption of proteins to surfaces (e.g., Smith et al., 1978), with salts possibly reducing electrostatic interactions with the anionically charged glass (Suelter and DeLuca, 1983). Although saliva naturally contains various ions (e.g., Na+, K+, Ca2+, Mg2+, HCO3−, PO43−) (Humphrey and Williamson, 2001), their concentration might be diminishingly low due to the high dilution with ultra-pure water, meaning that the beneficial effects of metallic ions such as sodium and calcium3 on enzymatic activity might vanish (Tatah and Oitoju, 2015). Furthermore, the presence of chloride ions (Cl−) close to the active site of the alpha-amylase is required for the catalytic activity of alpha-amylase (Payan, 2004). It is conceivable that in highly diluted saliva samples, however, the likelihood of the encounter between chloride ions and SAA is diminished, which may result in reduced sAA activities.

Notably, diluted saliva samples were not equally affected by this phenomenon (see Table 1). It is not surprising that saliva samples with initially relatively low sAA activity were more likely to be impacted by inactivation of enzymes due to adsorption or high dilution (Nakanishi et al., 2001). In addition, individuals may differ not only in their genetically determined amount of sAA activity (e.g., Santos et al., 2012; Yang et al., 2015) but also in their composition of saliva in general (e.g., diluted ions, bacteria composition), behavioral habits (e.g., smoking, diet) and health status, which may influence sAA activity (e.g., Rohleder and Nater, 2009; Straehler et al., 2017). Circadian rhythm of the salivary flow rate, saliva composition (Dawes, 1972) and sAA activity (Nater

3 The presence of calcium ions (Ca2+) plays a crucial role in maintaining the structural integrity and stability of alpha-amylase (Payan, 2004).
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Declaration of Competing Interest

The authors report no declarations of interest.

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