Complexity of the human whole saliva proteome

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Recent characterization of the whole saliva proteome led to contradictory pictures concerning the complexity of its proteome. In this work, 110 proteins were analysed by mass spectrometry allowing the identification of 10 accessions previously not detected on protein two-dimensional maps, including myosin heavy chain (fast skeletal muscle, IIA and IIB), phosphatidylethanolamine binding protein, secretory actin-binding protein precursor and triosephosphate isomerase. Further comparison with available data demonstrated simultaneously a low diversity in terms of variety of accessions and a high complexity in terms of number of protein spots identifying the same accession, the two thirds of identified spots corresponding to amylases, cystatins and immunoglobulins. This diversity may be of interest in the development of non invasive diagnostic tool for several disease.

Key words: Saliva, Protein processing, Proteomics, Mass spectrometry, Human.

Whole saliva is a mixture of secreted fluids having three main origins: the major and minor salivary glands (for *ca* 90%), and the gingival crevicular fluid (for *ca* 10%) by filtration through tight junctions, passive diffusion and exudation of plasma in the oral cavity (14, 16). Accordingly, it constitutes a quite complex bio-

logical fluid containing a variety of both inorganic and organic components, such as minerals, lipids, growth factors, hormones and an array of proteins (10). Whole saliva is known to have important physiological functions and to play a significant role in oral homeostasis (16). However, overall biochemical and molecular characterization of saliva remains quite limited, whilst such data would be highly desirable to increase our knowledge in terms of physiology. Further-

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more, saliva can be collected easily, in a non-invasive way. In this view, saliva could have an high potential for the development of novel diagnostic tests (5).

Whole saliva is composed of different components among which protein is most abundant. As a consequence, it is of crucial importance to better understand its physiological functions, which could offer important benefits of potential use in clinical medicine. For a long time, most available information about salivary proteins relied on dedicated techniques having no large scale capacity for protein identification (2, 3, 6, 17, 19). However, very recently, three works introduced a proteomic technology, combining resolute two-dimensional electrophoresis and mass spectrometry, to map whole saliva proteins. Accordingly, the saliva proteome was firstly shown to display a relatively simple pattern with 7 accessions identified in 10 protein spots (21). By opposition, in two other works, a much more complex situation was described (7, 20). In these cases, 20 to 35 accessions were identified within one hundred of protein spots. The most striking result correspond to redundant spots like α -amylase, cystatins, a prolactin inducible protein and zinc- α_2 -glycoprotein. Therefore, available proteomic characterizations lead to different pictures that are intriguing in several aspects. On a proteomic point of view, the occurrence of co-migrating spots is usually taken as frequent (8), and, on another hand, the observation of multiple spots for a same accession is attributed to post-translational modifications or event degradations. With respect to saliva, the observed proteome can be discussed in terms of post-translational modifications for a part of accessions. For others, like α -amylase, when the occurrence of isoforms is fre-

quent (2), redundancy can not be ascribed from aforementioned post-translational modification nor protein degradation. In this case, an additional possible mechanism would rely on bacterial degradation (7). This could deeply affect any strategy in search for biological markers in clinical applications.

The goal of this study was to investigate the complexity of the whole saliva proteome. For this purpose, two-dimensional maps displaying *ca.* 700 spots were established and characterized by MALDI-TOF MS.

Material and Methods

Chemicals.— Dry cover fluid, 180 mm immobiline dry strip gels and IPG buffer were from Amersham Biosciences (Orsay, France). Urea, thiourea, CHAPS, DTE, DTT, TRIZMA base, glycerol, SDS, iodoacetamide, ammonium persulfate, TEMED, glycine, agarose, β -mercaptoethanol, molecular weight markers, ammonium sulfate, α -cyano-4-hydroxycinnamic acid, HPLC grade acetonitrile and trifluoroacetic acid were purchased from SIGMA (L'Isle d'Abeau, France). Microsep 3K were from Pall (Ann Arbor, USA). 30% acrylamide/bis (37.5:1 w/w) and Coomassie blue G-250 were from Bio-Rad (Richmond, USA). The kit to measure protein concentration was from Pierce (Rockford, USA), the protease inhibitor cocktail from Roche (Meylan, France), modified sequencing grade trypsin from Promega (Charbonnières, France) and C18 Zip-TipTM from Millipore (Bedford, USA).

