Chapter 5

Proteomics of the Salivary Fluid

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72309

Abstract

Following the sequencing of the human genome, the mapping of the human proteome is the next task to being completed in order to gain knowledge on how proteins are involved in disease genesis, growth, therapy, and healing. As contrary to the genome, which is relatively static, the human proteome is significantly more complex and highly dynamic. Whilst the majority of the research is being focused on analyzing either the proteome of tumor tissues and tumor cells or the proteome of serum and plasma, little attention has been awarded to the analysis of proteomes in saliva or urine. The proteome in saliva can help providing important information on processes involving health issues in dentistry, head and neck cancers, gastric cancers or neurology, to name just a few. However, this is changing and the proteomics research community is increasingly focusing on deciphering the salivary proteome. So far, more than 3000 proteins have been identified in different studies and more is to come with new instrumentation and methods available. Some of the proteomics methods applied for analysis of salivary proteins will be discussed in this chapter.

Keywords: salival, proteomics, diagnostics, biomarkers, chromatography, mass spectrometry

1. Introduction

The raise of proteomics and the continuous development and improvement of analytical instruments such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS) have substantially fuelled the development of instrumental methods for analysis of proteins for both research and clinical questions [1–6]. Proteomics is not only addressing the efficient separation of peptides upon, mostly, tryptic digestion of proteins and their sensitive detection using mass spectrometry. Proteomics is a technology enabling significantly and profoundly better approach to investigating and understanding proteins' function and their posttranslational modifications [7–12]. Applying proteomics methods for analysis of clinical samples is especially important in time of personalized medicine, which tailors individualized treatment of each

patient based on specification of the diseases [3, 13–19]. Furthermore, (clinical) proteomics can be used as a method-of-choice for the screening of biomarkers used for early discovery and early diagnostics. Early diagnostics and early discovery, needless to say, will help decreasing patients' morbidity and mortality by detecting the disease at the stage when it can be effectively treated with less side effects and at significantly lower cost for the society. This approach can be very beneficial especially for diseases affecting large populations such as cardiovascular diseases, diabetes mellitus and other endocrinal diseases, glioblastoma and similar.

Proteomics can also be applied for the point-of-care diagnostic approaches where both medical professionals and patients can get rapid information and bed-side diagnosis. Of course, classic proteomics approaches with protein extraction from tissue or body fluids and overnight protein digestion cannot be applied; however, proteomics can provide information to be used with kits for point-of-care approach [20–24].

It has already been shown that saliva is a highly valid biological fluid that can be used for diagnostic applications [25–39].

Various number of components can be identified in saliva, which provides real-time data on the patient's condition. The substances found in saliva include but are not limited to DNA, RNA, proteins, metabolites, and microbiota from both oral and gastrointestinal origin. Sample collection is simple, cheap, and can be provided by patient at home without expensive equipment or medical personal needed on-site.

This manuscript will provide a short insight into different techniques applied for proteomics analysis of saliva starting with sample collection, protein precipitation and digestion, peptide separation and MS detection, and finally with data analysis.

2. Sample collection

For medical or biological analysis in general, the method how and when a sample is being collected is of utmost importance. Sample collection is one of the pre-analytical steps that need extraordinary caution and that can influence, badly in most cases, the complete process of sample analysis.

Saliva is mainly composed of water; however, there are a number of other substances being present. Mucins, proteins, DNA, RNA, enzymes, sugars, cell debris, and microbiota and their secretome can interfere with test performance. Therefore, an optimized sample collection process is needed, and a researcher must carefully and cautiously prepare the sample collection step, and the patient must be educated and trained if sample is being to be collected at home. Furthermore, steps like sample storage and transport to the laboratory must also be carefully planned and executed especially when longitudinal studies of the same patient or of the patients' groups are being performed, and especially in parts of the world where appropriate infrastructure is not always available [40–45].

Several methods can be applied for collection of saliva: (1) passive droll is the simplest approach but the saliva often has a high concentration of mucins and high viscosity and (2) the Salivette® Systems [46–48], the Greiner Bio-One Saliva Kit, and the recently introduced

RNA-Prosal [49]. All three sample collection systems have been used in the field, and publications describing their efficacy are available [50–52].

At the Proteomics Core Facility of the Medical University of Vienna Salivette® is being used for induced saliva sample collection by chewing cotton swabs. As mentioned previously, it is of great importance to carefully plan and perform sample collection. The patient or the donor must retain from consuming food, alcoholic beverage, and caffeine at least for 2 h before sample collection. Further, the patient shall briefly wash the mouth using water only. Saliva is being collected for 2 min during which the patient chews the cotton swab. This approach yields approximately 2.5 ml of saliva, which is sufficient for performing proteomics analysis. Some patients, however, need additional stimulation for saliva production and paraffin gum can be used in these cases to stimulate saliva flow and gain enough sample volume. In these cases, the use of Greiner Bio-One Kit helps obtaining more saliva than chewing the cotton swab; however, one shall be careful since this kit contains citric acid, which can lead to protein denaturation and protein loss during the sample collection.

As soon as the sample has been collected it shall be supplemented with enzyme inhibitors in order to suppress enzyme activity and protein degradation. A total protease inhibitor cocktail such as Roche's "Complete Protease Inhibitor Cocktail®" is being added to the sample following centrifugation and removal of cellular debris and prior to storage at −80°C.

It is of extreme importance to secure reproducible sample collection procedures and properly train the patients in cases of self-sampling to avoid sample contamination and alteration. Furthermore, conditions for proper sample transportation and handling until it is being processed must also be carefully considered and applied.

Table 1 shows the steps applied for sample collection and the preparative work.

