Proteomic Studies of Saliva: A Proposal for a Standardized Handling of Clinical Samples

François Chevalier, Christophe Hirtz, Sandrine Chay, Frédéric Cuisinier, Nicolas Sommerer, Michel Rossignol, Dominique Deville Périère

Clin Proteom (2007) 3:13–21
DOI 10.1007/s12014-007-9000-x

Proteomic Studies of Saliva: A Proposal for a Standardized Handling of Clinical Samples

François Chevalier · Christophe Hirtz · Sandrine Chay · Frédéric Cuisinier · Nicolas Sommerer · Michel Rossignol · Dominique Deville de Périère

Published online: 10 October 2007

Abstract
Background In recent years, differential analysis of proteins from human saliva, i.e., proteomic analysis, has received much attention mainly due to its unstressful sampling and its great potential for biomarker research. It is widely considered that saliva is a highly stable medium for proteins thanks to a large amount of antiprotease agents, even at ambient and physiological temperatures.
Objective To find the best protocol for the handling of samples, we have investigated the stability of saliva proteins stored at different temperatures (from −80 to 20°C) by one- and two-dimensional electrophoresis.
Results At 20°C, no major changes were observed on protein one-dimensional profiles following 1 day of storage; however, between 7 days and 30 days, the native alpha-amylase band decreased slightly to give several bands with molecular weight between 35 and 25 kDa. The same phenomenon appeared after 30 days of storage at 4°C. Two-dimensional analysis of salivary maps revealed degradation from day 7 of several protein groups for samples stored at 20°C.
Conclusion All these findings have to be carefully considered when saliva is collected for clinical proteomic analysis. We can conclude that, to maintain the optimum stability of saliva proteins, saliva samples should be collected on ice followed by the addition of protease inhibitor cocktail, centrifuged to remove insoluble material, and stored at −20 or −80°C.

Keywords Saliva · Stability · Storage · Protocol · Proteomic

Abbreviations

TCA	trichloroacetic acid
DTT	dithiothreitol
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI-TOF	matrix-assisted laser desorption/ionization
MS	time-of-flight mass spectrometry

Introduction

Saliva has become popular in recent decades as a medium for the measurement of numerous biomolecules [1–4]. The greatest advantage, when compared to blood sample collection, is that saliva is readily accessible and collectible. Consequently, it can be used in clinically difficult situations, such as in children, handicapped, and anxious patients, where blood sampling could be a difficult act to perform [5].

A wide range of biomolecules are now monitored in saliva [6, 7]. Proteins and peptides were extensively studied in saliva for biomarker research; these include the quanti-

François Chevalier and Christophe Hirtz contributed equally to this work.

F. Chevalier · C. Hirtz · S. Chay · N. Sommerer · M. Rossignol
Laboratory of Proteomic, INRA,
UR 1199 Montpellier, France

F. Chevalier · C. Hirtz · F. Cuisinier · D. D. de Périère
Laboratory of Physiology, UFR d'Odontologie,
Université Montpellier 1,
Montpellier, France

F. Chevalier (✉)
Plateforme de Protéomique,
Institut de Radiobiologie Cellulaire et Moléculaire, CEA - DSV,
18, route du panorama,
92265 Fontenay-aux-Roses Cedex, France
e-mail: francois.chevalier@cea.fr

fication of steroid hormones such as cortisol or progesterone [8–11], and the detection of various therapeutic or illicit drugs [12]. Tumor markers that were identified in saliva have been used to screen malignant diseases [13–15]. Antibodies against viruses and viral components can also be detected in saliva, therefore making it easier to diagnose various infections such as, for example, hepatitis A, B, or C; HIV; rubella; and dengue [16–19]. Identification of all saliva proteins [20–39] allowed comparative analysis of proteomic maps of healthy and ill people, which revealed differentially expressed proteins as potential biomarkers [26, 28, 32, 35, 36, 38].

On a recent summary about the proteomic technologies currently used, Hu et al. pointed out the critical challenges and perspectives for this emerging field [40]. The first point was the need to standardize the sample collection, preparation, and handling procedures. Indeed, to ensure comparable analyses of saliva and, thus, results between studies and labs, careful attention to methodological detail is required. Collection, storage conditions, and processing of saliva samples are key steps to guarantee reproducibility and validity of measurements [40–42].

In addition to salivary gland secretions (water, proteins, enzymes, electrolytes, and small organic molecules), blood derivatives from gingival crevicular fluid or oral wounds, bacteria, fungi, desquamated epithelial cells, expectorated bronchial and nasal secretions, or food debris have also been found in saliva [4]. Many of these components may have damaged molecules of interests for proteomic analysis. Careful control of temperature during saliva collection and salivary sample storage is crucial; additionally, most insoluble materials can be eliminated by centrifugation or filtration. The use of a protease inhibitor cocktail can help to reduce protein degradation during saliva sample collection and processing. A review of previous protocols used for proteomic analysis of saliva showed variations in several parameters such as temperature at sample collection, the addition of a protease inhibitor cocktail, the removal of insoluble material and the temperature of storage of saliva samples (Table 1). This can be explained by the fact that, for many decades, it was largely believed that saliva contains a high amount of protease inhibitors, which confers a great stability on proteins.

