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Original Article

Salivary Protein Profiling in Type 1 Diabetes Using Two-Dimensional Electrophoresis and Mass Spectrometry

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Abstract

Owing to its noninvasive collection, saliva is considered as a potent diagnostic fluid. The goal of this study was to investigate the modification of the salivary proteome occurring in type 1 diabetes to highlight potential biomarkers of the pathology. High-resolution two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were combined to perform a large-scale analysis. The proteomic comparison of saliva samples from healthy subjects and poorly controlled type 1 diabetes patients revealed a modulation of 23 proteins. Fourteen isoforms of α-amylase, one prolactin inducible protein, three isoforms of salivary acidic protein-1, and three isoforms of salivary cystatins SA-1 were detected as under expressed, whereas two isoforms of serotransferrin were over expressed in the pathological condition. The proteins under expressed were all known to be implicated in the oral anti-inflammatory process, suggesting that the pathology induced a decrease of non-immunological defense of oral cavity. As only particular isoforms of proteins were modulated, type 1 diabetes seemed to differentially affect posttranslational modification.

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Key Words: Saliva; type 1 diabetes; proteomics; isoforms; oral inflammation.

Introduction

Saliva is a biological fluid of great potential in clinical medicine *(1,2)* owing to opportunity of noninvasive, stress-free, simple, and safe collection. As modification of the salivary composition was demonstrated in pathological disorders, the interest to study systemic diseases that affect salivary gland function is growing *(3)*. For example, diabetes mellitus was shown to lead to marked dysfunction of the secretory capacity of the salivary glands and then may be responsible for the increased susceptibility to oral infection *(4)*. Type 1 diabetes is also known to affect oral homeostasis, and several studies showed the prevalence of gingival inflammation in diabetic patient. Both plaque and gingival indices, bleeding scores, probing depth, loss of attachment, and number of missing teeth were demonstrated to increase in diabetic patients *(5)*, whereas all measured parameters of periodontal status were affected in type 1 diabetic adolescents *(6)*.

Until recently, the study of salivary protein modification occurring in type 1 diabetes was mainly based on techniques targeting specific proteins. For instance, using enzymatic assays, salivary concentration of aminotransferases and lactate deshydrogenase was found to increase in type 1 diabetic patients when compared to healthy subjects *(7,8)*. Similarly, the use of enzyme-linked immunosorbent assay (ELISA) allowed the specific detection of glutamic acid decarboxylase autoantibodies in oral fluid of type 1 diabetic patients *(9,10)*. However, such approaches do not allow for a comprehensive characterization of the salivary composition. To obtain more systematic insights into changes in the whole saliva composition, protemic tools were recently introduced. The first bidimensional protein map, combined to mass spectrometry, was produced to compare the protein composition between human-acquired enamel pellicle and whole saliva. Although this work revealed a relatively simple protein profile and identified only 7 different proteins *(11)*, further investigation allowed the construction of a reference salivary protein map cumulating nearly 600 protein spots of which more than 300 were identified *(12–16)*. By comparison to other proteomes, the whole salivary proteome appears to recruit a relatively low number of different protein accessions but display extensive posttranslational modification of these proteins. A typical example concerns the salivary α-amylase, that is present simultaneously as well as under native, glycosylated, or truncated isoforms as under low-molecular-weight forms but showing both N- and C-terminal sequences, leading to hypothesize the occurrence of internal deletions *(17)*.

To date, differential comparison between proteome from healthy and diseased subjects was initiated to identify proteins that could be used as biomarkers *(18)*. However, owing to the emerging complexity of salivary proteome, and despite the absence of knowledge concerning its functional meaning, the occurrence of multiple isoforms raises the question of their potential use as biomarkers. The present work aimed at investigating modification of the salivary proteome occurring in type 1 diabetes, with special attention to track changes in patterns of isoforms for identified proteins. For this purpose, to separate, compare, and identify proteins between patients, we combined, respectively, high-resolution bidimensional gel electrophoresis, powerful image analysis, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/MS).