Step	Device	Temperature	Precautions
1. Sample collection	Salivette® (used in the Proteomics Core Facility at the Medical University in Vienna)	Human body	No food, alcoholic or caffeine beverages until 2 h before sample collection!
2. Centrifugation	Centrifuge	4°C	
3. Protein precipitation	Modified Wessel-Fluegge as described or Aceton	According to the protocol described	Sample should always be prepared on ice!
4. Depletion of high abundant proteins	Antihuman serum albumin and Anti-IgG columns and anti-amylase column	Ambient to 40°C	Pay attention to columns' loadability!
5. Enzymatic digest	Offline digest overnight	37°C	
6. (Multidimensional) HPLC separation and MS detection of tryptic peptides	Nano HPLC and mass spectrometer	Various combinations	Column capacity, compatibility of selected separation dimensions
7. Bioinformatics-Database search	Various platforms are available	Not relevant	Carefully select parameters, avoid very stringent but also very lax conditions
8. Verification and Validation			

Table 1. General description of sample preparation for proteomics analysis of salivary samples.

3. Sample preparation: depletion of abundant proteins and enzymatic digest

The enzymatic digest of salivary proteins does not differ from digestion of other proteins. Usually, trypsin is being used for proteolytic cleavage of proteins due to its relatively high specificity, availability, and ease of use. Furthermore, tryptic peptides are ideally suited for reversed phase HPLC separation and positive ionization using electrospray MS (ESI). Tryptic peptides are also ideal for using multidimensional separation approaches such as strong cation exchange (SCX), hydrophilic interaction liquid chromatography (HILIC), or electrostatic repulsion interaction chromatography (ERLIC) since they bear positive charges on N-terminus or Lys-residues.

Beside trypsin, other proteases can also be used alone or in combination with trypsin. The aim is either to achieve specific cleavage of proteins for special questions or to achieve smaller peptides and enhance their ionization, detection, and ultimately better sequence coverage for identified proteins. Mostly, proteins such as Lys-C and GluC are applied for pre-digestion before trypsin addition.

Protein digest begins with protein precipitation from saliva. A number of methods have been developed for protein precipitation such as: (1) alcoholic; (2) salting out; (3) applying strong acids (trichloroacetic acid); (4) using acetone; (5) using acetonitrile, etc. Protein precipitation shall help for removing DNA and RNA and their fragments from the sample and for removing lipids.

The Proteomics Core Facility of the Medical University in Vienna applies a modified Wessel-Fluegge [53] method for protein precipitation [54]. **Figure 1** shows the sample collection steps prior to protein reduction, alkylation, and addition of the protease.

In addition to trypsin, the use of proteases, e.g. LysC, will help generating smaller peptides prior to separation and detection [55, 56].

Figure 1. Sample collection steps prior to protein reduction, alkylation, and addition of the protease as performed at the Proteomics Core Facility of the Medical University of Vienna (https://www.sarstedt.com/en/products/diagnostic/salivasputum/product/51.1534.500/).

Unlike serum or plasma, saliva does not contain large range and amounts of high abundant proteins such as serum albumin or hemoglobin that can affect sensitivity and selectivity of detection. However, amylase and serum albumin are still proteins with the highest abundance in saliva and can also affect the detection of other low abundant species and should be removed from the sample [57, 58]. In human saliva, alpha-amylase makes about 60% of the abundance of all proteins present, and its removal will help identifying proteins of lower abundance such as cytokines, which can be used as putative biomarkers for different processes. Deutsch et al. [57] have shown a simple yet very effective method for removing alpha-amylase and gaining a deeper insight into saliva's proteome. The use of a simple potato starch resulted in sixfold reduction of the amount of alpha-amylase in the sample. Albumin removal can be facilitated by using a number of columns developed and based on immunoaffinity reactions [59].