Saliva storage protocols have been extensively analyzed for the stability of steroids [43], progesterone [44, 45], immunoglobulin A and lysozyme [46], RNA of hepatitis C virus, and blood group antigen detection [47, 48], but never for a proteome analysis, with the exception of Schipper et al. [39], who recently showed differences on surface enhanced laser desorption/ionization-time-of-flight (TOF) mass spectra of saliva samples as a function of storage temperature, centrifugation speed, and the use of protease inhibitors.

In the present paper, we propose to study the stability of the major salivary proteins stored at various storage temperatures (−80, −20, 4, and 20°C) by one-dimensional and bidimensional electrophoresis to estimate the rate of stability of saliva proteins and, in the case of degradation, which proteins or group of proteins were the most susceptible. The aim of this work is to establish a robust and standardized protocol for collection and storage of saliva that could be widely used for proteomic analysis of human saliva.

Materials and Methods

Collection of Saliva

Whole saliva was collected from a healthy subject 2 h after breakfast time. Before sample collection, the subjects brushed their teeth and rinsed their mouth with water. To help saliva production, individuals drank a glass of water 15 min before collection, and then, stimulated saliva was collected on ice for 5 min by chewing on paraffin wax and spitting intermittently. Saliva samples were centrifuged at 15,000×*g* for 15 min at 4°C to eliminate insoluble material. A sample was kept with insoluble material (without the centrifugation step) to investigate the influence of bacteria, mucine, and other insoluble material on protein stability.

Storage Kinetics of Saliva

Clear saliva was aliquoted (1 ml) into microcentrifuge tubes and stored at either 4°C or ambient temperature. At regular intervals (10 min, 1 h, 1 day, 7 days, 30 days), samples were placed in a −20°C freezer until further analysis by mono- and two-dimensional electrophoresis. A sample with protease inhibitor mixture (Complete®, Roche, Penzberg, Germany) was also prepared and placed at ambient temperature for 30 days. Control samples were also stored at either −80 or −20°C for 30 days.

Extraction of Salivary Protein

Proteins were first extracted using the trichloroacetic acid (TCA)/acetone method [49]. Briefly, proteins were precipitated using 90% acetone (v/v), 10% TCA (v/v), and 0.07% 2-mercaptoethanol (v/v). After incubation overnight at −20°C, insoluble material was centrifuged at 42,000×*g* for 10 min at 4°C. Pellets were washed three times with pure acetone containing 2-mercaptoethanol, air dried, and solubilized in 9 M urea, 4% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonic acid (w/v), 0.05% Triton X100 (v/v), and 65 mM dithiothreitol (DTT). Protein content was estimated using the Bradford method [50].

Table 1 A review of previous processing protocols used for proteomic analysis of saliva

Ref.	Saliva type	Temperature of collection	Addition of protease inhibitor	Removal of unsoluble material	Temperature of samples storage	Proteomic analysis
54	Stimulated parotid saliva	Ice	No	No	−20°C	HPLC and N-terminal sequencing
20	Whole saliva	Unknown	No	No	−20°C	SDS-PAGE and MALDI-TOF
21	Stimulated glandular and whole saliva	Ice	No	Centrifugation	−20°C	2DE and MALDI-TOF
22	Stimulated whole saliva	Ice	No	Centrifugation	No storage: direct analysis	2DE and MALDI-TOF
23	Unstimulated whole saliva	Unknown	No	Centrifugation	Unknown	2DE and MALDI-TOF-TOF
26	Unstimulated whole saliva	Ice	Yes	Centrifugation	−20°C	2DE and MALDI-TOF, Q-TOF, N-terminal sequencing and Western blotting
25	Whole saliva	Unknown	No	Centrifugation	No storage: direct analysis	HPLC and LC-MS and MALDI-TOF
55	Unstimulated whole saliva	Unknown	No	Centrifugation	Unknown	2D LC-MS
27	Stimulated parotid saliva	Ice	No	Centrifugation	−20°C	2DE and MALDI-TOF MALDI-TOF-TOF and Q-TOF
29	Whole saliva	Ice	Yes	Centrifugation	−80°C	Shotgun proteomics and 2DE and MALDI-TOF
30	Stimulated whole saliva	Ice	Yes	Centrifugation	−80°C	2DE and MALDI-TOF
33	Unstimulated whole saliva	Unknown	No	Centrifugation	Unknown	FFE and LC-MS/MS
31	Stimulated whole saliva	Ice	Yes	Centrifugation	−80°C	2DE and MALDI-TOF
32	Stimulated whole saliva	Ice	Yes	Centrifugation	−80°C	2DE and MALDI-TOF
34	Stimulated whole and parotid saliva	Unknown	No	No	−80°C	2DE and MALDI-TOF and LC-MS/MS
35	Unstimulated whole saliva	Unknown	No	Centrifugation	−80°C	2DE and MALDI-TOF
36	Parotid saliva	Ice	No	Centrifugation	−80°C	2D-DIGE and SELDI-TOF
37	Unstimulated whole saliva, parotid	Ice	No	Filtration	−80°C	2DE and MALDI-TOF
38	Unstimulated whole saliva	Ice	No	Centrifugation	No storage: direct analysis	2DE and MALDI-TOF
39	Stimulated and unstimulated whole saliva	Unknown	Yes	Centrifugation	Ice, −20°C and −80°C	SELDI-TOF

MS = mass spectrometry, SELDI = surface enhanced laser desorption/ionization

One-Dimension Electrophoresis

Saliva samples (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 11% acrylamide gels (1.5 mm thick) according to Laemmli [51]. Gels were stained with the silver staining procedure of Vorum [52].

