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Variation of human salivary alpha-amylase proteoforms in three stimulation models

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Abstract

Objectives To evaluate the sAA proteoforms' expression during different stimulation situations.

Materials and methods This study evaluated the salivary alpha-amylase (sAA) proteoforms' behavior by western blot (WB) analysis and high-resolution mass spectrometry (LC-MS/MS) in different situations that produce increases in sAA activity. For this purpose, six healthy women with a similar body mass index, age, and fit, underwent different sAA stimulation tests, such as acetic acid stimulation, psychological stress using the standardized Trier social stress test, and physical effort using the Cooper treadmill test.

Results The three models showed an increase in sAA activity. The WB demonstrated seven common bands observed in the six women (band one at 59 kDa, two at 56 kDa, three at 48 kDa, four at 45 kDa, five at 41 kDa, six at 36 kDa, and seven at 14 kDa), in which sAA protein was identified. The individual WB analysis showed that band two, which corresponded to the native non-glycosylated sAA proteoform, had a higher increase after the three sAA stimulation inducers, and this band was also the only proteoform correlated with sAA activity ($r = 0.56$, $P = 0.001$). In addition, when the label-free quantification analysis was performed, the different proteoforms showed different responses depending on the type of stimulation.

Conclusions This preliminary study showed that the diverse sAA proteoforms' expression depends on the different stimulation models.

Clinical relevance This study opens new perspectives and challenges for the use of the different alpha-amylase proteoforms as possible biomarkers in addition to the sAA activity.

Keywords Salivary alpha-amylase · Proteoforms · Inter-individual variability · Stress

Introduction

Salivary alpha-amylase (sAA; EC 3.2.1.1) is secreted by the salivary glands, mainly in the parotid (80% of the total sAA) but also by the submandibular, and the sublingual glands [1], and it increases in response to stress induction, as occurs in psychological and intense physical stress situations [2–4]. Previous studies suggest sAA as a surrogate marker of the autonomic nervous system (ANS) [1, 5, 6]. Usually, sAA is measured in saliva by its enzymatic activity using spectrophotometric assays, reported as international units of sAA activity per milliliter of sample analyzed (IU/mL) [1].

sAA is a complex enzyme composed of a main subset with a molecular weight (MW) around 56 kDa (the native or non-glycosylated form) and 59 kDa (the glycosylated form). A particularity of this enzyme is its high inter-individual variability in both basal activity and its response to stress, not only in humans [1, 7], but in other species such as horses [8] and pigs [9, 10]. It has been reported that the variability in humans could be due to a number of physiological, psychosocial, and environmental factors, such as salivary gland development related to age [7, 11], circadian rhythms [12], stress levels

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[13], eating habits [14], a fast-reacting biological response, habitual smoking, personal fitness, or psychopathologies [1]. In addition, the variability could be influenced by the fact that the *AMY1* gene, which codes for sAA, has one of the most variable copy number variation (CNV) loci in the human genome [7, 14, 15], with at least 12 distinct phenotypes for the sAA [16].

In addition to the two main forms, sAA has other proteoforms with lower MW, defined by Smith and Kelleher [17] as different molecular forms in which the protein product of a single gene can be found, that can be originated by genetic variations, alternatively spliced RNA transcripts and post-translational modifications [18, 19], or modifications due to post-secretion in the oral cavity [19]. We postulated that these proteoforms, in addition to varying between individuals in their basal distribution, can have a different response depending on the situation that induces increases in total sAA activity.

The aim of this study was to evaluate the sAA proteoforms' behavior during different stimulation situations, such as acetic acid stimulation [7], psychological stress (modelled by the standardized Trier social stress test [TSST]), and physical effort (Cooper treadmill test). For that purpose, western blot (WB) analysis using purified polyclonal antibodies against human sAA and the identification of sAA proteoforms using high-resolution mass spectrometry (LC-MS/MS) were performed on human saliva samples of subjects who underwent the different stimulations. This innovative approach will help to better understand the composition of human sAA and how different naturalistic stimulation situations can influence the sAA response.

Materials and methods

Participants and description of the stimulation models

Six healthy women (average age = 30.8 ± 5.2 years, body mass index [BMI] = 20.6 ± 1.5 kg/m^2) participated in three sAA stimulation models, as indicated in Fig. 1. All were informed about the procedure, sampling methods, and the objective of the experiment. Saliva collection from each model was undertaken on different days between 18:30 and 19:30 because this was the time of the day in which all participants were available. Although this is the period of the day in which the highest sAA basal level secretion has been described, this design allowed to use a homogenous period of time in all our experimental procedures and therefore to minimize the possible diurnal variations due to the fact of sampling at different times of the day [1].

The acetic acid stimulation was performed as previously described [7] (Table 1). Saliva samples were collected just before (Tb) and just after (T+0) and 15 min later (T+15) than the acid stimulation.

The psychological stress model performed was the TSST [20, 21] (Table 1). Saliva samples were taken while each participant rested in the isolation room 5 min before the interview (T−0), just after the arithmetic task (T+0), and 15 min later (T+15). To evaluate the subjective anxiety suffered during the psychological model, the Spanish version of the State-Trait Anxiety Inventory (STAI) [22, 23] was performed at T−0 and T+0. It is composed of two scales: the trait form (STAI-Trait) and the state form (STAI-State). Each scale consists of 20 items using a four-point graded response scale ranging from "almost never" or "not at all" (0) to "almost always" or "very much" (3), respectively. The scores of the items from each scale were added. Cronbach's alpha coefficient was used to evaluate the questionnaire [24].

The physical stress model performed was the Cooper treadmill test [25] (Table 1). The average 90% of maximal heart rate (MHR) in the six participants was 167.6 ± 3.2 beats/min. Heart rate data was collected using a Polar® RS800CX human heart rate monitor (Polar®, Finland) [26]. The aerobic fitness level [27] was tested by the maximum oxygen uptake (VO_2max) estimated as VO_2max (mL/kg/min) = (22.351 × distance covered in kilometers) − 11.288 after the Cooper treadmill test [25]. Saliva samples were taken just before the run (Tb), just after (T+0), and 15 min later (T+15).

Sample collection

Saliva was collected over 1 min by passive flow without tongue movements [1, 28], using 5 mL standard micro-centrifuge polystyrene tubes with round bottoms (12 × 75 mm). A volume higher than 400 μL was obtained in all samplings with this procedure. The passive flow collection was preferred instead of the stimulated collection by mastication method using salivettes because it has been described that any absorbent material could interfere in sAA measurements [29] and chewing stimulates mechanically sAA release; therefore, the different frequency or intensity of chewing could be a potential factor influencing sAA concentrations. To minimize any potential physiological effects in responses to sAA, 1 h before the beginning of the saliva collection, the participants were not allowed to eat, have coffee or caffeinated soft drinks, or consume dairy products; alcohol consumers and smokers were excluded from the test [1]. Each subject rinsed her mouth with water before saliva collection to avoid contamination of saliva samples with food components. In particular, in the acid stimulation model, the participants were asked to keep their tongue tips slightly upward when collecting acid-stimulated saliva to avoid it mixing with the acid filter paper. All samples were refrigerated or stored on ice