Saliva collection and protein extraction.— Parafilm-stimulated whole saliva was collected 2 hours after usual breakfast time (17) and complemented with a pro-

tease inhibitor cocktail. Saliva samples were centrifuged at 10,000 x g for 15 min, and the supernatant was frozen at -80°C.

Protein were precipitated using 90 % acetone (v/v), 10 % (v/v) TCA solution (100 % w/v) and 0.07% 2-mercaptoethanol (v/v). After incubation at -20°C for 2 hours, insoluble material was pelleted at 37000 g with an Allegra 64-R centrifuge (Beckman Coulter, CA, USA). Pellets were washed three times with pure acetone containing 0.07% 2-mercaptoethanol (v/v), air dried and solubilised in buffer containing 9 M urea, 4 % CHAPS (w/v), 0.05 % Triton X100 (v/v) and 65 mM DTT. Protein amount was estimated using the Bradford method (4).

Two-dimensional gel electrophoresis.— Precast IPG strips with nonlinear immobilized pH 3-10 gradient were rehydrated overnight with 200 mg of protein sample complemented with 0.0025% v/v bromophenol blue and 1 % v/v IPG buffer. Isoelectric focusing was carried out using the IPGphor™ isoelectric focusing system (Amersham Biosciences, Orsay, France) : 500 V (1 h), 1000 V (2 h), linear gradient from 1000 V to 8000 V, and a final phase at 8,000 V for 5 h, resulting in a total of ca 50,000 V.h. Thereafter, strips were equilibrated for 15 min in 9 M urea, 50 mM Tris HCl buffer at pH 8.8, 30 % v/v glycerol, 2 % SDS, 0.001 w/v bromophenol blue and 65 mM DTT, and finally for 15 min in the same solution except that DTT was replaced by 13.5 mM iodoacetamide. Then, proteins were separated on 12 % SDS-polyacrylamide gels at a constant voltage of 150 V overnight at 10 °C, using an Iso-DALT electrophoresis unit (Amersham Biosciences, Orsay, France). Gels were stained with silver nitrate or colloidal Coomassie blue. Computer analysis of 2D-PAGE images was

performed using Melanie II® software (Bio-Rad, Richmond, USA).

In-gel digestion.— The protocol of in-gel digestion is adapted from (11), using a Packard Multiprobe II liquid handling robot (Perkin Elmer, Courtaboeuf, France). Protein spots were excised from colloidal Coomassie blue stained gels, and washed successively with water, 25 mM ammonium bicarbonate, acetonitrile / 25 mM ammonium bicarbonate (1:1, v/v) and acetonitrile. Gel fragments were dried at 37 °C. The digestion was carried out at 37°C for 5 hours after addition of 10 µL of 0.0125 µg.µL⁻¹ trypsin in 25 mM 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. The tryptic peptides were desalted and concentrated to a final volume of 3 µL with C₁₈ Zip-Tip, and immediately spotted onto the MALDI target by the robot.

MALDI-TOF MS analysis.— The 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 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 700-3500 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 were used as internal standards for calibrating the mass spectra. Automatic annotation of monoisotopic masses was performed using Bruker's SNAPTM procedure.

Identification in database.— The MASCOT search engine software (Matrix Science, London, UK), running on a local server, was used to search the NCBI nr database. The following parameters were used for database search : mass tolerance of 50 ppm, a minimum of five peptides matching to the protein, and one missed cleavage allowed.