Two-Dimensional Gel Electrophoresis

Precast 18 cm IPG strips pH 4–7 (Amersham Biosciences, Orsay, France) were rehydrated directly overnight with protein samples (100 μg). Isoelectric focusing was carried out using the IPGphor™ isoelectric focusing system for a total of ca 70,000 V.h. Thereafter, strips were equilibrated for 15 min in 6 M urea, 50 mM Tris HCl buffer at pH 8.8, 30% glycerol (v/v), 2% SDS (w/v), and 65 mM DTT, and then for 15 min in the same solution with 65 mM iodoacetamide instead of DTT. Proteins were finally separated on 12% SDS-polyacrylamide gels, at constant voltage (150 V) and 10°C, using an Iso-DALT electrophoresis unit (Amersham Biosciences). Gels were stained with colloidal Coomassie blue [53]. Gel images were digitalized at 300 dpi with a GS 710 densitometer (Biorad, Hercules, CA, USA) and analyzed using the Progenesis software (Non-linear Dynamics, Newcastle upon Tyne, UK).

Image Analysis

Gel images were analyzed in triplicate with the Progenesis Workstation software v.2003.2 (Perkin Elmer Life Sciences, Cambridge, UK). An automatic averaged gel experiment was performed with single analytic gels using the control sample gels as reference. For each sampling time, eight areas were selected and compared. Images were first warped to the reference gel and areas were then matched. A combined algorithm warping and matching was used. The "progenesis background" was used as the background method and the spot volume was determined as percentage of total volume of all spots on respective gels.

Matrix-Assisted Laser Desorption/Ionization-TOF Mass Spectrometry Analysis

Spots were excised from gels after two-dimensional electrophoresis by hand and processed using a Packard Multiprobe II liquid handling robot (Perkin Elmer, Courtaboeuf, France). After washing with water, 25 mM ammonium bicarbonate, acetonitrile/25 mM ammonium bicarbonate (1:1, v/v) and acetonitrile, gel fragments were dried at 37°C. Protein digestion was carried out at 37°C for 5 h after addition of 0.125 μg trypsin. Resulting fragments were extracted twice with 50 μL of acetonitrile/water (1:1, v/v) containing 0.1% trifluoroacetic acid for 15 min. Pooled supernatants were concentrated to a final volume of ca 20 μL. Peptides were desalted and concentrated to a final volume of 3 μL with C18 Zip-Tip microcolumns and immediately spotted onto the matrix-assisted laser desorption/ionization (MALDI) target by the robot. Mass spectra were recorded in the reflector mode on a BiFlex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Automatic annotation of monoisotopic masses was performed using Bruker's SNAPTM procedure. The MASCOT search engine software (Matrix Science, London, UK) was used to search the NCBInr database. The following parameters were used: mass tolerance of 100 ppm, a minimum of five peptides matching to the protein, carbamidomethylation of cysteine as fixed modification, oxidation of methionine, and pyroglutamylation of glutamine as variable modifications and one missed cleavage allowed.

Results

Collection and Storage of Saliva Samples

Stimulated saliva was collected between 9 a.m. and 10 a.m. A male volunteer (age, 33 years) was asked to refrain from eating, drinking, smoking, or oral hygiene procedures, and a water mouth-rinse was required prior to sample collection. Stimulated saliva was collected on ice and clarified by centrifugation to eliminate mucine, cell debris, and insoluble material. A control sample was kept without centrifugation (unclear saliva). Samples (1 ml) of clear saliva were aliquoted into 1.5-ml microtubes and incubated at either −80, −20, 4, and 20°C. A control sample (0 min) was frozen at −20°C immediately after collection. The kinetics of storage was studied with samples kept at both 4 and 20°C for 10 min, 1 h, 1 day, 7 days, and 30 days. A cocktail of protease inhibitor was added to a sample maintained at both 4 and 20°C for 30 days.

One-Dimensional Electrophoresis Analysis of Stored Saliva

The same volume of clear or unclear saliva samples was separated by SDS-PAGE (Fig. 1). According to such one-dimensional electrophoresis, only profile changes of proteins of similar molecular weights could be observed.