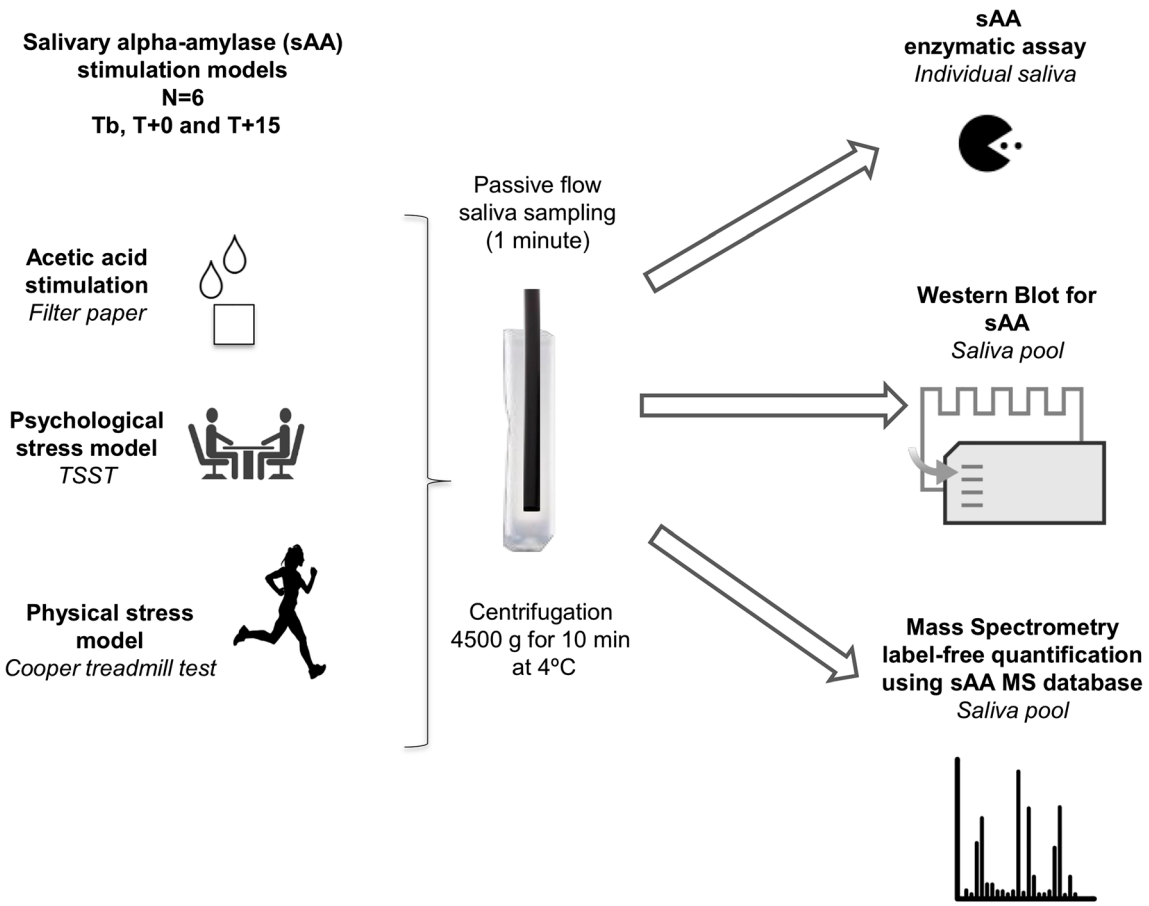

Fig. 1 Experimental workflow for the evaluation of the variation of human salivary alpha-amylase (sAA) proteoforms in three stimulation models

after their collection until arrival at the laboratory. At the laboratory, the saliva samples were centrifuged at 4500×*g* for 10 min at 4 °C to remove cells [28], and the supernatant was obtained. In order to minimize any bias possibly due to individual variations in sAA identification, a pool of saliva collected from the six individuals was prepared. Saliva samples were stored at − 80 °C until analysis, less than one month in all cases.

sAA enzymatic assay

sAA activity was measured using a colorimetric commercial kit (Alpha-Amylase, Beckman Coulter Inc., Fullerton, CA, USA) following the International Medicine (IFCC) method [1, 30], as previously reported and validated [31]. sAA was expressed only as U/mL [32].

Table 1 Description of the sAA stimulation models

sAA stimulation models	Description
Acetic acid stimulation	By placing a piece of filter paper (1 × 1 cm) containing acetic acid in the tip of each participant's tongue for 1min.
Psychological stress model	Each participant was invited to an isolated room where she was asked to prepare for a notional job interview for 5 min and then confronted with a committee consisting of two authoritatively and aloofly acting men investigators leading the 5-min interview session. Finally, the session was followed by a 5-min arithmetic task (countdown from 2043 in steps of 17) in front of the investigators.
Physical stress model	The participants run during 12 min the maximum possible distance at 90% of the expected maximal heart rate (MHR), which was defined as MHR = 208 − (0.7 × age).

SDS-PAGE and western blot for sAA

Eight mini polyacrylamide gels containing 0.1% (*w*/*v*) sodium dodecyl sulfate (SDS-PAGE) were performed, with a separating gel prepared in 12% (*w*/*v*) and a stacker gel prepared in 4% (*w*/*v*) according to the methodology described by Laemmli [33]. For total protein detection, one gel was prepared adding the times Tb from the acetic acid stimulation and T+0 in each situation. At each time, a pool was performed by mixing equal amount of saliva (100 μL) from all the six participants, and from each pool, saliva was added to the gel at 15.0 μg of total protein per lane. The gel was stained with 1% *w*/*v* Coomassie Brilliant Blue (Coomassie® Brilliant Blue R 250, Bio-Rad Laboratories S. A, CA, USA) for 24 h, followed by destaining with 5% *v*/*v* ethanol and 7% *v*/*v* acetic acid for 12 h. The other seven gels were prepared to carry out the WB using an indirect detection method [34], where in six gels, the proteins at Tb from the acetic acid stimulation and T+0 at each situation from each participant's saliva samples (one gel for each participant) and in one of them from the saliva pool described above were separated and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). Saliva samples for the WB analysis were added to the gels at 3.5 μg of total protein per lane. The rabbit polyclonal antibody against human sAA produced by the researchers' laboratory (described below) at 1:6000 dilution was used as a primary antibody, while horseradish peroxidase (HRP)-conjugated goat polyclonal antibody anti-rabbit (ab 6721, Abcam, Cambridge, UK) at 1:2000 was employed as a secondary antibody, which was detected using a Pierce ECL2 kit (Pierce, Thermo Fisher Scientific, USA) and an ImageQuant™ scanner (GE Healthcare, Uppsala, Sweden). Total proteins were determined using Lowry's method [35] in all saliva samples. Quantification of each sAA's protein band (μg) shown in the WB was estimated by comparing a natural human sAA protein (77875, Abcam, Cambridge, UK) of known quantity [7] and analyzed using ImageQuant™ TL 8.1 (GE Healthcare, Uppsala, Sweden).

In-gel digestion and mass spectrometric analysis

The pools made at Tb and T+0 in each situation were used for the identification analysis of sAA. The common bands observed in the pools, and also in the individual WBs, were excised from the preparative parallel SDS-PAGE gel and refrigerated for 24 h until analysis. They were then washed sequentially with 150 μL 50% EtOH, then 150 μL 50 mM AB (pH 8.4) and repeated once before drying on a speedvac. The bands were reduced (10 mM DTT, 56 °C, 1 h) and alkylated (55 mM IAA, 37 °C, 45 min), before dehydration (50% EtOH, 50 mM AB (pH 8.4)) and drying on a speedvac. The bands were recovered with a trypsin solution (12.5 mg/mL in 25 mM ammonium carbonate) and digested overnight at 37 °C. The digestion was stopped by adding 15 μL of pure formic acid (pH < 4). The generated tryptic peptides were desalted on BRAVO AssayMap (Agilent Technologies) with C18 Tips primed with 50 μL of 70% ACN/0.1% TFA, equilibrated with 50 μL of 0.1% TFA, loaded with sample, washed twice with 50 μL of 0.1% TFA, and eluted with 50 μL of 70% ACN/0.1% TFA. The samples were dried on a vacuum concentrator (Labconco, Kansas city, USA) and resuspended in 10 μL of *A* phase (*A* = 0.1% formic acid, 2% acetonitrile in water) for LC-MS/MS injection.