Results

Whole saliva generates complex proteome maps.— The whole saliva proteome was investigated by two-dimensional gel electrophoresis with the first goal to establish a map giving an overview of proteins present in saliva. For this purpose, pH 3-10 IPG gels and 12 % SDS-polyacrylamide gels were selected in order to separate proteins covering the whole pI range and a large MW range between 15 and 120 kDa. In addition, large format gels (18 x 20 cm) were used to improve the resolution. More than 700 protein spots were detected after silver staining of gels loaded with 100 mg of desalted protein (Fig. 1). These proteins covered all the MW range investigated but displayed mainly acidic and neutral pI. In order to characterize these proteins, micro-preparative gels were loaded with 500 mg salivary proteins and stained with colloidal Coomassie blue. The 100 most abundant spots were picked out, digested by trypsin and processed for MALDI-TOF MS fingerprinting. When fixing a mass tolerance

of 50 ppm, a minimum of five identified peptides per protein, and allowing no more than one missed cleavage, a total of 110 proteins was thus identified. These identifications showed a score that, in average, was 65 % higher than the significance threshold ($p = 0.05$) for the queried databases (Table I). In addition, the average sequence coverage amounted to 37 %. Together with the scores and the selected criteria, this argued for confident identification of proteins from their peptide mass fingerprints, including for the few proteins identified in the same spots due to co-migration.

In terms of sequences, the 110 characterized protein spots identified 26 different accessions in the database (Table I): one accession code for human salivary α -amylase (42 spots), one for serum albumin (4 spots), four for immunoglobulin chains (5 spots), two for myosin chains (6 spots), four for various cystatins (23 spots), one for calgranulin B (1 spot), one for lacrimal lipocalin (2 spots), one for secretory actin-binding protein (6 spots), one for α -enolase (2 spots), one for glutathione-S-transferase (3 spots), one for zinc α_2 -glycoprotein (5 spots), one for zinc finger protein (1 spot), one for serine proteinase inhibitor (1 spot) and one for an unknown protein (1 spot). Additionally, it should be noted that the following accessions were identified for the first time in saliva : myosin heavy chain (fast skeletal muscle, IIA and IIB), phosphatidylethanolamine binding protein, secretory actin-binding protein precursor and triosephosphate isomerase.

The saliva proteome includes a number of potentially processed proteins.— MS identification showed that 75 % of accessions were found in several spots. In addition, for *ca.* 60 % of proteins, the

Fig. 1. *Two-dimensional map of human salivary proteins.*

Proteins were resolved using pH 3-10 NL IPG and 12 % SDS-PAGE and gel was silver stained. Proteins subsequently identified by MALDI-TOF MS are indicated by respective spot numbers. Two different spot numbers are given for comigrating proteins.

Table I. *Identified proteins in whole saliva.*

Except when indicated protein name, accessions refer to human proteins, according to NCBI and/or SwissProt (*) databases. Sequence coverages are given as percentage (% cov.). Theoretical (theo.) and observed (obs.) MW and pI values were computed from the protein sequence or deduced from 2D gels.

Spot n°	Protein Name	Accession	% cov.	MW		pI	
				theo.	obs.	theo.	obs.
S002	serum albumin, chain A	1A06A	29	69366	68000	5.63	5.2
S003	serum albumin, chain A	1A06A	25	69366	68000	5.63	5.4
S005	serum albumin, chain A	1A06A	25	69366	68000	5.63	5.6
S006	serum albumin, chain A	1A06A	27	69366	68000	5.63	5.6
S007	serum albumin, chain A	1A06A	25	69366	68000	5.63	5.7
S024	zinc-alpha-2-glycoprotein, chain D (fragments)	1ZAGD	52	30547	44000	6.03	5.1
S025	zinc-alpha-2-glycoprotein, chain D (fragments)	1ZAGD	53	30547	43000	6.03	5.2
S026	zinc-alpha-2-glycoprotein, chain D (fragments)	1ZAGD	57	30547	42500	6.03	5.4
S027	zinc-alpha-2-glycoprotein, chain D (fragments)	1ZAGD	53	30547	42500	6.03	5.6
S028	zinc-alpha-2-glycoprotein, chain D (fragments)	1ZAGD	34	30547	34000	6.03	5.6
S029	myosin heavy chain IIB (fragment)	Q9JHR4	31	60994	45000	5.38	5.9