Materials and Methods

Chemicals

Immobiline dry strip gels (180 mm) were from Amersham Biosciences (Orsay, France), protease inhibitor cocktail from Roche (Meylan,

France), modified sequencing grade trypsin from Promega (Charbonnieres, France), C18 Zip-Tip™ from Millipore (Bedford, MA) and kit for protein concentration measurement from Pierce (Rockford, IL). Other high purity reagents were from Sigma (L'Isle d'Abeau, France) or Bio-Rad (Richmond, CA).

Subjects

The type 1 diabetes group encompassed eight patients with an average age of 35.6 ± 9.9 yr. The disease duration was superior to 5 yr and patients had a poorly controlled insulin-dependent diabetes (glycated hemoglobin HbA1C >8%). The control group was constituted of eight healthy individuals of similar age (34.7 ± 8.2 yr) showing no active disease, no history of drug treatment or therapy within the previous months, and no history of diabetes. Both groups did not manifest clinical signs of intraoral inflammation and had a good buccal hygiene.

Collection of Saliva

The study was approved by the Ethics Committee of the CHU of Montpellier. Whole saliva was collected 2 h after breakfast time, smooth tooth brushing, and rinsing mouth with water 15 min before collection. Stimulated saliva was collected for 5 min by chewing on paraffin wax and spitting intermittently in tubes containing a protease inhibitor cocktail and maintained on ice. After collection, samples were frozen at –20°C.

Protein Extraction

Samples were centrifuged at 10,000*g* and at 4°C for 15 min to eliminate mucines and avoid any oral debris. Proteins were precipitated using 90% acetone (v/v), 10% TCA (v/v), and 0.07% 2-mercaptoethanol (v/v). After incubation at –20°C for 2 h, insoluble material was centrifuged at 37,000*g*. Pellets were washed three times with pure acetone containing 2-mercaptoethanol, air-dried and solubilized in 9 *M* urea, 4% CHAPS (w/v), 0.05% Triton X-100 (v/v), and 65 m*M* DTT. Protein amount was estimated using the Bradford assay ***(19)***.

Two-Dimensional Gel Electrophoresis

Precast IPG strips (pH 3.0–10.0 NL) gradient were rehydrated overnight with 200 µg of protein sample. Isoelectric focusing was carried out using the IPGphor isoelectric focusing system (Amersham Biosciences, Orsay, France) for a total of ca. 50,000 V.h. Thereafter, strips were equilibrated for 15 min in 8 *M* urea, 50 m*M* Tris-HCl buffer at pH 8.8, 30% glycerol (v/v), 2% SDS (w/v), and 65 m*M* DTT, and finally for 15 min in the same solution excepted that DTT was replaced by 13.5 m*M* iodoacetamide. Proteins were finally separated on 12% SDS-polyacrylamide gels, at constant voltage (150 V) and at 10°C, using an Iso-DALT electrophoresis unit (Amersham Biosciences, Orsay, France). Gels were stained with colloidal Coomassie blue. Gel images were digitalized at 300 dpi with a GS 710 densitometer (Bio-Rad, Hercules, CA).

Image Analysis

Gel images were analyzed using the Progenesis Workstation software (Nonlinear Dynamics, UK). First, gels from healthy donors and diabetic patients were aggregated into two separate groups. The common area on all gels was selected for spot detection. For each group, the gel with the highest number of spots was autoselected as reference gel. Images of gels were first warped to this gel before matching spots. An average master gel was thus created for each group including all spots detected in individual gels. For comparative analysis, the intensity of spots was measured by the volume (expressed as percentage of total volume of all spots on respective gels) and data were submitted to Student's t-test for spot selection. One way ANOVA were also used for spot selection and led to similar conclusions.