For the mass spectrometric analysis, 7 μL of samples was injected on nanoRSLC Elute (Bruker). The NanoFlow LC was coupled to the QTOF MS instrument (Impact II, Bruker Daltonics) through a captive spray ion source operating with a nanobooster. In the LC part, the samples were desalted and preconcentrated on-line on a PepMap u-precolumn (300 μm × 5 mm, C18 PepMap 100, 5 μm, 100 Å). The peptides were transferred to an analytical column (75 μm × 500 mm; Acclaim Pepmap RSLC, C18, 2 μm, 100 Å) to perform separation. Peptides were separated using a 45 min LC gradient of acetonitrile (7-30 %) in 0.1% formic acid in water at a flow rate of 400 nL/min (50°C).

Data-dependent acquisition (DDA) was performed to identify peptides and a lock-mass (*m*/*z* 1222, Hexakis (1H, 1H, 4H-hexafluorobutyloxy) phosphazine) was used as an internal calibrator. Instant Expertise software selected as many as possible of the most intense ions per cycle of 3 s and active exclusion was performed after one spectrum during 2 min unless the precursor ion exhibited an intensity three times higher than the previous scan.

Peptide identification and label-free quantification

All MS/MS spectra were searched against *Homo sapiens* in the SwissProt database by using the Mascot v 2.4.1 algorithm (Matrix Science, http://www.matrixscience.com/) with the following settings: database: swissprot_2017_28_07; enzyme: trypsin; variable modifications: oxidation (M) and deamidated (N,Q); fixed modifications: carbamidomethyl (C); missed cleavages: 2; taxonomy: *Homo sapiens* (human); instrument type CID: ESI-QUAD-TOF; peptide tolerance: 10.0 ppm; MS/MS tolerance: 0.05 Da; peptide charge: 1+, 2+, and 3+; mass: monoisotopic; C13: 1; minimum peptide length: 5; peptide decoy: ON; adjust FDR [%]: 1; percolator: on; ions score cut-off: 12; ions score threshold for significant peptide IDs: 12.

Label-free quantification and comparison based on the MS1 profiles were performed by Skyline software. Skyline 2.6 was used to process the raw data. The peptides identified were recovered from the Mascot result. To perform this task, Mascot DAT was exported for each identification result. These files were combined by Skyline in the library generation step.

Peptide sequences, MSMS spectra, and retention times were contained inside the library. Skyline 2.6 (MacCoss Lab.) was used to generate these with a cut-off score of 0.95. Based on the list of peptides identified and retention times, masses were extracted from the raw data by profile analysis. The transition settings were adjusted to extract the monoisotopic mass for the precursor ion with a positive charge of 1/2/3/4 in the mass range of 300–1400 *m/z*.

MS1 filtering operated on TOF data, resolving power at 60,000, isotope analysis-based count, and on four peaks. For the chromatographic properties, the retention time tolerance was 10 min. At peptide level, peptides contained in the library were directly imported as compounds. Peaks were automatically integrated by Skyline. Manual checking was performed before exporting the results in an Excel file. The area under curve (AUC) was measured for every sAA peptide to perform the sAA MS profiles.

Statistical analysis

A Friedman test and Dunn's multiple comparisons test were used to determine if sAA activity values and salivary total proteins at different times in each situation were statistically different. A Wilcoxon matched-pairs signed rank test (2-tailed) was performed in the psychological situation to evaluate possible statistical differences in the STAI values at different times. Two-way ANOVAs of repeated measures and Tukey's multiple comparisons test were carried out to evaluate possible differences in each common band between the different situations (basal time, acetic acid stimulation, and psychological and physical stress models) and in each situation between the different bands, after a logarithmically transformation by applying the formula $\ln = \ln(x+1)$ [1, 32], that restored normality. A Spearman correlation test was performed between the sAA activity results and the estimated μg of each common band in the individual WBs from the six participants at Tb and at T+0 in the three sAA stimulation models. An r value of less than 0.3 was considered to be a negligible correlation, following the rule of thumb [36]. Finally, the inter-individual variability of the sAA activity and the estimated amount of the bands marked in the WB in each naturalistic situation were expressed as the CV value, calculated as the SD divided by the mean of the values of the different individuals and then multiplied by 100. An ordinary one-way ANOVA and Tukey's multiple comparisons test were used to verify differences between the inter-individual variability of the sAA activity and the estimated amount of the bands marked in the WB, after a logarithmically transformation. The significance level used in each case was $P < 0.05$. These statistical analyses were calculated using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Salivary alpha-amylase activity

Median and individual values in the different stimulations appear in Fig. 2. Median sAA activity values showed significant changes in the acetic acid stimulation ($F_2 = 10.3$, $P = 0.002$), the psychological stress model ($F_2 = 12.0$, $P < 0.001$), and the physical stress model ($F_2 = 10.3$, $P = 0.002$), with an increase at T+0 compared to Tb in the acetic acid stimulation ($P = 0.004$) and in the physical stress model ($P = 0.004$), and an increase in T+0 compared to T−0 in the psychological stress model ($P = 0.002$). The average inter-individual variation in the sAA activity in all the models used in our study was 50.2 ± 15.8% (Table 3). Total protein in saliva was also increased at T+0 in the psychological ($P = 0.010$) and physical ($P = 0.010$) model compared to Tb. However, it did not show significant changes in acetic acid stimulation ($P = 0.356$).

The psychological stress model showed significantly higher values ($Z = -2.2$, $P = 0.0313$) in the STAI at T+0 with respect to T−0 (Table 2). Cronbach's alpha coefficient was 0.70. The VO_2max estimated after the Cooper treadmill test was 36.5 ± 4.0 mL/kg/min.

Western blot analysis of sAA

Figure 3 shows the sAA WB image from the six participants' saliva pools. Seven bands always appeared in all the individual WB images (Online Resource 1, in which WBs of all the six participants are included) that we called common bands, which are bands one (at 59 ± 0.8 kDa), two (at 56 ± 1.0 kDa), three (at 48 ± 1.5 kDa), four (at 45 ± 2 kDa), five (at 41 ± 1.9 kDa), six (at 36 ± 3.1 kDa), and seven (at 14 ± 1.9 kDa). In addition, bands of different MW (i.e., 153 ± 11 kDa, 117 ± 8 kDa, 97 ± 1 kDa, 71 ± 1 kDa, 65 ± 1 kDa, 21 ± 1 kDa, 18 ± 1 kDa, 11 ± 1 kDa) appeared to be differentially expressed depending on the subject and the sAA stimulation, and we called them uncommon bands (Online Resource 1).

Identification of sAA using high-resolution mass spectrometry

Each of the seven common protein bands of the different stimulation models were excised from SDS-PAGE, digested with trypsin, and analyzed using high-resolution mass spectrometry. The sAA protein was identified in every band corresponding to the western blot and all peptides from sAA protein sequence were gathered and included for the label-free quantification analysis. sAA was identified with at least eight peptides and maximum 87 peptides, corresponding to 19.6 to 85.9% in terms of sequence coverage.