Spot n°	Protein Name	Accession	% cov.	MW		pI	
				theo.	obs.	theo.	obs.
S032	myosin heavy chain (fragment)	Q9JHR4	12	101955	45000	5.23	6.1
S039	alpha enolase	P06733	40	47008	47000	6.99	7.1
S041	alpha enolase	P06733	45	47008	45000	6.99	7.8
S061	cystatin SN precursor	UDHUP2	57	16351	32000	6.82	6.8
S071	myosin heavy chain 2A (fragment)	I51912	19	26881	28000	5.94	6.8
S072	triosephosphate isomerase	P00938	34	26522	26500	6.51	6.7
S077	IgG kappa chain (fragment)	BAA37169	43	23404	25000	6.92	7
S078	IgG kappa chain (fragment)	BAA37169	44	23404	25000	6.92	7
S079	phosphatidylethanolamine binding binding protein, chain A	1BD9A	60	20329	24000	7.18	7.2
S080	myosin heavy chain IIB (fragment)	Q9JHR4	11	60994	45000	5.38	5.9
S081	myosin heavy chain (fragment)	CAA27380	11	101955	36000	5.23	5
S084	Ig alpha-1 chain C region	A1HU	22	37631	37000	6.08	6.3
S085	myosin heavy chain (fragment)	Q9JHR4	11	101955	36000	5.23	5
S086	proteinase inhibitor, clade b	Q96J21	10	44594	35000	6.35	5
S089	Ig chain constant region alpha 1 (fragment)	CAC20453	16	37623	33000	6.08	6.2
S101	hypothetical 18.9 kda protein	Q96DA0*	31	18867	25000	5.38	4
S102	glutathione S-transferase P	P09211	61	23210	24000	5.44	5.5
S103	glutathione S-transferase P	P09212	61	23210	24000	5.44	5.7
S107	glutathione S-transferase P	P09211	46	23210	23000	5.44	6.2
S115	lacrimal lipocalin precursor	LCHUL	37	19250	17000	5.26	4.5
S117	secretory actin-binding protein precursor	SQHUAC	26	19238	18000	5.39	5.2
S118	secretory actin-binding protein precursor	SQHUAC	37	19238	18000	5.39	5.1
S119	secretory actin-binding protein precursor	SQHUAC	37	19238	20000	5.39	5.1
S120	secretory actin-binding protein precursor	SQHUAC	37	19238	20000	5.39	5.1
S121	lacrimal lipocalin precursor	LCHUL	23	19250	16000	5.26	4.6
S122	secretory actin-binding protein precursor	SQHUAC	37	19238	21000	5.39	5
S123	cystatin S precursor	UDHUP1	49	16204	15000	4.95	5
S125	secretory actin-binding protein precursor	SQHUAC	44	19238	12000	5.39	5.2
S126	cystatin S precursor	UDHUP1	42	16204	12000	4.95	5
S127	cystatin A	UDHUP2	32	16351	14000	6.82	5.2
S128	calgranulin B	P06702	5,71	13234	13000	5.71	5.5
S129	cystatin SN precursor	UDHUP2	43	16351	14000	6.82	6.1
S130	cystatin SN precursor	UDHUP2	38	16351	13000	6.82	5.8
S131	cystatin SN precursor	UDHUP2	42	16351	15000	6.82	5.7
S133	cystatin SN precursor	UDHUP2	41	16351	16000	6.82	6.5
S134	cystatin SN precursor	UDHUP2	51	16351	13000	6.82	6.5
S135	cystatin SN precursor	UDHUP2	48	16351	12000	6.82	6.5
S136	cystatin SN precursor	UDHUP2	38	16351	13000	6.82	6.8
S137	cystatin SN precursor	UDHUP2	38	16351	13000	6.82	6.8
S138	cystatin SN precursor	UDHUP2	51	16351	16000	6.82	6.8
S139	cystatin B	UDHUB	55	11167	11000	7.9	7.4
S140	cystatin B	UDHUB	61	11167	11000	7.9	7.4
S141	cystatin B	UDHUB	61	11167	11000	7.9	7.4
S142	cystatin SN precursor	UDHUP2	41	16351	15000	6.82	7.2
S143	cystatin SN precursor	UDHUP2	57	16351	15000	6.82	7.2
S144	cystatin SN precursor	UDHUP2	28	16351	12000	6.82	7.1
S145	Ig chain variable region	AAD30836	57	13816	12000	9.01	7.3
S150	myosin heavy chain (fragment)	Q9JHR4	11	101955	100000	5.46	5.5