In-Gel Digestion

Spots were excised from gels by hand. The protocol of in-gel digestion is adapted from **ref. 20** and was realized by a Packard Multiprobe II liquid handling robot (Perkin Elmer, Courtaboeuf, France). Protein excised spots from the stained gels were washed successively with water, 25 m*M* ammonium bicarbonate, HPLC grade acetonitrile/25 m*M* ammonium bicarbonate (1:1 v/v), and acetonitrile to remove remaining contaminants and to destain the proteins. The gel fragments were dried at 37°C. Digestion was carried out at 37°C for 5 h with 10 µL of 0.0125 µg, 1 µL trypsin (sequencing grade, modified; Promega) in 25 m*M* ammonium bicarbonate (pH 7.8). The resulting tryptic fragments were extracted twice with 50 µL of acetonitrile/water (1:1 v/v) containing 0.1% trifluoroacetic acid for 15 min. The pooled supernatants were concentrated to a final volume of ca. 20 µL by heating at 37°C for ca. 5 h. The tryptic peptides were desalted and concentrated to a final volume of 3 µL with Zip-TipTM C18 (Millipore), according to the manufacturer's protocol and immediately spotted onto the MALDI target by the robot.

MALDI Mass Spectrometry Analysis

α-Cyano-4-hydroxycinnamic acid matrix was prepared at half saturation in acetonitrile/water (1:1 v/v) acidified with 0.1% trifluoroacetic acid. 0.8 µL of each sample was mixed with 0.8 µL of the matrix and the mixture was immediately spotted on the MALDI target and allowed to dry and crystallize. The analyses were performed on a BiFlex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Reflector spectra were obtained automatically with the AutoXecute™ mode over a mass range of 700 to 3500 Da in the short-pulsed ion extraction mode using an accelerating voltage of 19 kV. Spectra from 200 laser shots were summed to generate a peptide mass fingerprint for each protein digest. Two peptide ions generated by the autolysis of trypsin (842.5099 and 2211.1046) were used as internal standards for calibrating the mass spectra. Automatic annotation of monoisotopic masses was performed using Bruker's SNAP procedure.

Identification by Database Search

We used the MASCOT search engine software (Matrix Science, London, UK) running on a local server to search NCBInr database. The following parameters were used for database search: (1) taxonomy search restrained to *mammalia*, (2) mass tolerance of 50 ppm, (3) a minimum of five peptides matching to the protein, (4) one missed cleavage allowed, (5) carbamidomethylation of cysteine as fixed modification, and (6) oxidation of methionine and pyroglutamylation of glutamine as variable modifications.

Results and Discussion

Proteome Comparison

Type 1 diabetes is a complex endocrine disease with a number of associated pathologies like neuropathy, cardiovascular disease, or oral pathologies **(21)** and care must be taken when selecting diabetic patients to minimize protein variation specifically induced by these associated pathology. To ensure homogeneity within the group, the schedule of conditions was restricted to 20- to 45-yr-old patients, showing diabetes type 1 since at least 5 yr old **(Table 1)**. In addition, as major biochemical changes are known in poorly controlled insulin-dependent diabetes **(22)**, we choose patients with poorly controlled insulin-dependent diabetes (HbA1C >8%; **Table 1**) to evaluate the impact of glucose metabolism on the salivary proteins synthesis.

As no previous information was available concerning the stability of saliva samples, preliminary experiments were performed on

Table 1
Clinical Details for Diabetic Patients[a]

Patient number	Sex	Age	Diabetes duration	HbA1c
9	M	24	5	13.7
10	M	45	12	10.7
11	M	26	6	11.1
12	F	45	10	10.5
13	F	39	8	9.1
14	M	42	20	10.2
15	M	42	37	8.1
16	M	22	8	12.0

[a]Patient 9–16 included sex (M refer to male and F to female), age (years), diabetes duration (years), and percentage of glycated haemoglobin (HbA1c).

various saliva samples. No changes in protein profile could be detected after storage up to 7 d at 4°C or up to 4 mo at –20°C (not shown). Therefore, in the present work, to ensure an increased protection against eventual degradation, samples were immediately frozen at –20°C after collection and processed within 10 d. To minimize experimental variation, standardized procedures were selected, including simultaneous processing of the two kinds of samples, and the overall reproducibility of the procedure was estimated. For this latter purpose, triplicates of samples were compared. Concerning the saliva protein concentration, no significant variation could be detected between the two studied groups, controlled and diabetic. As no presence of hemoglobin could be visually detected, a comparison on the basis of identical protein amounts loaded on gels can be allowed.