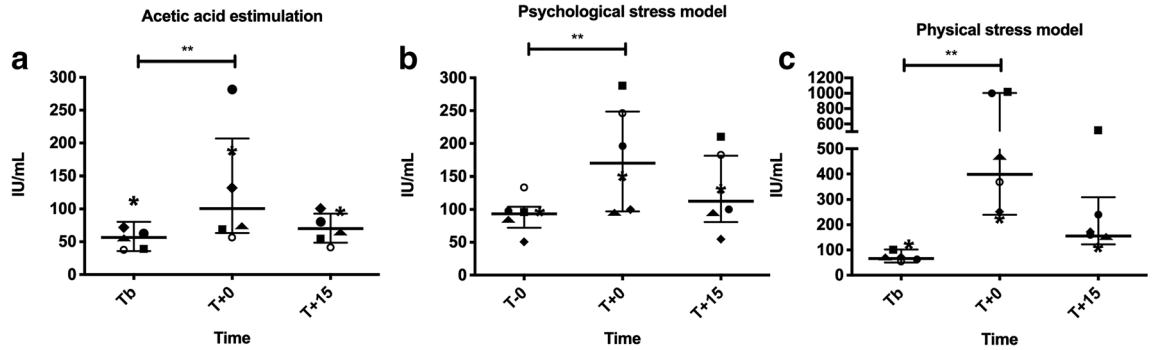

Fig. 2 Salivary alpha-amylase (sAA) results in three different stimulation models. **a** Acetic acid stimulation at basal time (Tb), just after (T+0), and 15 min later (T+15) the stimulation. **b** Psychological stress model by the standardized Trier social stress test (TSST) 5 min before the test (T−0), just after (T+0), and 15 min later (T+15). **c** Physical stress model by the Cooper treadmill test just before (Tb), just after (T+0), and 15 min later (T+15). Graphs show the individual values ($n = 6$) of each participant (black circle, subject one, white circle, subject two, asterisk, subject three, black triangle, subject four, black square, subject five, black diamond, subject six). Asterisks indicate significant post hoc difference (Dunn's multiple comparisons test) with respect the previous time: $^{**}P < 0.01$

Peptides identified in bands one, two, three, and four showed sAA peptides along the native sequence with a very good sequence coverage (> 80%). The sAA peptides in band five were identified only on C-terminus and missing on the N-terminus (0–209). The sAA peptides in band six were identified only on the N-terminus, but they were not detected on the C-terminus part of the sequence (306–510) that corresponds to a C-terminus truncated proteoform. The sAA peptides in band seven were found to be missing between the 154–266 position and 420–471 position. Additional, MS data on the identification of sAA proteoforms and the other proteins identified are specified in the Online Resource 2.

Table 2 The Spanish version of the State-Trait Anxiety Inventory (STAI) results from the six participants in the Trier social stress test (TSST) 5 min before the interview (T−0) and just after the arithmetic task (T+0). It is composed of the trait form (STAI-Trait) and the state form (STAI-State)

		STAI		
		State	Trait	Total
Subject 1	T−0	30	21	51
	T+0	31		52
Subject 2	T−0	21	14	35
	T+0	24		38
Subject 3	T−0	32	22	54
	T+0	34		56
Subject 4	T−0	27	36	63
	T+0	29		65
Subject 5	T−0	21	18	39
	T+0	22		40
Subject 6	T−0	26	32	58
	T+0	27		59

Variation of sAA in the different stimulation models and correlation with its activity

Estimated sAA quantification results from the individual WB analysis were showed in Fig. 4. Western blot analysis exhibited significant variations between sAA quantity in the different stimulation models ($F_{6,120} = 165.9$, $P < 0.001$). Bands one and two were significantly more abundant ($P < 0.001$) than the bands three, four, five, six, and seven at Tb and at T+0 in all sAA stimulation models. The non-glycosylated sAA band (band two) was the only one that showed changes that were different depending on the situation evaluated ($F_{3,105} = 4.6$, $P < 0.05$), since it showed significant higher increases in the amount at T+0 in the psychological stress model (1.43 median-folds, $P = 0.047$) and in the physical stress model (1.93 median-folds, $P < 0.001$) compared to Tb (Fig. 4), and at T+0 in the physical stress model compared to the acetic acid stimulation (1.61 median-folds, $P = 0.016$) and to the psychological stress model (1.35 median-folds, $P = 0.048$) (Fig. 4). In addition, this non-glycosylated sAA band (band two) was the only one that was found to be correlated with sAA activity ($r = 0.56$, 95% confidence interval [CI] 0.15–0.81, $P = 0.001$). Although the estimated sAA amount from bands one, two, three, five, six, and seven had lower average inter-individual variability than the activity results, it was only significant for the band one ($P = 0.031$) (Table 3).

Label-free quantification of sAA using high-resolution mass spectrometry

Figures 5 and 6 display the four different features for sAA proteoforms' identification. Only the bands one, four, six, and seven were showed because are representative of the main sAA proteoforms identified: native, C-terminus truncated, and