Spot n°	Protein Name	Accession	% cov.	MW		pI	
				theo.	obs.	theo.	obs.
S151	cystatin SN precursor	UDHUP2	42	16351	14000	6.82	5.3
S152	cystatin SN precursor	UDHUP2	32	16351	14000	6.82	5.4
S169	cystatin SN precursor	UDHUP2	42	16351	160000	6.82	8
S177	myosin heavy chain (fragment)	Q9JHR4	29	101955	95000	5.46	5.8
S205	cystatin S precursor	UDHUP1	37	16204	13000	4.95	4.8
S206	cystatin S precursor	UDHUP1	44	16204	14000	4.95	4.8
S207	cystatin S precursor	UDHUP1	44	16204	14000	4.95	4.8
S015	salivary α -amylase	1SMD/P04745*	51	55746	56000	6.34	6.1
S016	salivary α -amylase	1SMD/P04745*	50	55724	59000	6.45	6.1
S033	salivary α -amylase	1SMD/P04745*	26	55746	45000	6.34	5.3
S034	salivary α -amylase	1SMD/P04745*	45	55746	43000	6.34	5.5
S035	salivary α -amylase	1SMD/P04745*	45	55746	46000	6.34	5.6
S036	salivary α -amylase	1SMD/P04745*	51	55746	45000	6.34	5.6
S037	salivary α -amylase	1SMD/P04745*	45	55746	46000	6.34	5.7
S038	salivary α -amylase	1SMD/P04745*	30	55746	47000	6.34	6.8
S040	salivary α -amylase	1SMD/P04745*	46	55746	46000	6.34	6.9
S042	salivary α -amylase	1SMD/P04745*	52	55746	46000	6.34	7.2
S043	salivary α -amylase	1SMD/P04745*	49	55746	46000	6.34	7.4
S044	salivary α -amylase	1SMD/P04745*	18	55746	44000	6.34	6.9
S045	salivary α -amylase	1SMD/P04745*	47	55746	45000	6.34	7.2
S046	salivary α -amylase	1SMD/P04745*	56	55746	45000	6.34	7.5
S047	salivary α -amylase	1SMD/P04745*	28	55746	46000	6.34	7.1
S162	salivary α -amylase	1SMD/P04745*	36	55746	46000	6.34	6.9
S050	salivary α -amylase	1SMD/P04745*	47	55746	43000	6.34	6.8
S051	salivary α -amylase	1SMD/P04745*	48	55746	42000	6.34	6.9
S052	salivary α -amylase	1SMD/P04745*	39	55746	39000	6.34	7.4
S053	salivary α -amylase	1SMD/P04745*	40	55746	39000	6.34	7.5
S054	salivary α -amylase	1SMD/P04745*	43	55746	36000	6.34	7.4
S055	salivary α -amylase	1SMD/P04745*	16	55746	37000	6.34	6.8
S056	salivary α -amylase	1SMD/P04745*	50	55746	34000	6.34	6.4
S057	salivary α -amylase	1SMD/P04745*	49	55746	33000	6.34	6.5
S060	salivary α -amylase	1SMD/P04745*	43	55746	32000	6.34	6.7
S062	salivary α -amylase	1SMD/P04745*	56	55746	32000	6.34	7.1
S063	salivary α -amylase	1SMD/P04745*	53	55746	30000	6.34	7.1
S064	salivary α -amylase	1SMD/P04745*	17	55746	28000	6.34	6.8
S068	salivary α -amylase	1SMD/P04745*	44	55746	30000	6.34	6.4
S069	salivary α -amylase	1SMD/P04745*	40	55746	29000	6.34	6.3
S070	salivary α -amylase	1SMD/P04745*	39	55746	29000	6.34	6.5
S088	salivary α -amylase	1SMD/P04745*	31	55746	32000	6.34	6.1
S093	salivary α -amylase	1SMD/P04745*	47	55746	28000	6.34	6.4
S100	salivary α -amylase	1SMD/P04745*	34	55746	28000	6.34	5.7
S104	salivary α -amylase	1SMD/P04745*	27	55746	28000	6.34	5.6
S108	salivary α -amylase	1SMD/P04745*	19	55746	25000	6.34	6.5
S110	salivary α -amylase	1SMD/P04745*	20	55746	23000	6.34	6.2
S111	salivary α -amylase	1SMD/P04745*	31	33074	21000	7.56	5.9
S112	salivary α -amylase	1SMD/P04745*	19	33074	18000	7.56	5.6
S113	salivary α -amylase	1SMD/P04745*	22	33074	21000	7.56	5
S114	salivary α -amylase	1SMD/P04745*	18	55746	22000	6.34	4.7
S116	salivary α -amylase	1SMD/P04745*	17	55746	21000	6.34	4.5