A large diversity of molecular weights was observed with the initial sample frozen immediately after collection (Fig. 1a, lane 1). Major bands were observed close to 50 and 15 kDa, corresponding to native alpha-amylase and cystatins, respectively. Minor and diffuse bands were observed between 50 and 20 kDa, corresponding to an abundant variety of salivary proteins. According to the storage kinetics, no major changes were observed on protein profiles of samples stored for 60 min at ambient temperature (Fig. 1a, lane 3). At 1 day, the band intensity just below the native alpha-amylase seemed to be less intense. At 7 days, the intensity of the native alpha-amylase band (57 kDa) decreased slightly, resulting in several bands between 35 and 25 kDa. This phenomenon seemed to be enhanced after 30 days storage at ambient temperature (Fig. 1a, lane 6). The addition of protease inhibitors was not able to block such degradation; no real difference was observed at 30 days between samples containing cocktail inhibitors or not.

In contrast, when saliva was stored at 4°C, no such degradation was observed until 7 days (Fig. 1b, lane 4). From days 7 to 30, the protein profile seemed to be slightly less intense in the molecular weight range between 50 and 25 kDa, but without any band appearance. The addition of protease inhibitors successfully blocked such degradation at day 30 (Fig. 1b, lane 6). In contrast, when insoluble material was not removed, saliva proteins were completely disappeared at either 4 or 20°C (Fig. 1a, lane 8, and Fig. 1b, lane 7, respectively). In this case, proteins were certainly degraded by proteases from bacteria and cells of the oral cavity. The storage of saliva samples for 30 days at −20 and −80°C allowed a complete preservation of saliva proteins, lanes 8 and 9, respectively (Fig. 1b).

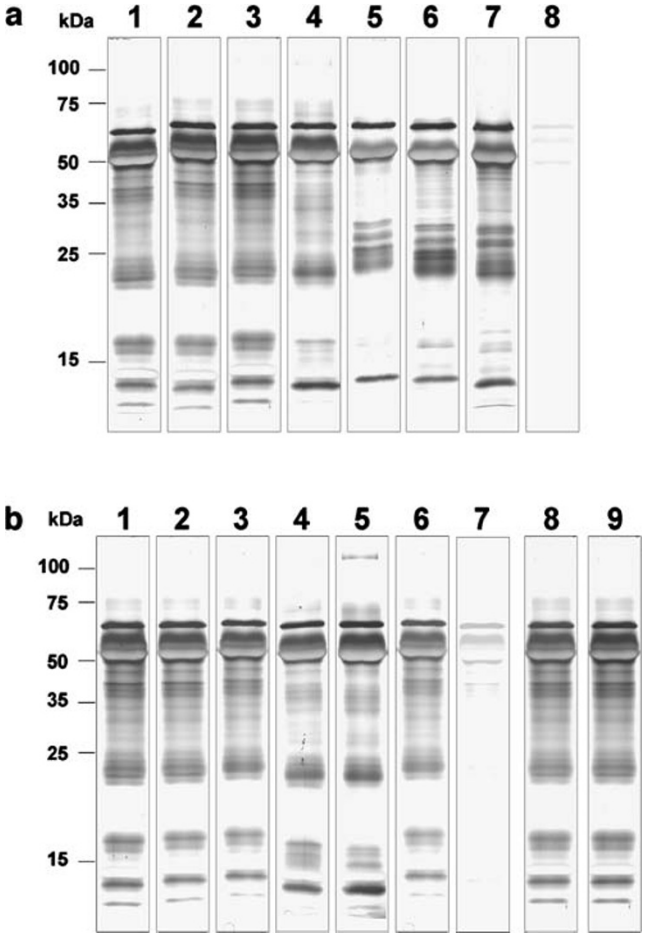

Fig. 1 One-dimensional SDS-PAGE separation of salivary proteins. **a** *lanes 1* to *6*: clear saliva stored at 20°C for 0 min (control sample), 10 min, 60 min, 1 day, 7 days, and 30 days; *lane 7*: clear saliva stored at 20°C for 30 days with a protease inhibitor cocktail; *lane 8*: unclear saliva (without centrifugation) stored at 20°C for 30 days. **b** *lanes 1* to *5*: clear saliva stored at 4°C for 10 min, 60 min, 1 day, 7 days and 30 days; *lane 6*: clear saliva stored at 4°C for 30 days with a protease inhibitor cocktail; *lane 7*: unclear saliva (without centrifugation) stored at 4°C for 30 days; *lane 8*: clear saliva stored at −20°C for 30 days; *lane 9*: clear saliva stored at −80°C for 30 days