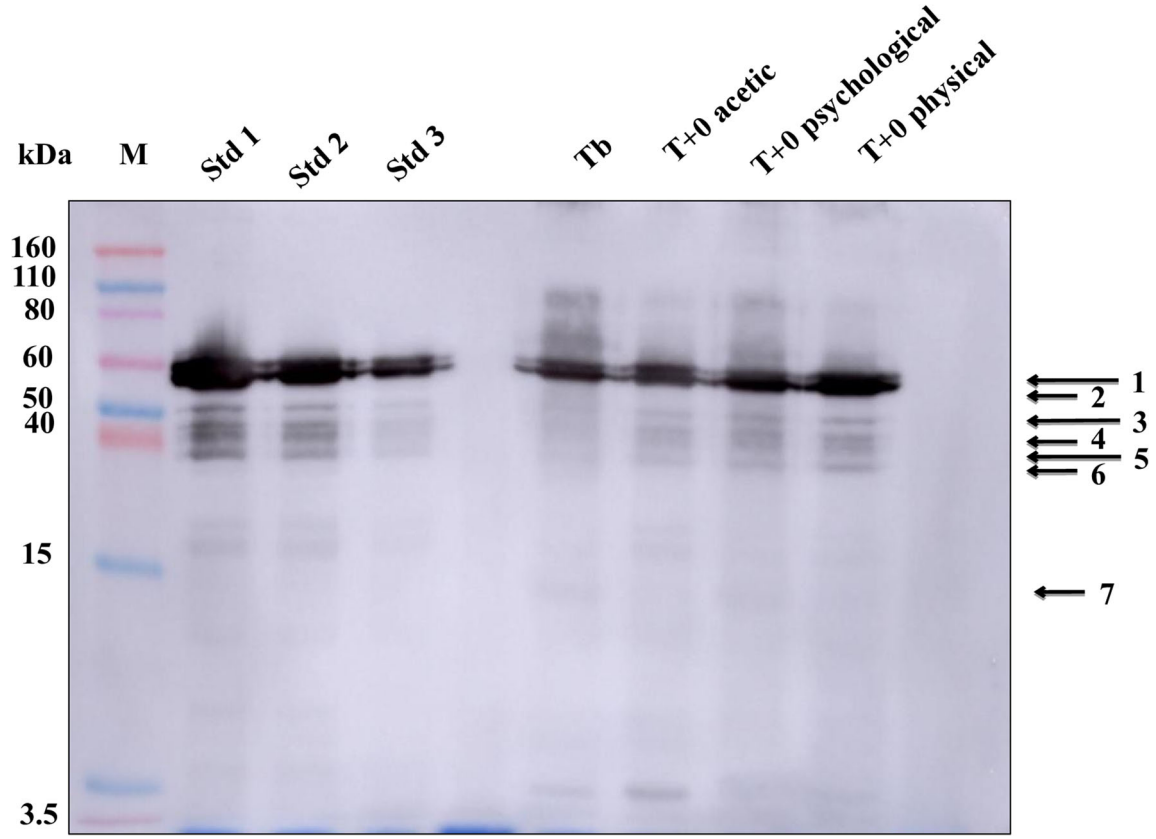

Fig. 3 Western blot for sAA detection from a six participants' saliva pool in three different stimulation models: acetic acid stimulation at basal time (Tb) and just after (T+0), psychological stress model by the Trier social stress test (TSST) just after (T+0), and physical stress model by the Cooper treadmill just after (T+0). The standards (Std) were natural commercial purified human sAA protein (77875, Abcam, Cambridge, UK) of known quantity (Std 1 = 3.5 μg; Std 2 = 1.75 μg; Std 3 = 0.44 μg). Molecular weight markers (Novex Sharp Pre-Stained, Invitrogen, Carlsbad, California). Numbers on the side (1–7) label the common bands that always appeared in the individual WB images from the six participants

alternative proteoforms (i.e., exhibiting peptides along the protein sequence but exhibited a lower molecular mass). The physical stress model seemed to lead at T+0 to an overexpression of the native form of sAA (59 kDa, band one) compared

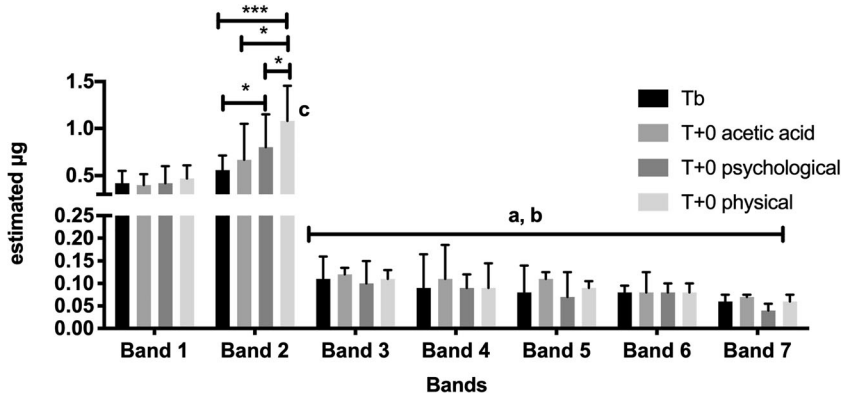

Fig. 4 Estimated μg of each common band marked in the individual western blot images from six participants in four different salivary alpha-amylase (sAA) stimulations. Bars show the median values with the interquartile range at basal time (Tb) and after acetic acid stimulation (T+0 acetic acid), psychological stress model by the Trier social stress test (TSST) (T+0 psychological), and physical stress model by the Cooper treadmill test (T+0 physical). Asterisks indicate significant post hoc differences (Tukey's multiple comparisons test) between the sAA stimulations in each band: $*P < 0.05$; $***P < 0.001$. The letters show significant post hoc differences (Tukey's multiple comparisons test) between the bands in each sAA stimulation: a = $P < 0.001$ with respect the band one at Tb and at T+0 in all sAA stimulations; b = $P < 0.001$ with respect the band two at Tb and T+0 in all sAA stimulations; c = with respect the band one at T+0 in the acetic acid stimulation ($P < 0.001$), in the psychological stress model ($P < 0.001$), and in the physical stress model ($P < 0.001$)

Table 3 Inter-individual variability (%) of salivary alpha-amylase (sAA) measured by enzymatic activity (U/L) and the estimated amount of the common bands marked in the individual WBs (μg), in the three different situations

Inter-individual variability (%)									
		sAA activity (IU/L)	Estimated amount of the common bands marked in the individual WBs (μg)						
			1	2	3	4	5	6	7
Acetic acid stimulation	Tb	47.1	32.8	28.6	54	62.8	61.3	42.9	31.2
	T+0	68.9	26.1	34.9	50.3	56.6	38	51.5	26.5
	T+15	37.3	nm[a]	nm	nm	nm	nm	nm	nm
Psychological stress model	T−0	26.8	nm	nm	nm	nm	nm	nm	nm
	T+0	44.6	31.1	42.6	50.5	44.7	51.3	47.5	79.4
	T+15	45.7	nm	nm	nm	nm	nm	nm	nm
Physical stress model	Tb	41.5	nm	nm	nm	nm	nm	nm	nm
	T+0	67.7	17.9	36.5	44.6	51.5	29.2	33.2	49.8
	T+15	72.1	nm	nm	nm	nm	nm	nm	nm
	Mean	50.2	27*	35.6	49.8	53.9	44.9	43.8	46.7
	SD	15.8	6.7	5.7	3.9	7.7	14.2	7.9	24

Asterisk indicates significant post hoc difference (Tukey's multiple comparisons test) with respect sAA activity inter-individual variability: $*P < 0.05$

[a] nm, not measured

to the acetic acid stimulation at T+0, psychological stress model at T+0, and Tb, respectively (Fig. 5a). In addition, the native sAA isoform 56 kDa (band two) and the sAA proteoform 48 kDa (band three) showed the same characteristics as band one. Peptides identified in sAA proteoform 45 kDa (band four) showed that N-term peptides (15–209) were less abundant for acetic acid stimulation at T+0 compared to other stimulations. It was found to be overexpressed for the physical stress model at T+0 compared to the psychological stress model at T+0, acetic acid stimulation at T+0, and Tb, respectively (Fig. 5b). sAA peptides from the proteoform 41 kDa (band five) were identified all along the protein sequence. N-term peptides (15–209) seemed to be less abundant than central and C-term peptides for every stimulation model at T+0 compared to Tb, thus, exhibiting a different profile. Proteoforms were found to be overexpressed for the physical stress model at T+0 compared to acetic acid stimulation at T+0, Tb, and the psychological stress model at T+0, respectively. The proteoform 36 kDa (band six) was found to be under expressed in the acetic acid stimulation at T+0 compared to the psychological and physical stress models at T+0, and to Tb, respectively (Fig. 6a). Finally, the N-terminus part of sAA proteoform 14 kDa (band seven) exhibited overexpressed for the acetic acid stimulation at T+0 compared to the physical stress and psychological stress models at T+0, and to Tb. While the C-terminus part exhibited a different pattern with overexpression of sAA proteoform for the physical stress model at T+0 compared to the psychological stress model at T+0, acetic acid stimulation at T+0, and Tb (Fig. 6b).

Discussion

sAA is being widely studied as a tool to evaluate stress in psychological and physical situations in humans [1–4, 37] and animals [8–10, 38]. However, more knowledge about this enzyme would be desirable to better understand the cause of the high variability in its response. In this paper, proteomics studies have been conducted on the sAA of a group of healthy women during different naturalistic situations, such as an acetic acid stimulation, considered to be a mechanical gustatory stimuli [7], a psychological social stress model [37], and a physical stress model, in order to evaluate the expression of the different sAA proteoforms.