observed MW differed by more than 10 % from its theoretical MW as computed from the corresponding accessions. In the same way, for ca 40 % of proteins, the observed pI differed by more than 10 % from the theoretical one. On another hand, such patterns were observed as well with fresh saliva, that was analysed immediately after collection, as with samples stored for up to 20 days at -80°C or for up to 20 days at room temperature before analysis. In addition, although quantitative variations were found among donors, similar qualitative patterns were found for several healthy donors differing by age and gender (data not shown). Taken together, these observations suggested that the protein composition of whole saliva was stable but quite complex suggesting that a major proportion of the detected proteins could be subjected in vivo to post-translational modification or processing.

Discussion

Saliva is usually taken as a complex medium, that was recently characterized by proteomic approach (7, 9, 20, 21), allowing the cumulated identification of up to 35 accessions.

In the present work, 26 were detected including 10 novel accessions leading to an enriched picture of the saliva proteome. A main feature raising from the 320 protein spots that were characterized in these works (7, 9, 20, 21), is that the saliva proteome contains a relatively low number of different accessions, but, conversely, spread out over a large number of protein spots (Table II).

Accordingly, 3 kinds of proteins account for two thirds of spots (α -amylase, 32 %; cystatins, 23 %; immunoglobulins, 10 %) and 3 for another 15 % (albu-

min, a zinc- α_2 -glycoprotein and a prolactin-inducible protein). On this point of view, our results amplify the most recent saliva proteomic characterization (7, 9, 20) and do not support the previous conclusion that major proteins, such as α -amylase and albumin, display little if any modification (21).

Nevertheless, a very large redundancy of protein spots is also clear for proteins, such as, for instance, the cystatin family of cysteine protease inhibitors. In this case, a total of 9 accessions is presently identified in 93 protein spots. This diversity is in agreement with recent report based on different separation technique and identifying the presence of several modified or possibly truncated forms from the cystatin family in saliva (15).

