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— Current Topics —

Taste and Health: New Frontiers in Oral Physiology and Rehabilitation

The Current Status and Future Prospects of the Salivary Proteome

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Saliva performs many biological functions that are instrumental in maintaining oral health. Digestive enzymes, such as alpha-amylase and antimicrobial proteins, such as lysozyme and cystatin, are present at high concentrations in human saliva. It is a readily available atraumatic body fluid, useful in the diagnosis of disease, aging and oral health. Large scale analysis of salivary proteins is important for biomarker discovery. However, the abundance of some salivary proteins and contamination with proteins from food and mouth bacteria obscures identification of medium and low copy salivary proteins. In this review, we summarize current knowledge of the salivary proteome using mass spectrometry based technologies.

Key words —— saliva, proteome, mass spectrometry, diagnosis, biomarker

INTRODUCTION

Saliva is produced and secreted from salivary glands in the oral cavity. Humans have three major salivary glands: parotid glands (PG), submandibular glands (SMG) and sublingual glands (SLG), and minor salivary glands. The different salivary glands secrete different compositions of proteins. A major objective of the comprehensive analysis and identification of salivary proteins is not only to determine salivary protein function, but also to determine its potential in diagnostics for human health.[1–3] Whole saliva contains various enzymes derived from exocrine and non-exocrine fluid, including oral bacteria, therefore the whole saliva proteome is affected by post-translational modifications by many kinds of enzymes, not only in the salivary gland but also after glandular secretion. In addition, whole saliva contains many proteins derived from gingival crevicular fluid, desquamated epithelial cells and microbial cells. Therefore, changes to the saliva proteome provides information about the whole body and oral health: whole saliva contains proteins that can be informative for disease detection in oral health,[4–11] in particular for oral cancer,[12] head and neck squamous cell carcinoma[13] and Sjögren's syndrome.[14–16] Saliva, as a diagnostic fluid, has received a lot of attention recently, due to its relatively simple and non-invasive collection,[2] which is an important advantage, especially in sample collection from children and elderly people. In this review, we summarize current knowledge about the salivary proteome using mass spectrometry (MS) based technologies.

FUNCTIONS OF SALIVARY PROTEINS

Whole saliva is a mixture of secretion fluid from major and minor salivary glands and gingival crevice fluid. A large number of proteins are contained in saliva, such as mucins, amylase, peroxidases, lactoferrin and lysozymes, although it is also contaminated with proteins from bacteria and food. Saliva proteins have a number of important functions, such as ion storage, lubrication action, buffer-

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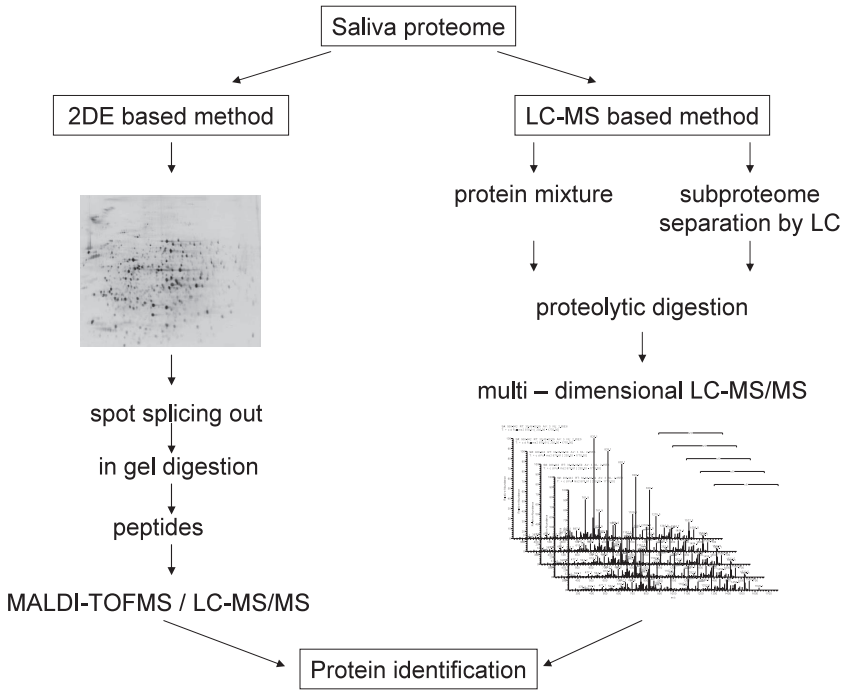

Fig. 1. General Proteomic Strategies Using 2DE Based and LC-MS Based Methods

ing action, clarification, antimicrobial activity, digestive processes, microorganism aggregation and tooth surface protection. Amylase is the most abundant protein in saliva, accounting for approximately 20% of salivary proteins. It is necessary for food digestion that salivary amylase hydrolyzes starch into limit dextrin. Salivary proline–rich proteins (PRPs) and mucins are important for pellicle formation, which protects the tooth surface from erosion and abrasion. The histatin family of proteins has antifungal activity. Many antimicrobial proteins present in saliva are necessary to maintain oral health. The antimicrobial effects are divided into two categories: immunoglobulins and others (non-immune factors). IgA, IgM and IgG, which are secretory immunoglobulins, are all present in salivary fluid for host defense. The non-immune factors include peroxidases, lactoferrin, histatins, cystatins, mucins and lysozyme proteins and growth factors: nerve growth factor,[17] epidermal growth factor,[18,19] and transforming growth factor-β. Large scale identification of the salivary proteome is expected to find unknown salivary proteins.