The variation coefficient for the number of detected spots amounted to 5 and 88% of spots could be matched on every gel. For these matched spots, the average variation coefficient for intensity was of 35%. This showed that protein separation was robust and suggested that significant variations could be identified for fold changes of a factor 2.

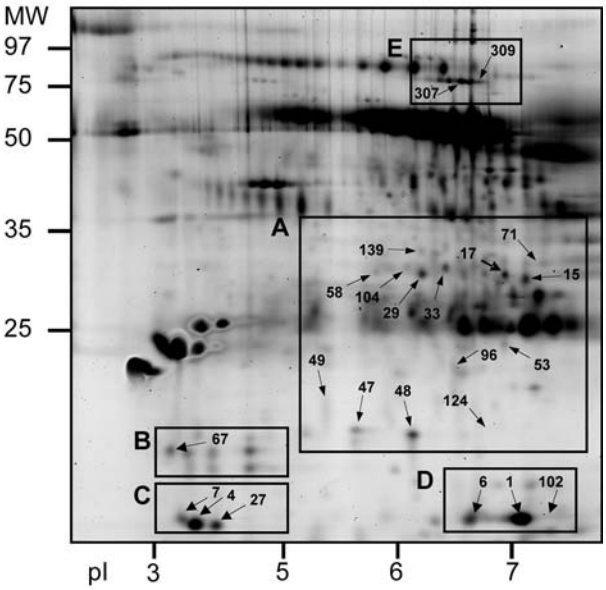

Fig. 1. Two-dimensional map of human salivary proteins from diabetic type 1 patient. Two hundred micrograms of proteins were resolved using pH 3.0–10.0 NL IPG and 12% SDS-PAGE and gel was stained with colloidal Coomassie blue. Spots in **A–E** were identified by MALDI-TOF/MS as α-amylase **(A)**, prolactin inducible protein **(B)**, salivary acidic protein-1 **(C)**, salivary cystatin SA-1 **(D)**, and serotransferin **(E)**.

A typical two-dimensional gel is shown on **Fig. 1**. Similar numbers of spots were detected on individual gels from healthy subjects (243 ± 36 spots) and diabetic patients (255 ± 55 spots). Master gels for the two groups were found to share 214 spots that were selected for subsequent statistical analysis. As illustrated on **Fig. 2**, it should also be emphasized that not every gel displayed all these spots (like spot 27) and that intrapopulation differences in the abundance of some spots were also observable (like for spot 4). Nevertheless, statistical analysis showed that 23 spots displayed significantly different accumulation levels between the two groups **(Fig. 1)**, with fold changes exceeding, respectively, 7 and 10 for spots, respectively, over-accumulated and under-accumulated in saliva of diabetic