In the case of α -amylase, 42 characterized protein spots identified accession in the database. Alpha amylase is encoded by a multigene family (1) and constitutes one of the most studied and strongly characterized salivary protein (12, 22). The protein is well known to occur into two major forms, one with 56 kDa MW corresponding to the unglycosylated protein, and another one with 59 kDa MW corresponding to a glycosylated state (3). The two α -amylase spots identified as these two forms, differed by 3 kDa and are part of horizontal series of spots displaying identical MW but different pI. According to the usual interpretation, all these spots could be assumed to exemplify the various isoforms of both glycosylated and unglycosylated α -amylases ranging into a pI range between 5 and 8 (2, 21). In this frame, nearly 30 spots might be associated to unglycosylated forms, and at least 20 spots to glycosylated forms. Thus, α -amylase appears to display a diversity which is far larger than previously reported (7, 20, 21). It should be noted that a

Table II. *Present knowledge of the saliva proteome.*

Data are from proteomic characterization (21, Yao *et al.*, 2003; 7, Ghafouri *et al.*, 2003; 20, Vitorino *et al.*, 2004; 9, Hu *et al.*, 2005) of the whole saliva by 2D gel electrophoresis and MALDI-TOF MS. Protein names in bold refer to those which were identified only in this work.

Protein Name	Yao <i>et al.</i>	Ghafouri <i>et al.</i>	Vittorino <i>et al.</i>	Hu <i>et al.</i>	present work
alpha-amylase	3	27	5	38	42
alpha-enolase			1	1	2
beta2-microglobulin		1	1		
Actin beta				4	
Alpha fetoprotein				6	
Anti Entamoeba histolytica immunoglobulin kappa light chain				1	
Anti HBs antibody light-chain Fab (fragment)				1	
Anti TNF-alpha antibody light-chain Fab				1	
Apolipoprotein A1		2	1		
Beta galactosidase precursor				1	
Ig M heavy chain (fragment)				1	
Calgranulin A	1	1	1		
Calgranulin B		4	2		1
Carbonate dehydratase VI precursor				6	
carbonic anhydrase VI			1		
Cystatin A			1		1
Cystatin B			1		3
Cystatin C			1		
Cystatin D		3	1		
Cystatin S		9	1	2	5
Cystatin SA	1	8	1	1	1
Cystatin SA-III	1		1		
Cystatin SN	2	2	3	3	16
cystein-rich secretory protein 3			1		
Cytokeratin 16				1	
Cytokeratin 2e				1	
Cytokeratin 4				1	
Cytokeratin 6Aa				3	
Desmin				1	
DnaK-type molecular chaperone				1	
DnaK-type molecular chaperone HSP70				1	
Fatty acid binding protein		1	1	1	
Fibrinogen beta chain precursor				2	
Fructose-bisphosphate aldolase				1	
Glutathione S-Transferase P		1	1	1	3
Glutathione synthase				1	
Glyceraldehyde-3-phosphate dehydrogenase				2	
Hemopexin precursor				2	
histatin 1			1		
histatin 3			1		
Hypothetical 18.9 kDa protein					1
Ig alpha-1 chain C region				3	
Ig alpha-2 chain C region				1	
Ig heavy chain constant region alpha 1 (fragment)				3	
Ig mu chain C region				1	
Iga1 chains a and b, heavy, chains c and d, light,chain A				3	