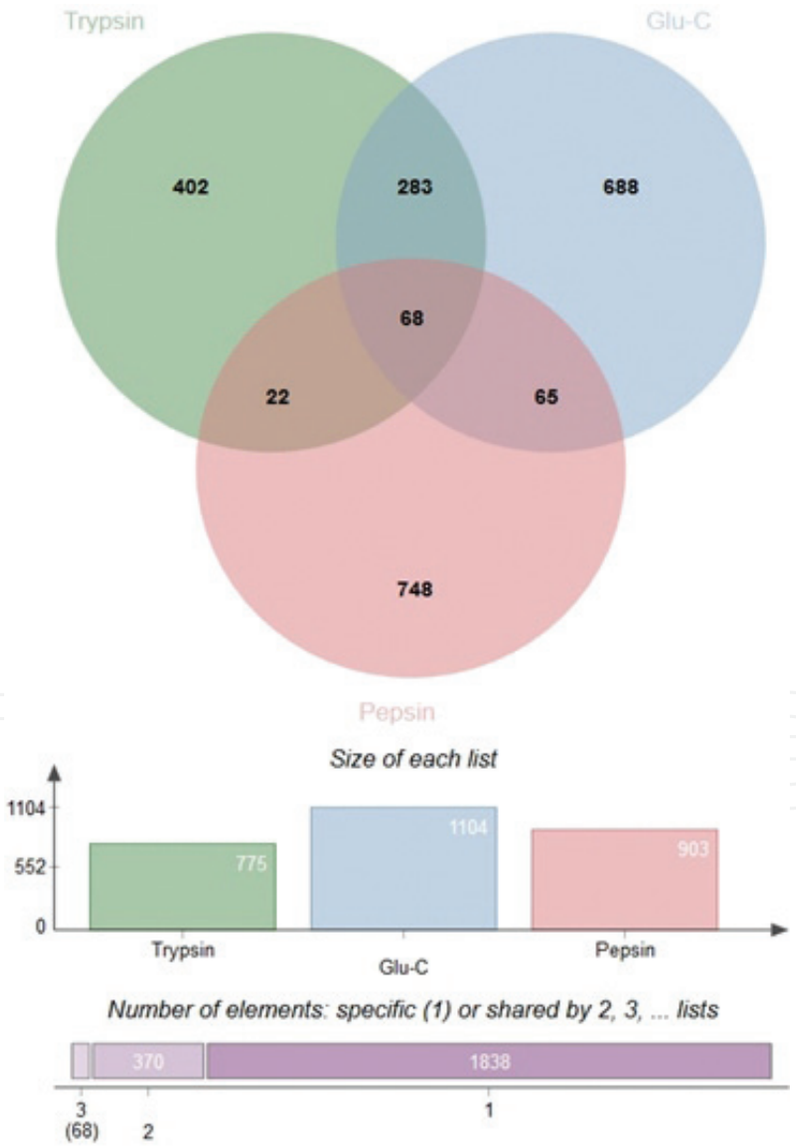


Figure 2. Comparison of the number of identified proteins from human saliva by applying different proteases. Courtesy of Zofia Świątczak (Master Thesis).

Enzymatic digest of salivary proteins does not differ from enzymatic digests used for other biological samples. Based on previous experience and results, trypsin is the most widely used protease for protein digest resulting with peptides suited for subsequent separation using cation exchange columns or anion exchange columns, reversed phase separation, and mass spectrometric detection. A comparison of results obtained using different digestion methods for salivary samples obtained from healthy donors is shown in **Figure 2**.

As shown, the highest number of proteins was identified upon applying a combined digestion approach and using GluC and trypsin. However, the choice of proteases used also depends on analytical problem to be addressed as, e.g. for detection of glycosylated proteins, which might require additional proteases to be applied.

4. Chromatographic separation of digested proteins

Upon tryptic digestion of proteins, resulting peptides are being separated on a chromatographic column prior to mass spectrometric detection and subsequent bioinformatics analysis. Separation of peptides is being performed either using one-dimensional approach or the multidimensional separation by combining two or more separation technologies prior to MS detection and analysis.

For the one-dimensional chromatographic separation approach, peptides are being injected onto the reversed phase nano HPLC column where they are separated according to their hydrophobic interaction with the stationary phase [60]. **Figure 2** shows an exemplary base peak chromatogram (BPC) for one-dimensional analysis separation of salivary peptides. Usually, long separation gradients are selected for one-dimensional separation in order to provide the best possible conditions for peptide separations and large number of identifications.

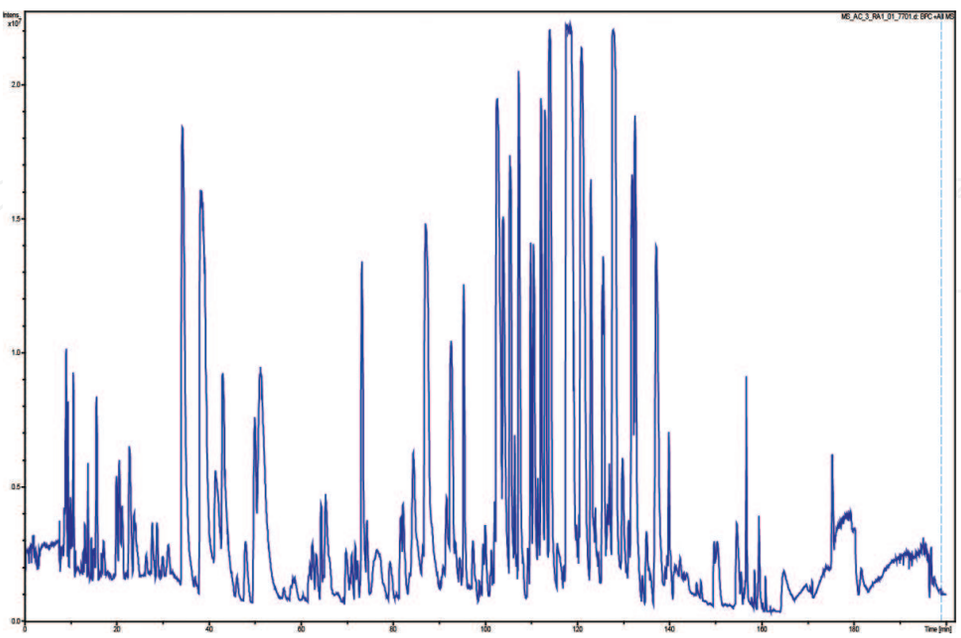


Figure 3. Base Peak Chromatogram (BPC) of GluC-tryptically generated peptides, which were separated on a reversed phase nano HPLC column.

The separation shown in **Figure 3** was performed using a 180-min gradient and a total analysis runtime of 210 min. Thus, the total amount of available time must be considered when performing this kind of analysis.

The use of multidimensional separation methods will increase the number of peptides detected and the number of identified proteins, and, in addition, protein's sequence coverage will be