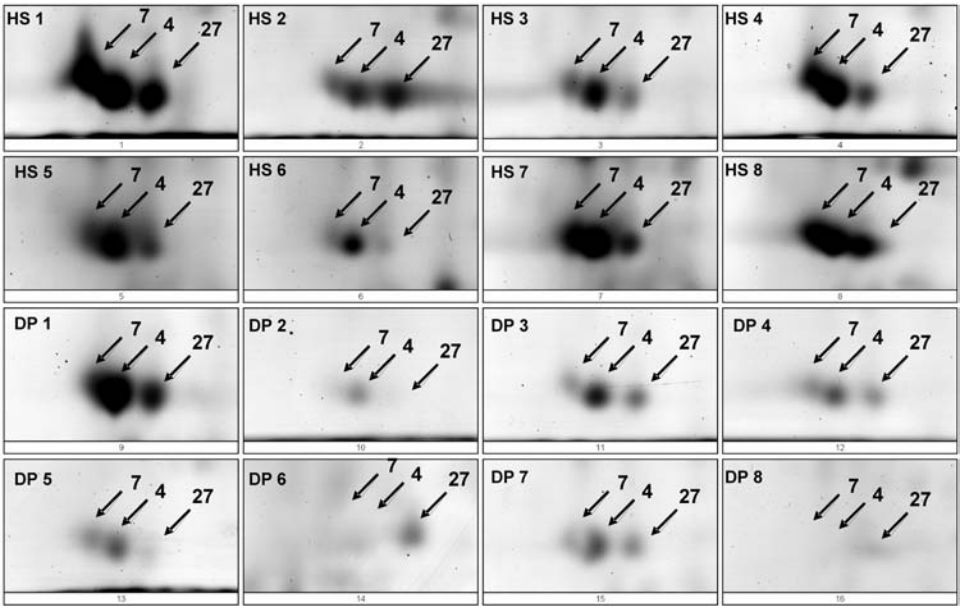

Fig. 2. Intrapopulation variability of protein accumulation. Example of salivary acidic protein-1 isoforms showing quantitative differences for diabetic patients (DP) and healthy subjects (HS).

patients (Table 2). Protein identification by MALDI-TOF/MS showed that they corresponded to five different accessions in databases, of which four were identified in multiple spots. Therefore, the salivary proteome appears to differ in diabetic patients, but the differences both concern a relatively limited part of the proteome (ca. 10% of spots) and are mainly restricted to some isoforms of a few proteins. However, as the analysis was performed only on spots detected on master gels from both groups, the possibility of larger variations, through specific presence of given spots in only one group, cannot be ruled out.

Proteins Under-Accumulated in Saliva From Diabetic Patients Are Involved in Oral Anti-Inflammatory Processes

The largest group (Fig. 1A) included 14 spots of α-amylase (Swiss-Prot P04745). These spots were detected in nearly all subjects and showed an average fivefold under-accumulations in diabetic patients (Table 2). α-Amylases belong to the family of α-1,4-glucan-4-glucanohydrolases that catalyze the cleavage of α-D-(1-4) glycosidic bonds in starch and related carbohydrates (23). It constitutes one of the major secretory products of the pancreas and salivary glands (24). Amylases would also be involved in anti-inflammatory reactions, such as those caused by the release of histamine and similar substances, and α-amylase is used, among other medicines, in the treatment of some lung problems including asthma and emphysema. Furthermore, in solution, α-amylase has been shown to bind with high affinity to oral viridans streptococci, an interaction that may lead to the clearance and/or adherence of these bacteria in the oral cavity (25). Therefore, it could be speculated whether the under-accumulation of α-amylase spots in diabetic patients could be related to changes in oral anti-inflammatory status. In this view, it must be pointed out that all under-accumulated α-amylase spots exhibit a molecular weight lower than that of native α-amylase (56 kDa). The occurrence of such

low-molecular-weight isoforms was reported in several recent works (*12–14,16,17*). According to these data (*16,17*), the 14 spots shown here to be under-accumulated in diabetic patients would correspond to about 30% of the previously described low-molecular-weight isoforms. This suggests that the disease would affect selectively only a part of α-amylase isoforms, the biological role of such isoforms still remains to be investigated.