Two-Dimensional Electrophoresis Analysis of Stored Saliva

To obtain more detailed information, two-dimensional electrophoresis was used to analyze changes in specific protein groups. Based on the results of one-dimensional analysis, the times at which protein degradation was most affected were selected for the two-dimensional electrophoresis separation: in addition to control samples (0 day and 30 days with protease inhibitors), we selected samples stored at 20°C for 1, 7, and 30 days to be analyzed. The 30 major protein spots were selected on two-dimensional gels (Fig. 2) and identified by peptide mass fingerprinting (Table 2). To compare protein amount during storage times, we decided to group proteins from the same family and/or gel area (Fig. 2 and Table 2). All the areas were calculated as a mean of three replicates of the same extract and were matched together to analyze the evolution of relative intensity according to the storage time (Fig. 3). Areas A to D were strongly affected by storage time at 20°C. Area A, corresponding to serum albumin, disappeared on the bidimensional map from 1 day and only half of the spot intensity was maintained at 30 days when a protease inhibitor cocktail was added to the sample. In the case of area B, the quantity of salivary acidic prolin rich protein (PRP) decreased dramatically up to 7 days at ambient temperature, with only a small amount of the corresponding spots still observable at days 7 and 30. The same result was obtained with spots of area C, corresponding to prolactin inducible proteins and salivary acidic protein-1. Spot identified as salivary cystatins SA-1 (area D) had disappeared totally by day 7, showing its low stability at 20°C. The addition of a protease inhibitor cocktail did not affect the degradation of these proteins after 30 days storage at ambient temperature. The protease inhibitor cocktail tablets used (Complete Mini®, Roche) were designed to inhibit mainly serine-, cysteine- and metallo-proteases. Nevertheless, the relatively quick degradation of proteins could be attributed to residual protease activity or to the presence of acidic proteases such as aspartic protease.

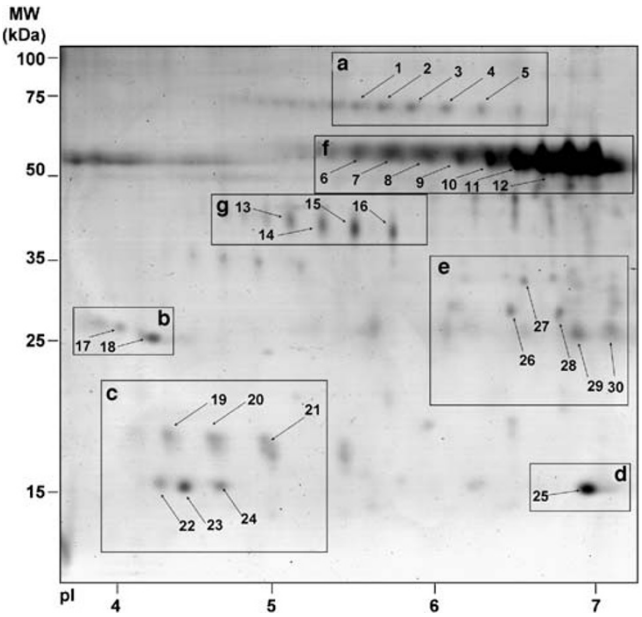

Fig. 2 Two-dimensional electrophoresis of 100 μg of salivary proteins extracted from the control sample (0 min). Proteins were resolved using pH 4–7 IPG and 12% SDS-PAGE, and the gel was stained using Coomassie blue. The 30 most abundant proteins were reported and identified by peptide mass fingerprint MALDI-TOF (i.e., see Table 2)

Table 2 Identification of the 30 most abundant spots from the two-dimensional gel of whole saliva (see Fig. 2), by peptide mass fingerprint MALDI-TOF

No	Group	Protein name	Ref.	% Cov.	MW (kDa) theoretical	Observed	pI theoretical	Observed
1	A	Serum albumin	P02768	29	66.5	68	5.67	5.5
2	A	Serum albumin	P02768	25	66.5	68	5.67	5.6
3	A	Serum albumin	P02768	25	66.5	68	5.67	5.8
4	A	Serum albumin	P02768	27	66.5	68	5.67	6
5	A	Serum albumin	P02768	25	66.5	68	5.67	6.2
6	F	Salivary-amylase	P04745	42	55.9	59	6.34	5.5
7	F	Salivary-amylase	P04745	33	55.9	59	6.34	5.8
8	F	Salivary-amylase	P04745	28	55.9	59	6.34	6
9	F	Salivary-amylase	P04745	51	55.9	59	6.34	6.1
10	F	Salivary-amylase	P04745	50	55.9	59	6.34	6.3
11	F	Salivary-amylase	P04745	51	55.9	59	6.34	6.5
12	F	Salivary-amylase	P04745	50	55.9	59	6.34	6.7
13	G	Zinc-alpha-2-glycoprotein	P25311	52	32.1	42	5.58	5.1
14	G	Zinc-alpha-2-glycoprotein	P25311	53	32.1	42	5.58	5.3
15	G	Zinc-alpha-2-glycoprotein	P25311	57	32.1	42	5.58	5.5
16	G	Zinc-alpha-2-glycoprotein	P25311	53	32.1	42	5.58	5.7
17	B	Salivary acidic PRP	P02810	36	15.4	26	4.63	4.1
18	B	Salivary acidic PRP	P02810	42	15.4	25	4.63	4.2
19	C	Prolactin inducible	P12273	38	13.5	18	5.47	4.2
20	C	Prolactin inducible	P12273	50	13.5	18	5.47	4.5
21	C	Prolactin inducible	P12273	43	13.5	18	5.47	5
22	C	Salivary acidic protein 1	P01036	27	14.2	16	4.83	4.2
23	C	Salivary acidic protein 1	P01036	29	14.2	16	4.83	4.4
24	C	Salivary acidic protein 1	P01036	34	14.2	16	4.83	4.7
25	D	Salivary cystatin SA-1	P01037	40	14.3	16	6.92	7
26	E	Salivary-amylase	P04745	34	55.9	30	6.34	6.4
27	E	Salivary-amylase	P04745	22	55.9	35	6.34	6.5
28	E	Salivary-amylase	P04745	35	55.9	30	6.34	6.7
29	E	Salivary-amylase	P04745	43	55.9	28	6.34	6.8
30	E	Salivary-amylase	P04745	27	55.9	27	6.34	7.1