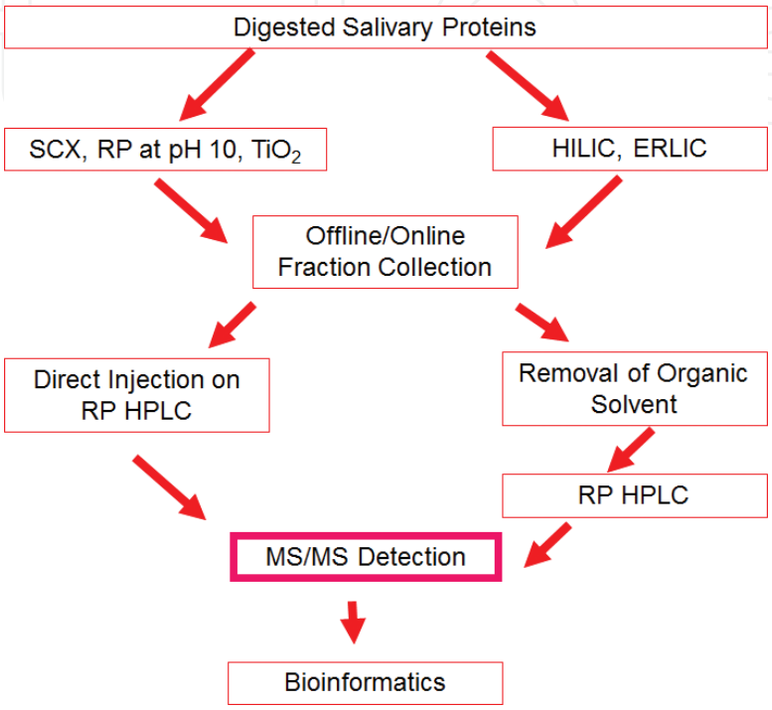


Figure 4. A number of combinations of different techniques can be used for separation of peptides and proteins in a proteomics approach.

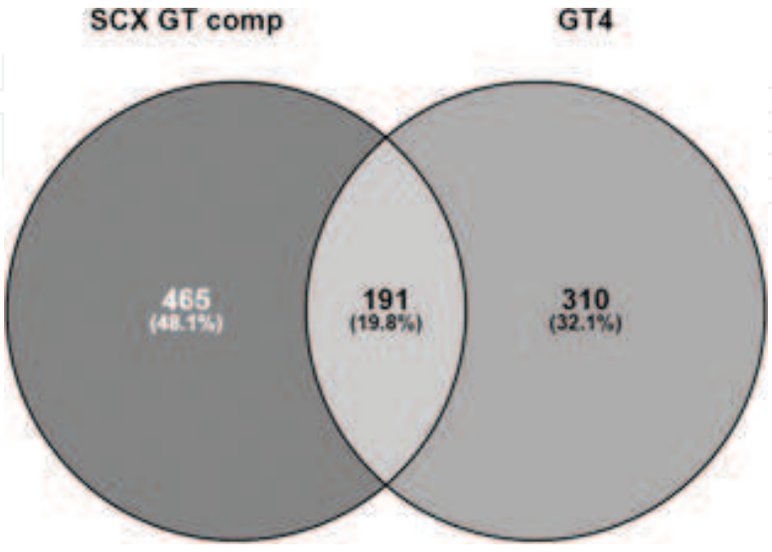


Figure 5. Two-dimensional separation approach for salivary peptides enables identification of higher number of proteins. Data courtesy of Zofia Świątczak (Master Thesis).

improved for identified proteins. Improved sequence coverage for identified proteins is one of the major challenges for proteomics analysis with high importance since it enhances chances for detection of posttranslational modifications (PTM) such as phosphorylation, glycosylation, methylation, etc., which are important as drug targets.

Different types of separation approaches can be used for the two-dimensional approach: strong cation exchange columns, weak anion exchange columns, reversed phase columns at high pH, and other combinations are possible [61–63]. A schematic of possible combinations of chromatographic approaches is shown in **Figure 4**.

Generally, the use of multidimensional separation will result in increased number of identifications, and **Figure 5** shows the comparison of the number of detected proteins upon applying the two-dimensional chromatographic separation with strong cation exchange column used for the first separation dimension.

5. Mass spectrometric detection and bioinformatics analysis

Upon separation, peptides are being detected using mass spectrometry and analyzed by comparing experimental data and databases of in-silico digested proteins. Several MS approaches have been applied for detection of salivary peptides: Electrospray Time-of-Flight (ESI-ToF), MALDI-Time-of-Flight (MALDI-ToF), ESI-Orbitrap analysis, ESI-Quadrupole ToF, etc.

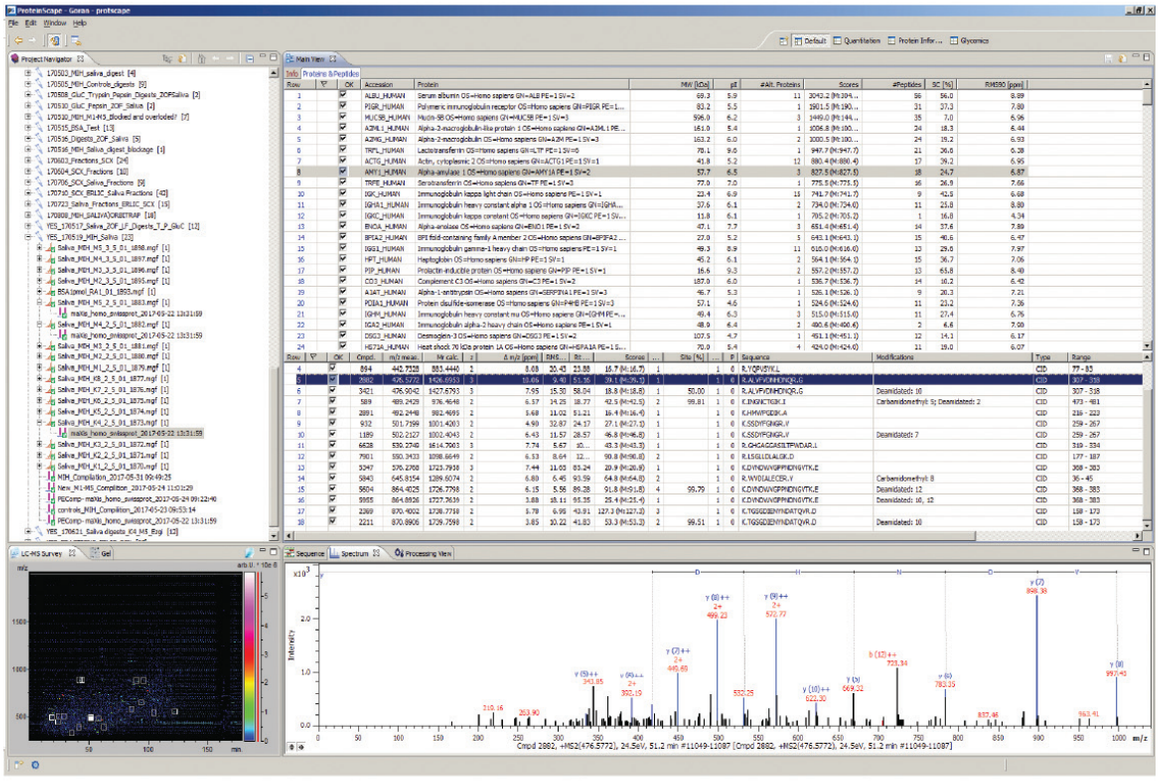
Depending on MS type and selected instrumental method, posttranslational modifications of proteins can also be identified and thoroughly analyzed thus enabled a deeper insight into the proteome. The majority of top-down analysis, i.e. analysis of undigested proteins is performed using MALDI mass spectrometers, and the majority of analysis for digested proteins (peptides) is performed using electrospray (ESI) ionization and ToF and Orbitrap mass analyzer.