The second group of proteins under-accumulated in type 1 diabetes was composed of isoforms of two different salivary cystatins: three spots identifying the salivary acidic protein-1 (Swiss-Prot P01036; **Fig. 1C**) and three spots of salivary cystatin SA-1 (Swiss-Prot P01037; **Fig. 1D**). As for α-amylase, these spots were detected in nearly all subjects but average under-accumulation in diabetic patients was two-times lower for salivary acidic protein-1 than for cystatin SA-1 or α-amylase (**Table 2**). Salivary cystatins constitute a large group of proteins that are potent inhibitors of cystein peptidases. Salivary acidic protein-1 belongs to the cystatin S subgroup, inhibits papain and ficin, and is involved in the prevalence of periodontal disease (*26*). Cystatin SA-1 is part of the cystatin SN subgroup that differs from cystatins S by their specificity. Beside the control of proteolytic events in vivo, both subgroups are known to be involved in the regulation of inflammatory processes (*27*) with examples concerning the nonimmunological defense against oral microorganisms like *Streptococcus salivarus* (*28*) and the regulation of inflammatory processes in the oral cavity and ocular tissues (*25*). On this basis, it can be hypothesized that type 1 diabetes could induce a specific decrease in nonimmunological defense of the oral cavity. Interestingly, these under-accumulated cystatins cover most of the isoforms previously detected for the acidic protein-1 and the cystatin SA-1 in the saliva proteome (*16*), but other subgroups of cystatins appear to remain unaffected in diabetic patients. By this way, and conversely to α-amylase where only a part of isoforms is sensitive to diabetes, the disease would exert a limited control of the accumulation of cystatins by affecting most products of only some cystatin genes.

The last spot under-accumulated in diabetes type 1 identified a prolactin-induced protein (PIP) (Swiss-Prot P12273; **Fig. 1B**). This spot was detected in only half diabetic patients where it showed an average fivefold under-accumulations (**Table 2**). The PIP is believed to originate from a limited set of tissues, including breast and salivary glands, and was previously used as a clinical marker for the diagnosis of metastatic tumors of unknown origin (*29*). It was also proposed to be involved in the nonimmunological host defense by binding to bacteria (*30*). On another hand, only one spot of PIP was shown here to be responsive to diabetes, whereas the protein was observed in several spots (*16*). Therefore, this suggests that, as pointed above for α-amylase, only a part of PIP isoforms would be affected by the disease.

It is striking that, for all the under-accumulated proteins, previous data point to their possible involvement in nonimmunological anti-inflammatory processes of oral cavity. Although no oral complication was observed in the diabetic patient group, this suggests that a generalized decrease in the nonimmunological defense could be associated to the disease and possibly account for the prevalence of oral infections that is known in patients with type 1 diabetes. In this view, the decrease in the defense would result from complex regulations concerning both specific isoforms (in the case of α-amylase and PIP) and the overall secretion (for some subgroups of cystatins). Numerous reports describe the occurrence of multiple isoforms for these proteins (12–14, 16, 17, 19, 24, 25, 36–38, 40–44). However, both the molecular basis for these regulations by diabetes and their functional consequences remain to be elucidated.

Table 2
Protein Spots Showing Significant Differences ($p < 0.05$) in Their Accumulation in Saliva From Diabetic Patients and Healthy Subjects[a]

Spot number	Protein name	Mascot PMF score	Number of matching peptides	Area	Accession Swiss prot	MW (kDa)	pI	Matchs HS	Matchs DP	Fold change	p value
1	Salivary cystatin SA-1	182	10	D	P01037	14.3	6.92	8	8	–1.9	0.041
4	Salivary acidic protein-1	132	14	C	P01036	14.2	4.83	8	7	–2.0	0.049
6	Salivary cystatin SA-1	165	9	D	P01037	14.2	4.83	8	8	–2.3	0.032
7	Salivary acidic protein-1	158	14	C	P01036	14.2	4.83	8	7	–3.6	0.018
15	Salivary α-amylase	210	22	A	P04745	55.9	6.34	8	8	–2.6	0.016
17	Salivary α-amylase	140	15	A	P04745	55.9	6.34	8	8	–2.3	0.013
27	Salivary acidic protein-1	202	17	C	P01036	13.5	5.47	8	8	–2.6	0.031
29	Salivary α-amylase	185	19	A	P04745	55.9	6.34	8	8	–3.8	0.012
33	Salivary α-amylase	236	21	A	P04745	55.9	6.34	8	8	–3.7	0.025
47	Salivary α-amylase	176	14	A	P04745	55.9	6.34	8	7	–2.8	0.041
48	Salivary α-amylase	179	14	A	P04745	55.9	6.34	8	8	–3.8	0.033
49	Salivary α-amylase	248	19	A	P04745	55.9	6.34	7	3	–8.3	0.019
53	Salivary α-amylase	161	15	A	P04745	55.9	6.34	6	6	–8.2	0.009
58	Salivary α-amylase	142	11	A	P04745	55.9	6.34	7	7	–6.5	0.019
67	Prolactin-inducible protein	155	8	B	P12273	13.5	5.47	7	4	–5.9	0.040
71	Salivary α-amylase	127	14	A	P04745	55.9	6.34	6	5	–4.3	0.040
96	Salivary α-amylase	140	12	A	P04745	55.9	6.34	8	8	–3.8	0.010