Protein reference (Ref.) corresponds to the Swiss-Prot/NCBI accession number; sequence coverage (% cov.) is given as percentage

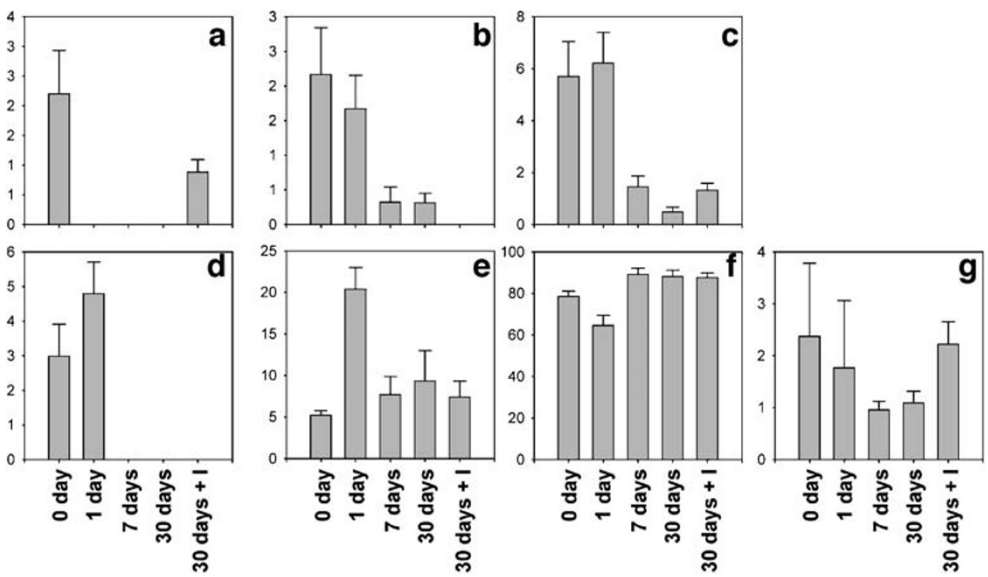

Fig. 3 Total protein amount of spot groups from the two-dimensional separation (i.e., see Fig. 2) as a function of storage time at ambient temperature. **a**–**g** groups a–g

In contrast, the opposite result was obtained with areas E, F, and G, in which no real degradation was observed. The corresponding proteins, alpha-amylase (groups E and F) and zinc-alpha-2 glycoprotein (group G), seemed to resist degradation up to 30 days storage at ambient temperature. Nevertheless, after 1 day of storage, a 15% decrease of area F spot intensities was observed with a concomitant increase of area E spots. It could be hypothesized that native alpha-amylase was degraded to constitute a part of alternative alpha-amylase forms. These lower-molecular-weight forms of alpha-amylase were previously described as a complex but stable protein pattern [31]. Certainly a part of these alternative alpha-amylase spots remained from the degradation of the native alpha-amylase form [3, 4], but another part, which constitutes the stable pattern [31], seemed not to be affected by degradation. The example of these alternative alpha-amylase spots clearly demonstrated the importance of a strong control of protease actions to understand and compare proteomes.

Concluding Remarks

As saliva is now frequently used as a diagnostic biological fluid, our work demonstrated the necessity for a well-controlled storage protocol of saliva samples to ensure quantitative and reproducible clinical analysis. Of course, storage temperatures such as −20 and −80°C are now commonly used to store biospecimens, but such storage conditions could be considered as excessive owing to the fact that saliva contains a high amount of protease inhibitors which can prevent protein degradation.

Our results clearly showed the weak stability of several salivary proteins. According to one- and two-dimensional electrophoresis studies (Table 3), the stability of clear saliva was preserved until 1 day at 20°C and 7 days at 4°C. After 30 days at 20°C, almost half of the major salivary proteins were degraded even if a protease inhibitor cocktail was added to the sample.

According to these results, and in agreement with Schipper et al. [39], the protocol used by Hu et al. [29] seemed to be the best to maintain the optimum stability of saliva proteins. In case of a clinical comparison with a pathological condition, control saliva samples should be collected from a healthy nonsmoking subject, in the morning, at least 2 h after eating, and the mouth should be rinsed with water. After collection, saliva samples should be stored in a freezer at −20°C, and during sampling, saliva should be kept on ice with a protease inhibitor cocktail and centrifuged to remove insoluble material and then stored at −80°C.

Acknowledgments The authors gratefully acknowledge the financial and technical supports from the University of Montpellier 1 and from the Proteome Platform of Montpellier-LR Genopole. We would like to thank Dr. Nuala Rynne for her help in the redaction of the manuscript.