The analysis of obtained raw data is performed by searching protein databases such as SwissProt, Uniprot, NR (by NCBI), and user-generated databases. A number of commercially available software packages such as Mascot (Matrix Science, London), ProteinScape (Bruker, Germany), Proteome Discoverer (Thermo Scientific, Bremen, Germany), and of free available software such as The Global Proteome Machine (www.thegpm.org), MaxQuant (http://www.coxdocs.org/doku.php?id=maxquant:start), PeptideShaker (http://www.uib.no/en/rg/probe/65218/peptideshaker), Skyline (https://skyline.ms/project/home/begin.view?), OpenMS (https://www.openms.de/), and other packages. The choice of the software to be used strongly depends not only on personal preferences but also on data to be analyzed and the information needed to be extracted.

Figure 6 shows a screenshot of two software packages preferably used at the Proteomics Core Facility at the Medical University of Vienna, Peptide Shaker and ProteinScape.

In addition to database search and protein identification, the analysis of the pathways where proteins are being over- or underexpressed and the analysis of interactions with other proteins have been performed using free software such as DAVID® (https://david.ncifcrf.gov/), STRING (https://string-db.org/), Reactome (http://reactome.org/) or commercially available MetaCore® (http://lsresearch.thomsonreuters.com/) or similar.

Peptide Shaker



ProteinScape

Figure 6. Screenshots showing analysis of a salivary sample by applying two distinct software packages. Note that identified proteins have been listed based on their scores, which can be calculated using different algorithms.

Term	RT	Genes	Count	%	P-Value	FDR
Salivary secretion	RT		37	1.2	6.3E-9	8.4E-6
Glycolysis / Gluconeogenesis	RT		27	0.9	4.5E-6	6.0E-3
Carbon metabolism	RT		35	1.2	9.6E-5	1.3E-1
ABC transporters	RT		18	0.6	2.2E-4	2.9E-1
Glutamatergic synapse	RT		34	1.1	2.7E-4	3.6E-1
Biosynthesis of antibiotics	RT		54	1.8	3.3E-4	4.3E-1
Amoebiasis	RT		32	1.1	3.4E-4	4.5E-1
Oocyte meiosis	RT		32	1.1	5.8E-4	7.7E-1
Calcium signaling pathway	RT		45	1.5	1.5E-3	1.9E0
Platelet activation	RT		34	1.1	3.2E-3	4.2E0
Pathogenic Escherichia coli infection	RT		17	0.6	4.2E-3	5.4E0
Biosynthesis of amino acids	RT		22	0.7	4.4E-3	5.7E0
Regulation of actin cytoskeleton	RT		49	1.6	4.9E-3	6.4E0
Vascular smooth muscle contraction	RT		31	1.0	5.3E-3	6.9E0
Chemokine signaling pathway	RT		44	1.5	5.6E-3	7.2E0
Circadian entrainment	RT		26	0.9	5.9E-3	7.5E0
Endocytosis	RT		57	1.9	7.2E-3	9.2E0
Renin secretion	RT		19	0.6	8.8E-3	1.1E1
Pancreatic secretion	RT		25	0.8	8.9E-3	1.1E1
Pentose phosphate pathway	RT		11	0.4	1.1E-2	1.3E1
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	RT		20	0.7	1.3E-2	1.6E1
GnRH signaling pathway	RT		24	0.8	1.3E-2	1.6E1
Tight junction	RT		33	1.1	1.3E-2	1.6E1
Oxytocin signaling pathway	RT		37	1.2	1.3E-2	1.6E1
Estrogen signaling pathway	RT		25	0.8	1.9E-2	2.3E1
Adrenergic signaling in cardiomyocytes	RT		34	1.1	1.9E-2	2.3E1
Dilated cardiomyopathy	RT		22	0.7	2.0E-2	2.3E1
Cholinergic synapse	RT		27	0.9	2.3E-2	2.7E1
Aldosterone synthesis and secretion	RT		21	0.7	2.6E-2	2.9E1
ECM-receptor interaction	RT		22	0.7	2.9E-2	3.2E1