Protein Name	Yao <i>et al.</i>	Ghafouri <i>et al.</i>	Vittorino <i>et al.</i>	Hu <i>et al.</i>	present work
Immunoglobulin k-chain		1	1	1	2
Immunoglobulin A secretory chain		6			1
Immunoglobulin A a-chain C region		6	1		1
Immunoglobulin chain constant region a1					1
Immunoglobulin chain variable					1
Immunoglobulin J chain		3	1		
Interleukin receptor antagonist protein		3	1		
Lacrimal lipocalin precursor		4	1		2
lactoferrin			1		
Lipocortin I				2	
L-lactate dehydrogenase, chain H				1	
Lysozyme C		1	1		
Myosin heavy chain fast skeletal muscle					5
Myosin heavy chain IIA					1
Myosin heavy chain IIB					2
parotid secretory protein			1		
Phosphatidylethanolamine binding protein					1
Phosphatidylinositol transfer protein alpha isoform				1	
Phosphoglycerate kinase				1	
PLUNC			1		
Prolactin inducible protein		11	1	4	
Proteasome subunit, alpha type 3				1	
Protein-glutamine gamma-glutamyltransferase				2	
Hepatocellular carcinoma associated protein				1	
Secretory actin-binding protein precursor					6
Secretory component precursor				6	
Sequence 19 from Patent				4	
Sequence 48 from Patent				1	
Sequence 50 from Patent				1	
Serine/cysteine proteinase inhibitor, clade B					1
Serum albumin	1	4	1	9	5
SNC66 protein				1	
Statherin	1		1		
Stratifin				1	
Transferrin precursor				4	
Transketolase				1	
Triosephosphate isomerase					1
Zinc- α 2-glycoprotein		5	2	6	5

similar situation was recently described in plants, for α -amylase from germinating barley seeds (18).

For most α -amylase spots that are observed at various MW lower than expected, other mechanisms must be taken into account. One such source of diversity of α -amylase patterns was

recently assumed to rely on degradations resulting in truncated forms, either in the N-terminal or in the C-terminal region, with molecular weights spanning between 19 kDa and 35 kDa (21). Since protease inhibitors were added during the collection and storage, it should be emphasized that the possible degradations observed

for α -amylase pattern could occur in the oral cavity, despite the abundance of protease inhibitors in saliva (cystatins, clade B; *ca.* one quarter of identified spots; 7, 9, 21, 22). Perhaps the most intriguing situation corresponds to spots that possess peptides in both the N-terminal and C-terminal regions, but display MW on gel that is 25 % to 50 % lower than expected, suggesting the occurrence of internal deletions. In the absence of complementary knowledge about alternative processing mechanisms in proteins, the elucidation of the origin of such internal deletions remains a challenge.

In conclusion, with respect to the contradiction recently raised by the first proteomic characterizations of saliva (7, 9, 20, 22), our data identify several proteins previously not reported in saliva and argue in favour of a complex proteome. This complexity appears to result from two opposite features: a relatively limited diversity of observed accessions, but counterbalanced by a large redundancy of corresponding spots. A prominent illustration is given by α -amylase that occurs in several spots. This unexpected complexity could be the result of the interplay of (post-) transcriptional processes and many post-translational events, including internal deletions by a presently unknown mechanism. Moreover, this diversity could offer the basis for using saliva in non invasive diagnostic of disease.

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MERER y DEVILLE DE PÉRIÈRE. *Complejidad del proteoma de la saliva humana*. J. Physiol. Biochem., **61** (3), 469-480, 2005.

Las recientes caracterizaciones del proteoma salival completo han llevado a resultados contradictorios. En este trabajo, se han analizado 110 proteínas por espectrometría de masas, lo que ha permitido la identificación de 10 nuevas no detectadas anteriormente en los mapas proteómicos bi-dimensionales. Incluyen cadena pesada de miosina (músculo esquelético rápido, IIa y IIb); proteína de unión a fosfatidiletanolamina, precursor de la proteína secretora de la unión a la actina y trisafosfato isomerasa. Una comparación más precisa con los datos de los estudios precedentes demuestra una baja diversidad en la variedad de accesos y una alta complejidad en el número de bandas correspondientes al mismo acceso. Los dos tercios de las bandas identificadas corresponden a amilasas, cistatinas e inmunoglobulinas. Esta diversidad puede ser de interés en el desarrollo de técnicas de diagnósticos no invasivas.

Palabras clave: Saliva, Maduración proteica, Proteómica, Espectroscopía de masas, Humanos.

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