(Continued)

Table 2 *(Continued)*

Spot number	Protein name	Mascot PMF score	Number of matching peptides	Area	Accession Swiss prot	MW (kDa)	pI	Matchs HS	Matchs DP	Fold change	*p* value
102	Salivary cystatin SA-1	141	8	D	P01037	14.3	6.92	8	8	–10.8	0.047
104	Salivary α-amylase	214	17	A	P04745	55.9	6.34	8	7	–5.6	0.026
124	Salivary α-amylase	231	19	A	P04745	55.9	6.34	8	7	–10.3	0.033
139	Salivary α-amylase	285	22	A	P04745	55.9	6.34	8	6	–6.0	0.014
307	Serotransferrin	189	17	E	P02787	75.2	6.70	3	2	7.1	0.000
309	Serotransferrin	204	18	E	P02787	75.2	6.70	3	3	5.3	0.023

[a]The area column refers to the box of localization in **Fig. 1**. The significance threshold of the Mascot database search restrained to *mammalia* is of 64. Negative fold changes refer to under-accumulated spots in saliva of diabetic patients. *p* value was obtained using Student's t-test. MW corresponds to the theorical molecular weight of the proteins.

Over-Accumulated Proteins in Saliva From Diabetic Patients

Two spots of serotransferrin (Swiss-Prot P02787; **Fig. 1E**) were found to display an average sixfold over-accumulation in saliva of diabetic patients. However, these spots were observed in only a minority of subjects **(Table 2)**, suggesting the possible occurrence of different responses according to the patients. Transferrins are iron binding transport proteins expressed in the liver and secreted in plasma *(31)*. The accumulation of these proteins in blood was previously described for diabetic patients in response to oxidative stress *(32,33)*. At first sight, this result appears to be surprising whereas serotransferrin was not described to be secreted into saliva. A hypothesis of its presence in saliva could be a blood contamination. However, considering that we used a standard protocol described in other salivary proteome publication *(11)*, and that we have carefully verified the colorless of our saliva samples, this hypothesis is improbable. Also, we can notify that a number of plasma proteins are known to be transferred into saliva by exudation of plasma in the oral cavity *(34,35)*. Moreover, if the presence of serotransferrin in saliva is rare, it has already been reported in nonstimulating whole saliva investigated using two-dimensional liquid chromatography and mass spectrometry *(15,36)*. Considering that the amount of plasmatic protein in saliva is proportional to the blood concentration of free protein *(37)*, we can hypothesize that the accumulation of transferrin in saliva might be related to an increase in free plasmatic serotransferrin in diabetic patients.

Concluding Remarks

The present work shows, for the first time, that the salivary proteome of diabetic patients differs from healthy subjects. It is noteworthy that type 1 diabetes appears to induce relatively limited and subtle changes in the saliva proteome. Moreover, beside the rare positive markers observed, these changes offer more functional information than candidate markers. Of physiological interest, lower abundances of given α-amylase, cystatin, and PIP isoforms are demonstrated to occur

concomitantly in diabetic patients. According to available data concerning the role of these proteins in anti-inflammatory processes, this situation is proposed to reveal a decrease of the nonimmunological defense of the oral cavity. On another hand, this work illustrates also that the proteomics technology has the power to evidence events undetectable when using global protein approaches, the latter lacking to address individual isoforms. In this view, the saliva proteomics can be speculated simultaneously to constitute an efficient diagnostic tool and to open new challenges for deciphering the function of responsive isoforms.

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