Although it is recommended to collect saliva during 2 or 5 min by the passive flow collection method [1], with our procedure, only 1 min was enough to obtain the volume needed for all the assays performed in our study. Regarding the three models used in our study, the acetic acid stimulation resulted in an increase of sAA activity. Yang et al. [7] reported an increase of sAA activity after citric acid stimulation in Chinese children, but not in adults. For this reason, the authors used other kind of acid as gustatory stimulus (acetic acid). According to our results, the nature of the acid used (citric acid vs acetic acid) could be the reason why sAA increased significantly in adults in our study since the same method that Yang et al. [7] used was employed. Regarding the model of psychological stress, although we used a simplified version of the TSST, it produced increases of the sAA activity levels in all the participants, as well as in their subjective anxiety (indicated by high STAI values) suffered during the

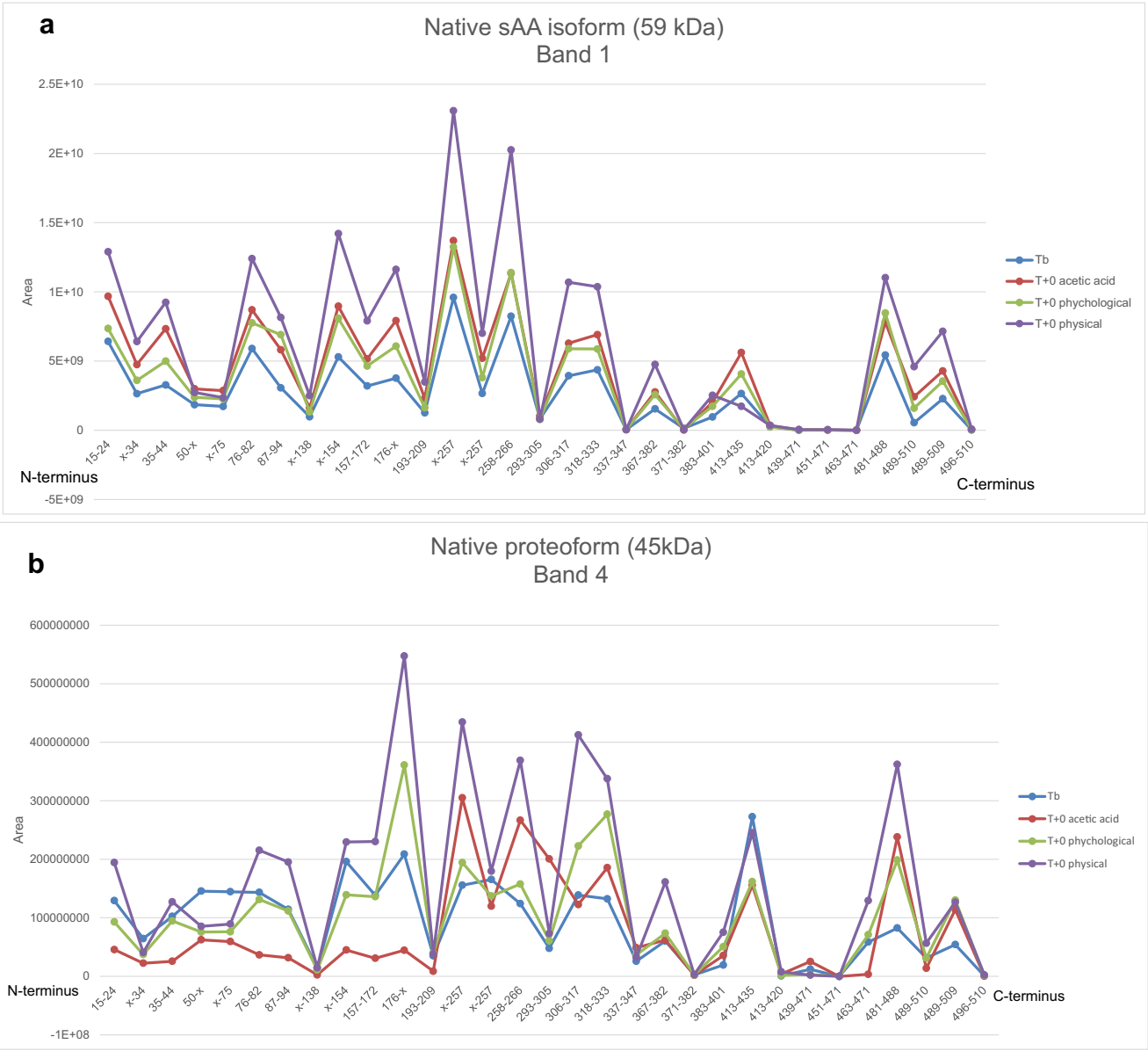

Fig. 5 Salivary alpha-amylase (sAA) peptide features of the identified peptides using LC-MS/MS. Peptides identified were plotted based on MS pics area and placed on the sAA protein sequence. **a** Corresponding to native glycosylated sAA proteoform at 59 kDa. **b** Corresponding to sAA proteoform at 45 kDa exhibiting peptides along the protein sequence but differing by molecular weight

psychological situation, which produced an acceptable Cronbach's alpha coefficient level [39]. It is widely described that sAA activity increases after different psychological stressors [2] such as social stressors induced by laboratory tests as the TSST [2, 37]. The Cooper treadmill test gave each individual in our study a similar average VO_2max for the respective age group, according to the American Heart Association Cardiorespiratory Fitness Classification [27]; therefore, the subjects evaluated had a similar fit. The physical model led to increases in sAA, being in coherence with the findings about increases of sAA activity in intense exercise [4] such as in bicycle ergometer exercises during 90 min or in treadmill running with a peak of 70% VO_2max in endurance-trained males. Although other salivary proteins could increase in psychological and physical stimulations since salivary total proteins increased significantly at T+0 in the psychological and physical models compared to Tb, sAA accounts for 40 to 50% of the total salivary gland-produced protein [2]. So it could be indicated that when there is an increase of total proteins in saliva, the main protein that increases would be sAA. Overall, it could be concluded that the three experimental models used in our study produced the expected effect. Nonetheless, high inter-individual variability in the activity levels was detected in all the situations despite the homogeneity of the experimental group, as previously reported [37]. In fact, this inter-individual variation was also