Table 3 Stability of saliva proteins according to the one-dimension and two-dimensional electrophoresis experiments

Temperature of samples storage	Duration of storage	Addition of protease inhibitor	Removal of unsoluble material	Degradation of proteins[a]	Protein groups sensible[b]
20°C	0	−	+	−	None
20°C	10 min	−	+	−	/
20°C	60 min	−	+	−	/
20°C	1 day	−	+	+/−	A
20°C	7 days	−	+	+	A, B, C, D, G
20°C	30 days	−	+	+	A, B, C, D, G
20°C	30 days	+	+	+	A, B, C, D, G
20°C	30 days	−	−	+++	/
4°C	10 min	−	+	−	/
4°C	60 min	−	+	−	/
4°C	1 day	−	+	−	/
4°C	7 days	−	+	+/−	/
4°C	30 days	−	+	+/−	/
4°C	30 days	+	+	−	/
4°C	30 days	−	−	+++	/
−20°C	30 days	−	+	−	/
−80°C	30 days	−	+	−	/

[a] One-dimensional electrophoresis experiment
[b] Two-dimensional electrophoresis experiment

References

1. Ferguson DB. Current diagnostic uses of saliva. J Dent Res 1987;66:420–4.
2. Mandel ID. The diagnostic uses of saliva. J Oral Pathol Med 1990;19:119–25.
3. Hofman LF. Human saliva as a diagnostic specimen. J Nutr 2001;131:1621S–5S.
4. Kaufman E, Lamster IB. The diagnostic applications of saliva—a review. Crit Rev Oral Biol Med 2002;13:197–212.
5. Aps JK, Martens LC. The physiology of saliva and transfer of drugs into saliva. Forensic Sci Int 2005;150:119–31.
6. Haeckel R, Hanecke P. The application of saliva, sweat and tear fluid for diagnostic purposes. Ann Biol Clin 1993;51:903–10.
7. Lawrence HP. Salivary markers of systemic disease: noninvasive diagnosis of disease and monitoring of general health. J Can Dent Assoc 2002;68:170–4.
8. Vining RF, McGinley RA. The measurement of hormones in saliva: possibilities and pitfalls. J Steroid Biochem 1987;27: 81–94.
9. Ellison PT. Measurements of salivary progesterone. Ann N Y Acad Sci 1993;694:161–76.
10. Aardal E, Holm AC. Cortisol in saliva—reference ranges and relation to cortisol in serum. Eur J Clin Chem Clin Biochem 1995;33:927–32.
11. Castro M, Elias PC, Martinelli CE, Antonini SR, Santiago L, Moreira AC. Salivary cortisol as a tool for physiological studies and diagnostic strategies. Braz J Med Biol Res 2000;33:1171–5.
12. Kintz P, Cirimele V, Ludes B. Detection of cannabis in oral fluid (saliva) and forehead wipes (sweat) from impaired drivers. J Anal Toxicol 2000;24:557–61.
13. Tavassoli M, Brunel N, Maher R, Johnson NW, Soussi T. p53 antibodies in the saliva of patients with squamous cell carcinoma of the oral cavity. Int J Cancer 1998;78:390–1.
14. Streckfus C, Bigler L, Tucci M, Thigpen JT. A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. Cancer Invest 2000;18:101–9.
15. Drake RR, Cazare LH, Semmes OJ, Wadsworth JT. Serum, salivary and tissue proteomics for discovery of biomarkers for head and neck cancers. Expert Rev Mol Diagn 2005;5:93–100.
16. Stuart JM, Majeed FA, Cartwright KA, Room R, Parry JV, Perry KR, et al. Salivary antibody testing in a school outbreak of hepatitis A. Epidemiol Infect 1992;109:161–6.
17. Malamud D. Saliva as a diagnostic fluid. Br Med J 1992;305: 207–8.
18. Perry KR, Brown DW, Parry JV, Panday S, Pipkin C, Richards A. Detection of measles, mumps, and rubella antibodies in saliva using antibody capture radioimmunoassay. J Med Virol 1993;40:235–40.
19. Cuzzubbo AJ, Vaughn DW, Nisalak A, Suntayakorn S, Aaskov J, Devine PL. Detection of specific antibodies in saliva during dengue infection. J Clin Microbiol 1998;36:3737–9.
20. Chen YC, Li TY, Tsai MF. Analysis of the saliva from patients with oral cancer by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2002;16:364–9.
21. Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J Biol Chem 2003;278:5300–8.
22. Ghafouri B, Tagesson C, Lindahl M. Mapping of proteins in human saliva using two dimensional gel electrophoresis and peptide mass fingerprinting. Proteomics 2003;3:1003–10.
23. Vitorino R, Lobo MJ, Duarte JA, Ferrer-Correia AJ, Domingues PM, Amado FM. Identification of human whole saliva protein components using proteomics. Proteomics 2004;4:1109–15.
24. Messana I, Loffredo F, Inzitari R, Cabras T, Giardina B, Onnis G, et al. The coupling of RP-HPLC and ESI-MS in the study of small peptides and proteins secreted in vitro by human salivary glands that are soluble in acidic solution. Eur J Morphol 2003;41:103–6.
25. Messana I, Cabras T, Inzitari R, Lupi A, Zuppi C, Olmi C, et al. Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. J Proteome Res 2004;3: 792–800.
26. Huang CM. Comparative proteomic analysis of human whole saliva. Arch Oral Biol 2004;49:951–62.
27. Hardt M, Thomas LR, Dixon SE, Newport G, Agabian N, Prakobphol A, et al. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry 2005;44:2885–99.
28. Hu S, Denny P, Denny P, Xie Y, Loo JA, Wolinsky LE, et al. Differentially expressed protein markers in human submandibular and sublingual secretions. Int J Oncol 2004;25:1423–30.
29. Hu S, Xie Y, Ramachandran P, Ogorzalek Loo RR, Li Y, Loo JA, et al. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics 2005;5:1714–28.
30. Hirtz C, Chevalier F, Sommerer N, Centeno D, Egea JC, Rossignol M, et al. Complexity of the human whole saliva proteome. J Physiol Biochem 2005;61:469–80.
31. Hirtz C, Chevalier F, Centeno D, Rofidal V, Egea JC, Rossignol M, et al. MS characterization of multiple forms of alpha-amylase in human saliva. Proteomics 2005;5:4597–607.
32. Hirtz C, Chevalier F, Sommerer N, Raingeard I, Bringer J, Rossignol M, et al. Salivary protein profiling in type1 diabetes using two-dimensional electrophoresis and mass spectrometry. Clin Proteomics 2006;2:117–28.
33. Xie H, Rhodus NL, Griffin RJ, Carlis JV, Griffin TJ. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol Cell Proteomics 2005;4:1826–30.
34. Neyraud E, Sayd T, Morzel M, Dransfield E. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. J Proteome Res 2006;5:2474–80.
35. Yang LL, Liu XQ, Liu W, Cheng B, Li MT. Comparative analysis of whole saliva proteomes for the screening of biomarkers for oral lichen planus. Inflamm Res 2006;55:405–7.
36. Ryu OH, Atkinson JC, Hoehn GT, Illei GG, Hart TC. Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. Rheumatology 2006;45:1077–86.
37. Walz A, Stuhler K, Wattenberg A, Hawranke E, Meyer HE, Schmalz G, et al. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. Proteomics 2006;6:1631–9.
38. Giusti L, Baldini C, Bazzichi L, Ciregia F, Tonazzini I, Mascia G, et al. Proteome analysis of whole saliva: A new tool for rheumatic diseases—the example of Sjogren's syndrome. Proteomics 2007;7:1634–3.
39. Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W. SELDI-TOF-MS of saliva: methodology and pre-treatment effects. J Chromatogr B 2007;847:45–53.
40. Hu S, Loo JA, Wong DT. Human saliva proteome analysis. Ann N Y Acad Sci 2007;1098:323–9.