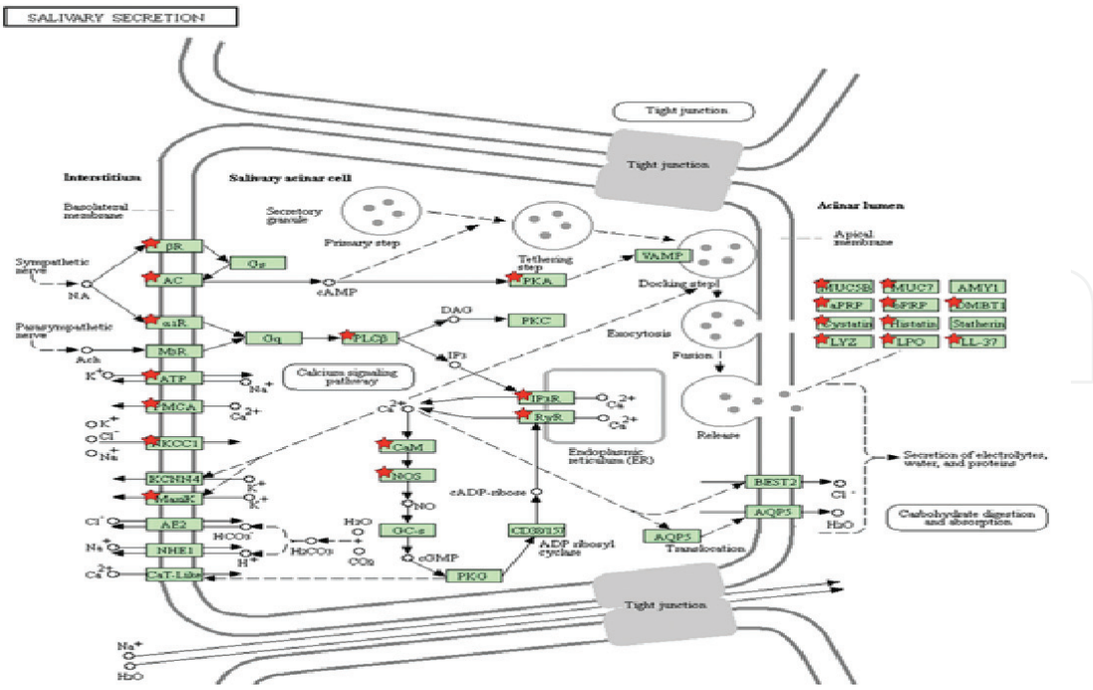


Figure 7. Pathway analysis of salivary proteins using DAVID® resulted in expected output and identification of *salivary secretion* as the pathway with the highest number of expressed proteins. Data courtesy of Zofia Świątczak (Master Thesis).

An exemplary result of the pathway analysis of a salivary sample using DAVID® is shown in **Figure 7**.

Finally, the analysis of all generated data and extracted information shall enable detection of putative biomarkers for diseases and therapy monitoring.

6. Application of salivary proteomics analysis for clinical research

As already mentioned, analysis of the salivary proteome can be applied to study a large area of conditions and diseases. The most intensively studied area of saliva as a diagnostic tool was its appliance for dental [21, 57, 64, 65], oral cancer [31, 66], diabetes [67, 68] or gastric cancer [58]. Furthermore, salivary proteomics was also applied for studying neurological and psychiatric disorders [69].

6.1. Application of salivary proteomics for diagnostics of oral diseases

The use of proteomics for diagnostics and treatment of oral diseases has been described in a number of publications. Jancsik et al. [70] describe the use of salivary proteomics to identify squamous oral cancer in diabetes patients. Authors have performed an additional sample homogenization, which is rarely described in other approaches to analyze saliva. Following analysis was performed by applying 2D gel separation of proteins and MS analysis using MALDI-TOF without the previous chromatographic separation and fractionation of proteins. It is known that inflammatory processes have a well-documented carcinogenetic role. Patients suffering from type-2 diabetes have also a higher risk of inflammatory diseases in the gastrointestinal tract such as ulcerative colitis or Crohn's disease. These patients have also a higher risk of developing gastrointestinal cancer. It was shown that the incidence of developing benign tumors, leukoplakia, and malignancies was significantly increased in the group of patients with diabetes than in the healthy control group. The authors have shown a discovery of several putative biomarkers such as, e.g. Annexin A8-like, Annexin A8-like 1, Tyrosine kinase, AX969656, Protein kinase, Peroxiredoxin-2, and Annexin A2. Annexins are known to be overexpressed in colorectal cancer but also to have altered in tumorigenesis in several types of tumor. Furthermore, loss of Annexin A1 has been found to be an early event in esophageal squamous cell carcinoma. Obviously, these results show that diabetic patients have a higher risk of developing esophageal squamous cell carcinoma than the control healthy group and close monitoring shall be applied for early detection.

Delaleu et al. [71] have performed a particularly interesting and thorough investigation of the salivary proteome from patients suffering from Sjörgen's syndrome. Salivary proteome was analyzed using a 187-plex capture antibody-based assay, and the salivary proteomic biomarker profiles were generated from patients with primary Sjörgen's syndrome, patients with rheumatoid arthritis, and from asymptomatic controls. Authors were able to characterize putative biomarkers by detecting significant changes in 61 and 55 proteins, respectively, in samples of patients compared to that of donors without the diagnosis of Sjörgen's syndrome. Authors were able to detect, based on 4-plex and 6-plex biomarker signatures, markers

including interleukin-4 (IL-4), IL-5, and clusterin. Accurate prediction of an individual's group membership was achieved for at least 94% of cases.