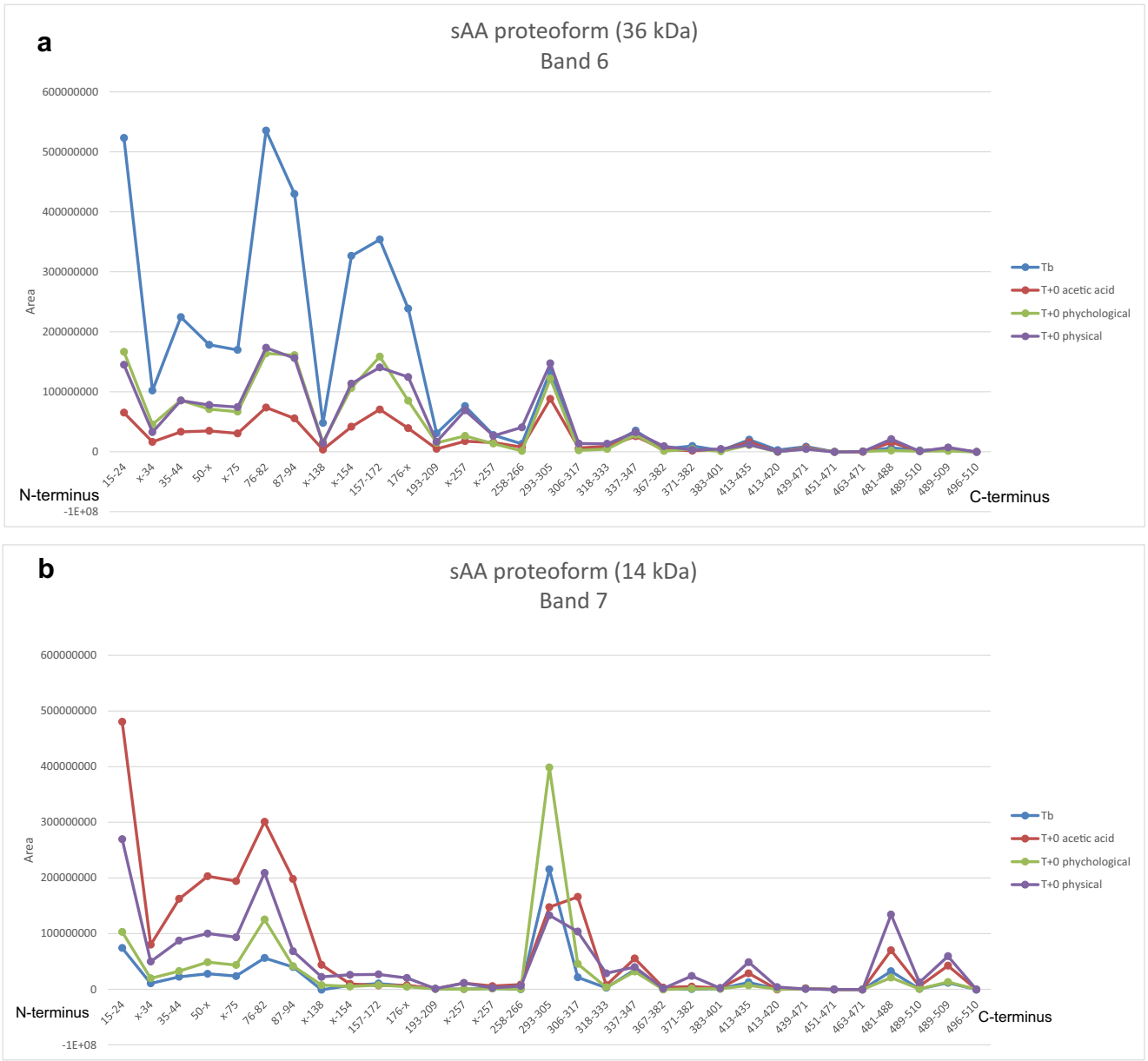

Fig. 6 Salivary alpha-amylase (sAA) peptide features of the identified peptides using LC-MS/MS. Peptides identified were plotted based on MS pics area and placed on the sAA protein sequence. **a** Corresponding to sAA proteoform at 36 kDa with C-terminus truncation. **b** Corresponding to sAA proteoform at 14 kDa exhibiting peptides in N-terminus and C-terminus region but differing by molecular weight

observed at basal level. This result evidences that sAA activity could be influenced by a variety of factors (age, somatic health, eating habits, sleep, or stress levels) that could be unique for each individual [7, 40, 41].

A high-resolution mass spectrometry analysis of sAA proteoforms in a pool of saliva from six female subjects allowed us to identify different sAA proteoforms. Our results suggest the presence of a different subset of proteoforms already described in human saliva [19, 42]. We identified a sAA native form in the WB bands corresponding to the glycosylated and non-glycosylated amylase at 59 and 56 kDa as expected. These two main sAA native forms are well known and have already been described [19]. We also identified C-terminus truncated sAA proteoforms (e.g., band six) and N-terminus truncated sAA proteoforms (e.g., band five). Hirtz et al. [42] suggested that these truncations should occur in the oral cavity, despite the presence of protease inhibitors in saliva and should be a specific process, impacting particularly the sAA terminal sequence. We also identified alternative sAA proteoforms (e.g., band four) with the presence of peptides along the whole protein sequence but exhibiting a lower MW (45 kDa), whose origin is still not explained.

When the individual WB analysis was performed, it showed higher estimated amounts of the native non-

glycosylated sAA proteoform (band two) in all the three models of stimulation compared to Tb, and this proteoform was found to be moderately correlated with the sAA activity, whereas other bands were not correlated. Probably, this contributed to the significant correlations between sAA activity and concentration previously described [32]. In addition to the non-glycosylated band, changes in other bands, such as bands one (native glycosylated form), three, four, six, and seven, were observed by the label-free quantification. Yang et al. [7] demonstrated a significant increase of the glycosylated levels after a citric acid stimulation in adults and children. Overall, the different sAA proteoforms seem to be regulated depending on the stimulation nature.

Although this preliminary study's results should be taken with caution due to the low sample size, it opens a new field for the evaluation of possible selected sAA proteoforms as potential biomarkers of stress, since the inter-individual variability in the estimated amounts from WB images for some bands is lower than the activity results.

These studies have some limitations. We did not measure the pH of saliva samples. This is of importance in the case of acetic acid stimulation, since a low pH could interfere with sAA activity values [1]. However, Yang et al. [7], using the same method that we have used to stimulate sAA release in the present study, described values of pH higher than 6.4, which is considered as the lower limit of pH that does not produce changes in amylase activities. Considering that quantification of the sAA proteoforms was performed using a label-free quantification analysis, we speculate that the use of a isobaric tag, such as tandem mass tags (TMTs) [43] or isobaric tags for relative and absolute quantitation (iTraq) [44], could provide more accurate quantification. In addition, further studies in a larger population and in a mixed sex group should be performed to confirm our results in both genders.

Conclusions

This preliminary study shows for the first time that the diverse sAA proteoforms express differentially depending on the different stimulation models. This opens new perspectives and challenges for the use of alpha-amylase proteoforms, specially the non-glycosylated proteoform as a potential non-invasive biomarker of psychological and physical situations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This project has been approved by the Murcia University Ethics Committee with the number 1349/2016.

Informed consent All were informed about the procedure, sampling methods, and the objective of the experiment and signed a consent form. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

References

1. Rohleder N, Nater UM (2009) Determinants of salivary α-amylase in humans and methodological considerations. Psychoneuroendocrinology 34:469–485. https://doi.org/10.1016/j.psyneuen.2008.12.004
2. Nater UM, Rohleder N (2009) Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. Psychoneuroendocrinology 34:486–496. https://doi.org/10.1016/j.psyneuen.2009.01.014
3. Granger DA, Kivlighan KT, El-Sheikh M, et al (2007) Salivary alpha-amylase in biobehavioral research—recent developments and applications. Ann N Y Acad Sci 1098:122–144. https://doi.org/10.1196/annals.1384.008
4. Koibuchi E, Suzuki Y (2014) Exercise upregulates salivary amylase in humans. Exp Ther Med 7:773–777. https://doi.org/10.3892/etm.2014.1497
5. Petrakova L, Doering BK, Vits S, Engler H, Rief W, Schedlowski M, Grigoleit JS (2015) Psychosocial stress increases salivary alpha-amylase activity independently from plasma noradrenaline levels. PLoS One 10:1–9. https://doi.org/10.1371/journal.pone.0134561
6. Grassi G, Esler M (1999) How to assess sympathetic activity in humans. J Hypertens 17:719–734
7. Yang Z, Chen L, Zhang M et al (2015) Age differences of salivary alpha-amylase levels of basal and acute responses to citric acid stimulation between Chinese children and adults. Front Physiol 6:1–11. https://doi.org/10.3389/fphys.2015.00340
8. Fuentes-Rubio M, Fuentes F, Otal J, Quiles A, Tecles F, Cerón JJ, Hevia ML (2015) Measurements of salivary alpha-amylase in horse: comparison of 2 different assays. J Vet Behav: Clin Appl Res 10:122–127. https://doi.org/10.1016/j.jveb.2014.12.008
9. Contreras-Aguilar MD, Escribano D, Martínez-subiela S et al (2018) Changes in alpha-amylase activity, concentration and isoforms in pigs after an experimental acute stress model: an exploratory study. BMC Vet Res 14:1–8
10. Fuentes M, Tecles F, Gutierrez A et al (2011) Validation of an automated method for salivary alpha-amylase measurements in pigs (Sus scrofa domesticus) and its application as a stress biomarker. J Vet Diagn Investig 23:282–287
11. Ghezzi EM, Ship JA (2003) Aging and secretory reserve capacity of major salivary glands. J Dent Res 82:844–848. https://doi.org/10.1177/154405910308201016
12. Nater UM, Rohleder N, Schlotz W, Ehlert U, Kirschbaum C (2007) Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology 32:392–401. https://doi.org/10.1016/j.psyneuen.2007.02.007
13. Almela M, Hidalgo V, Villada C, van der Meij L, Espín L, Gómez-Amor J, Salvador A (2011) Salivary alpha-amylase response to acute psychosocial stress: the impact of age. Biol Psychol 87:421–429. https://doi.org/10.1016/j.biopsycho.2011.05.008

14. Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, Werner J, Villanea FA, Mountain JL, Misra R, Carter NP, Lee C, Stone AC (2007) Diet and the evolution of human amylase gene copy number variation. Nat Genet 39:1256–1260. https://doi.org/10.1038/ng2123
15. Mandel AL, des Gachons CP, Plank KL et al (2010) Individual differences in AMY1 gene copy number, salivary alpha-amylase levels, and the perception of oral starch. PLoS One 5:1–9. https://doi.org/10.1371/journal.pone.0013352
16. Donald Merritt A, Karn R (1977) The human alpha-amylases. Adv Hum Genet 8:135–234
17. Smith LM, Kelleher NL (2013) Proteoform: a single term describing protein complexity. Nat Methods 10:186–187. https://doi.org/10.1038/nmeth.2369
18. Karn RC, Schulkin J, Meritt A, Newell R (1975) Genetic and post-translational mechanisms determining human amylase isozyme heterogeneity. Press, Academic, New York
19. Hirtz C, Chevalier F, Centeno D, Rofidal V, Egea JC, Rossignol M, Sommerer N, Deville de Périère D (2005) MS characterization of multiple forms of alpha-amylase in human saliva. Proteomics 5:4597–4607. https://doi.org/10.1002/pmic.200401316
20. Campbell J, Ehlert U (2012) Acute psychosocial stress: does the emotional stress response correspond with physiological responses? Psychoneuroendocrinology 37:1111–1134. https://doi.org/10.1016/j.psyneuen.2011.12.010
21. Nater UM, La Marca R, Florin L et al (2006) Stress-induced changes in human salivary alpha-amylase activity—associations with adrenergic activity. Psychoneuroendocrinology 31:49–58. https://doi.org/10.1016/j.psyneuen.2005.05.010
22. Guillén Riquelme A (2014) Validación de la adaptación española del State-Trait Anxiety Inventory en diferentes muestras españolas
23. Bieling PJ, Antony MM, Swinson RP (1998) The state-trait anxiety inventory, trait version: structure and content re-examined. Behav Res Ther 36:777–788. https://doi.org/10.1016/S0005-7967(98)00023-0
24. Bardo J, Hughey J (1978) Examination of the biasing properties of Cronbach coefficient alpha under conditions of varying shapes of data distribution: a Monte Carlo simulation. Percept Mot Skills 46:308–310
25. KH C (1968) A means of assessing maximal oxygen intake: correlation between field and treadmill testing. JAMA 203:201–204
26. Giles D, Draper N, Neil W (2016) Validity of the Polar V800 heart rate monitor to measure RR intervals at rest. Eur J Appl Physiol 116:563–571. https://doi.org/10.1007/s00421-015-3303-9
27. AHA (1972) Exercise testing and training of apparently healthy individuals: a handbook for physicians. American Heart Assoc.
28. DeCaro JA (2008) Methodological considerations in the use of salivary α-amylase as a stress marker in field research. Am J Hum Biol 20:617–619. https://doi.org/10.1002/ajhb.20795
29. Bosch JA, Veerman ECI, de Geus EJ, Proctor GB (2011) Alpha-amylase as a reliable and convenient measure of sympathetic activity: don't start salivating just yet. Psychoneuroendocrinology 36:449–453. https://doi.org/10.1016/j.psyneuen.2010.12.019
30. van der Heiden C, Bais R, Gerhardt W, et al (1999) IFCC methods for measurement of catalytic concentration of enzymes—part 9. IFCC method for alpha-amylase {[}1,4-alpha-D-glucan 4-glucanohydrolase, EC 3.2.1.1]. Clin Chim Acta 281:S5–S39
31. Tecles F, Fuentes-Rubio M, Tvarijonaviciute A, Martínez-Subiela S, Fatjó J, Cerón JJ (2014) Assessment of stress associated with an oral public speech in veterinary students by salivary biomarkers. J Vet Med Educ 41:37–43. https://doi.org/10.3138/jvme.0513-073R1
32. Contreras-Aguilar MD, Escribano D, Martínez-Subiela S, Martínez-Miró S, Rubio M, Tvarijonaviciute A, Tecles F, Cerón JJ (2017) Influence of the way of reporting alpha-amylase values in saliva in different naturalistic situations: a pilot study. PLoS One 12:1–13. https://doi.org/10.1371/journal.pone.0180100
33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
34. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets—procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354. https://doi.org/10.1073/pnas.76.9.4350
35. Noble JE, Bailey MJA (2009) Quantitation of protein. In: Burgess, RR and Deutscher M (ed) Guide to protein purification, Second edi. Elsevier Academic Press Inc., 525 B Street, Suite 1900, San Diego, CA 92101-4495 USA, pp 73–95
36. Hinkle, D.E; Wiersma, W.; Jurs S G. (2003) Applied statistics for the behavioral sciences, 5th Ed. Boston Mass: Houghton Mifflin., London
37. Strahler J, Skoluda N, Kappert MB, Nater UM (2017) Simultaneous measurement of salivary cortisol and alpha-amylase: application and recommendations. Neurosci Biobehav Rev 83:657–677. https://doi.org/10.1016/j.neubiorev.2017.08.015
38. Contreras-Aguilar MD, Tecles F, Martínez-Subiela S, Escribano D, Bernal LJ, Cerón JJ (2017) Detection and measurement of alpha-amylase in canine saliva and changes after an experimentally induced sympathetic activation. BMC Vet Res 13:1–6. https://doi.org/10.1186/s12917-017-1191-4
39. George D, Mallery P (1995) SPSS/PC+ step by step: a simple guide and reference. Wadsworth Publishing Company, Belmont
40. Zubieta JK, Heitzeg MM, Smith YR et al (2003) COMT val(158) met genotype affects mu-opioid neurotransmitter responses to a pain stressor. SCIENCE 299:1240–1243. https://doi.org/10.1126/science.1078546
41. Uhart M, McCaul ME, Oswald LM et al (2004) GABRA6 gene polymorphism and an attenuated stress response. Mol Psychiatry 9:998–1006. https://doi.org/10.1038/sj.mp.4001535
42. Bank RA, Hettema EH, Muijs MA et al (1992) Variation in gene copy number and polymorphism of the human salivary amylase isoenzyme system in Caucasians. Hum Genet 89:213–222
43. Thompson A, Schaefer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone RAW, Mohammed AKA, Hamon C (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75:1895–1904. https://doi.org/10.1021/ac060310l
44. Ross PL, Huang YLN, Marchese JN et al (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3:1154–1169. https://doi.org/10.1074/mcp