41. Nimmagudda RR, Ramanathan R, Putcha L. A method for preserving saliva samples at ambient temperatures. Biochem Arch 1997;13:171–8.
42. Landi MT, Caporaso N. Sample collection, processing and storage. IARC Sci Publ 1997;142:223–36.
43. Groschl M, Wagner R, Rauh M, Dorr HG. Stability of salivary steroids: the influences of storage, food and dental care. Steroids 2001;66:737–41.
44. Banerjee S, Levitz M. On the processing of saliva samples for progesterone assay. Steroids 1983;42:539–47.
45. Banerjee S, Levitz M, Rosenberg CR. On the stability of salivary progesterone under various conditions of storage. Steroids 1985;46:967–74.
46. Ng V, Koh D, Fu Q, Chia SE. Effects of storage time on stability of salivary immunoglobulin A and lysozyme. Clin Chim Acta 2003;338:131–4.
47. Roy KM, Bagg J, McCarron B. The effect of saliva specimen collection, handling and storage protocols on hepatitis C virus (HCV) RNA detection by PCR. Oral Dis 1999;5:123–7.
48. Kim W, Kim YK, Chung SC, Lee SW, Kho HS. Detection of ABH blood group antigens in the saliva of Koreans and their stability according to storage of saliva samples. Forensic Sci Int 2002;129:58–63.
49. Chevalier F, Martin O, Rofidal V, Devauchelle AD, Rossignol M. Proteomic investigation of natural variation between Arabidopsis ecotypes. Proteomics 2004;4:1372–81.
50. Bradford MA. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
51. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
52. Mortz E, Krogh TN, Vorum H, Gorg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. Proteomics 2001;1:1359–63.
53. Chevalier F, Rofidal V, Vanova P, Bergoin A, Rossignol M. Proteomic capacity of recent fluorescent dyes for protein staining. Phytochem 2004;65:1499–506.
54. Perinpanayagam HE, Van Wuyckhuyse BC, Ji ZS, Tabak LA. Characterization of low-molecular-weight peptides in human parotid saliva. J Dent Res 1995;74:345–50.
55. Wilmarth PA, Riviere MA, Rustvold DL, Lauten JD, Madden TE, David LL. Two-dimensional liquid chromatography study of the human whole saliva proteome. J Proteome Res 2004;3:1017–23.