Winck et al. [72] analyzed the salivary proteome in order to decipher the immune response in oral cancer based on the salivary proteome and the extracellular vesicles isolated from saliva. The authors were able to identify significant differences in processes related to inflammatory and humoral immune responses, to peptidase inhibitor activity, iron coordination, and protease binding. Based on identifications achieved, the two classes of individuals (healthy versus patients with Oral Squamous Cell Carcinoma) were distinguished with 90% accuracy based solely on the proteomics data. The authors have used the label-free approach to quantify the identified proteins. Although both groups of peptides share the great majority of identified proteins, some identified proteins were present only in the healthy or only in the group diagnosed with cancer. Authors described that out of many differentially expressed proteins, only the protein peptidyl-prolyl cis-trans isomerase A (also known as cyclophilin-A) was statistically significant in the analysis of the mean survival time of patients, with reduced abundance of PPIA being a factor that may predict poor prognosis of OSCC patients.

6.2. Application of salivary proteomics for diagnostics of diabetes

Sedentary lifestyle paired with unhealthy food, environmental derogation, and stress situations have led to significant increase in diabetes patients worldwide. Early detection of biomarkers would enable more efficient therapy and possible delay of the diseases onset or even a prevention of the outbreak. Caseiro et al. [67] have described the use of proteomics to study Diabetes mellitus (DM) type-1. Authors have performed a quantitative proteomics analysis using the chemical proteomics approach and chemical labeling using iTRAQ. Here, sample from patients diagnosed with diabetes and from healthy subjects was pooled and processed prior to LC-MS/MS measurement and bioinformatics analysis of generated data. In addition to performing iTRAQ labeling and quantitation, authors have chosen for separating peptides by applying two-dimensional separation using high pH reversed phase chromatography. It is remarkable that authors also identified endogenous salivary peptides that are mostly ignored using MALDI-TOF and combined the results to identify more than 400 proteins. Authors used the data obtained to evaluate protein expression for patients with retinopathy, nephropathy, and no complications with the salivary proteome of healthy donors. Identification of the bactericidal/permeability increasing protein-like 1 (BPI) and pancreatic adenocarcinoma upregulated factor (PAUF) in the saliva of all diabetics clearly suggests that the activation of the immune system in type 1 DM is the most prominent process. One of the proteins, BPI, is an essential component of the innate immune system with bacteriostatic and bactericidal effects against gram-negative bacteria through lipopolysaccharides binding. The PAUF is an endogenous ligand of Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4), and it is also involved in the inflammatory response, which seems to be more pronounced and more prominent in patients with retinopathy and nephropathy considering the high salivary levels of alpha-2-macroglobulin, defensin alpha 3 neutrophil-specific, leukocyte elastase inhibitor, matrix metalloproteinase-9, and neutrophil elastase.

As authors also performed analysis of the peptidome, interesting data were obtained that supported the hypothesis that Diabetes Mellitus (DM)-related proteins have higher susceptibility

to proteolysis and evidenced an increased content of some specific protein fragments in saliva, which have been shown to be related with bacterial attachment and the accumulation of phosphopeptides. Especially, the accumulation of phosphopeptides seems to be involved in tooth protection against erosion and the level of their expression and phosphorylation might be a measure for dental damage that can be sustained in diabetes patients. The proteolytic fragments from bPRP1, bPRP2, and aPRP, in particular, might be considered for monitoring the disease pathogenesis with potential use for as early detection markers.

Rao et al. [73] applied multidimensional HPLC-MS/MS proteomics analysis for investigation of salivary proteins in patients with Diabetes Mellitus type-2. A Strong Cation Exchange separation of tryptic peptides from saliva of diabetic, prediabetic, and healthy subjects was performed prior to reversed phase separation and MS/MS detection. More than 400 proteins were identified and characterized and label-free quantitation was applied. As with other analysis, proteins involved with metabolic and inflammatory processes were detected in the saliva of subjects with diabetes. An important finding of the study was achieved in the prediabetic saliva: Salivary biomarkers of established Diabetes Mellitus were identified by proteomic profiling to be also differentially abundant in the saliva of patients with impaired glucose tolerance (IGT) alone and IGT + IFG (impaired fructose tolerance). These results were further confirmed by direct Western immunoblot and ELISA analyses.

The authors showed that the relative increase of some of these putative markers is associated with progression of prediabetes to the diabetic state. Therefore, systematic analysis of these putative biomarkers in prediabetic saliva, as well as their variability in individual samples, by immunoassays is of extreme importance for early acting and treatment of patients, which can prevent cardiovascular complications and mortality in diabetes patients.

An important aspect of patients suffering from DM type-2 is the severe retinopathy that can lead to blindness. Chee et al. [74] performed quantitative proteomics analysis of salivary proteins from patients diagnosed with DM type-2 without retinopathy, which served as controls and patients with DM type-2 and retinopathy. Quantitative proteomics analysis was performed by applying iTRAQ labeling and peptide separation before MS/MS detection was performed on a 50-cm nano column. Authors identified more than 300 proteins but have selected only the fully labeled pairs for quantitative analysis, a total of 119 proteins. Authors identified that increased proteins were predicted to be defense proteins and metabolic proteins suggesting that the expression of salivary defense and metabolic proteins is related to diabetic retinopathy. These results confirmed the report by Fernandez-Real et al. [75] that defense response proteins were elevated in type-2 diabetic patients and this gradually led to surging of metabolic proteins.

6.3. Application of salivary proteomics for psychiatric and neurological diseases

Fields of neurology and psychiatry urgently need new biomarkers for objective and earlier diagnoses of conditions attributed to the central nervous system (CNS). Proteomics and other "omics" technologies are being increasingly applied for these discoveries. Henskens et al. [76] performed analysis of salivary proteins already in 1996 for patients treated with different medications for epilepsy. This work was not performed by using nowadays technology, however, several salivary proteins in saliva from epileptic patients, who were medicated with

different antiepileptic drugs (namely phenytoin, valproate, and carbamazepine), were found to be increased and were compared with protein levels in the saliva of healthy control subjects. It was also found that, for all patient groups, the specific amylase activity was increased up to twofold. On the other side, absolute and relative concentrations of cystatin S were diminished in all samples, but particularly strong in patients using either valproate or phenytoin. These data suggest that use of antiepileptic drugs over long periods may cause a decrease of salivary proteins such as sIgA and cystatins, which are involved in the protection of the oral cavity against microbial infections and, therefore, these patients suffer more complications related to gingiva and oral cavity in general.

Ngounou Wetie et al. [77] have investigated the use of salivary proteins as possible markers for early onset of Autism Spectrum Disorders (ASDs). Authors have identified increased levels of apolipoproteins apoA1 and apoA4 and of serum paraoxonase/arylesterase 1 (PON1) in ASD sera compared to healthy controls in blood serum and have tested the hypothesis that levels of these peptides might also be elevated in saliva. Authors found statistically significant differences in expression of a number of salivary proteins such as elevated prolactin-inducible protein, lactotransferrin, Ig kappa chain C region, Ig gamma-1 chain C region, Ig lambda-2 chain C regions, neutrophil elastase, polymeric immunoglobulin receptor and deleted in malignant brain tumors 1. Identifications made support the hypothesis that immune system disturbances may be present in individuals with ASDs.

Castagnola et al. [78, 79] have applied a proteomics approach for studying the naturally occurring peptidome of human saliva in children diagnosed with ASDs. The study revealed that naturally occurring peptides in the saliva of children with ASD can bear multiple phosphorylations. The phosphorylation level of four specific salivary phosphopeptides, identified in this study, statherin, histatin 1, and acidic proline-rich proteins for both entire and truncated isoforms, was found to be significantly lower in autistic patients, with hypophosphorylation of at least one peptide observed in 18 ASD subjects (66%). Authors suggest that different phosphorylation and hypophosphorylation of salivary peptides suggest potential asynchronies in the phosphorylation of other secretory proteins. These proteins could be relevant in the development of central nervous system during embryonic development or in early infancy. Furthermore, obtained results suggest that naturally occurring salivary phosphopeptides might help to detect and discriminate a subgroup of ASD patients.

6.4. Application of salivary proteomics for dentistry

Saliva has a continuous and intensive contact and interaction with human teeth and plays an important role in cleaning the tooth surface and antimicrobial defense. Salivary proteomics and its role in dentistry have been studied in a number of experiments. Some of these have addressed the role of salivary proteins in edentulous patients diagnosed with DM. Byrd et al. and Border et al. [80, 81] have addressed the problem of edentulous patients with Denture stomatitis (DS) and DM type-2.

Denture stomatitis refers to an inflammatory condition of the mucosal tissue underneath the denture, which could lead to severe health problems. Clinical classification of DS distinguishes

three types: type 1 (DS I), type 2 (DS II), and type 3 (DS III), referring to clinically localized mild, localized moderate, and generalized tissue inflammation [80]. Authors have performed a quantitative proteomics analysis based on label-free quantitation and using two different MS platforms—an Orbitrap instrument and a Time-of-flight instrument. Interestingly, proteins were detected as differentially expressed between the two LC/MS systems. Protein expression was also different depending on the severity of DS. Authors have observed different levels of protein expression between different stages of DS and between the number of identified and quantified proteins for different disease stages and have identified serum proteins in the saliva of patients with DS III, e.g. ceruloplasmin, hemoglobins, serotransferrin, and albumin, which suggest that DS III patients experience higher level of inflammation and protein leakage from blood into saliva.

An interesting approach has been undertaken by Kaczor-Urbanowicz et al. [82] to study tooth absorption during an inflammatory process caused by orthodontic tooth movement. The orthodontically induced inflammatory root resorption (OIIRR) occurs as a consequence (the most prevalent and unavoidable) of orthodontic tooth movement. Authors have applied 2D-gel separation for salivary proteins upon depletion of abundant proteins (amylase, serum albumin, and IgG), and separated the tryptic peptides using HPLC followed by MS/MS detection. Identified proteins were quantified using the label-free approach. Authors were able to identify more than 700 proteins, which were revealed by quantitative MS of which different numbers were identified for different groups of patients. The strength of this study lays with the depth of the analyzed proteome and the significance of the results. Authors performed the bioinformatic analysis for proteins, which were found to be more than threefold increased. Different patient groups revealed different results although they all have been diagnosed with the tooth resorption. The moderate-to-severe root resorption young group revealed 38 functional clusters associated with acute and dynamic processes and in the moderate-to-severe root resorption adult group, other 16 functional clusters were found and those were related to less dynamic and slower processes. For the young group, these processes included the regulation of acute inflammatory response, defense response, response to stress, response to wounding or healing as opposed to apoptosis, glycoproteins expression, cell adhesion, signal peptides, etc. in the adult group with moderate-to-severe processes. Finally, a number of new putative biomarkers were identified, and these might be used to produce a clinical test that would serve along with radiography to perform a fast and more reliable diagnosis.

7. Conclusion

In summary, salivary proteomics is an upcoming approach for both basic and clinical research with a significant potential for use in fast diagnostic approaches. Not only the analysis of salivary proteins but also the analysis of endogenous peptides in saliva and their posttranslational modifications shall be addressed and targeted. Although a number of studies have been published and more are to come, more research is required to validate the discovered putative biomarkers so far.

Current proteomics approach for the analysis of oral fluid is not yet suited for daily routine in clinical diagnostics. However, it can help discovering biomarkers for which immunological tests such as ELISA can be developed.

Furthermore, it is essential to develop and curate a comprehensive database for the salivary proteome and establish standard conditions for sample collection and processing until the MS analysis.

The space was scarce in this chapter to address more of the clinical approach of salivary proteomics, but the researcher is encouraged to stay focused and follow the further development.

Current development shows that, without any doubt, this process will continue and will yield more biomarker candidates in the future.

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