PROTEOME ANALYSIS OF SALIVA

Proteomic technologies have great tools for disease diagnosis and biomarker discovery.[20,21] The major application of proteomic techniques is the search for biomarkers in specific tissues, cell culture and blood.

Many kinds of technologies have been utilized in candidate biomarker discovery, including two-dimensional gel electrophoresis (2DE), liquid chromatography–tandem mass spectrometry (LC-MS/MS) and protein antibody array (Fig. 1). Using 2DE in combination with MS, whole salivary proteins were separated and 5–9 proteins were identified.[22–28] 2DE technologies have many advantages, they provide information about protein modifications and proteolysis and produce quantitative values. Recently, advances in MS technologies and high resolution nanoflow LC have produced MS-based proteomics known as shotgun proteomics. This strategy involves protein digestion before or after purification by chromatography.[29–31] More recently, MS-based methods combined with multi dimensional chromatography (Fig. 2) have been applied in the study of extremely complex protein/peptide mixtures, including cancer tissues and blood.[32,33] Wilmarth *et al.* reported the successful use of applied two-dimensional liquid chromatography (2DLC)[34] and combined capillary isoelectric focusing/nano-RPLC[35,36] to greatly extend the human saliva proteome. Due to technology improvements, they were able to identify additional 102 and 1382 distinct proteins in a single whole saliva sam-

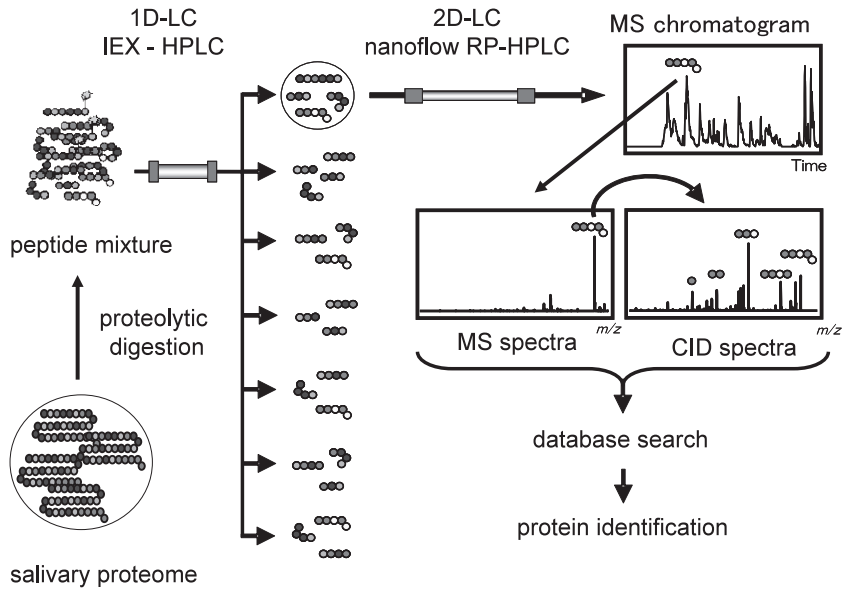

Fig. 2. Scheme of Shotgun Proteomics Using 2DLC-MS/MS Technology

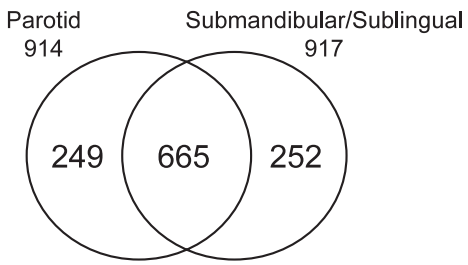

Fig. 3. Reported Number of Identified Human Salivary Proteins Collected from the Parotid and Submandibular/sublingual Ducts in the Salivary Proteome Knowledge Base

This figure is based on data from Denny *et al.* (2008) and the SPKB (http://hspp.dent.ucla.edu/cgi-bin/spkbcgi-bin/main.cgi).

ple, respectively.

More recently, three groups based at the University of California San Francisco, University of California Los Angeles and the Scripps Research Institute/University of Rochester, became involved in a salivary proteome consortium to build a human salivary protein catalogue, funded by the National Institute of Dental and Craniofacial Research (NIDCR). The three groups independently studied the human PG and SMG/SLG proteome using different samples, sample preparation methods and MS-based analysis.[37, 38] The huge amount of data can be accessed from the Salivary Proteome Knowledge Base (PSKB, http://hspp.dent.ucla.edu/cgi-bin/spkbcgi-bin/main.cgi) as a saliva diagnostic atlas. PSKB is database and management system for human salivary proteomics, integrated with other databases, such as transcriptome, metabolome and microRNA in the Sarivanomic Knowledge Base (SKB, http://www.hspp.ucla.edu). The consortium made a healthy human saliva protein catalogue, which identified 914 proteins from the PG and 917 from the SMG/SLG (Fig. 3). All identified proteins can be accessed via the PSKB database from the PSKB homepage.

QUANTITATIVE PROTEOMICS OF THE SALIVA PROTEOME FOR BIOMARKER DISCOVERY

Quantitative protein profiling of relative protein abundance between different samples is an important technology in determining candidate disease biomarkers and to increase our knowledge in systems biology. Generally, three major methods have been used in quantitative proteome profiling (Fig. 4). The first method is 2DE based, in particular, 2D difference gel electrophoresis (2D-DIGE),[39] which is a powerful tool in quantitative proteome profiling. This method involves prelabeling two different protein extracts, using different fluorescent dyes, Cy3 and Cy5. Both samples are then mixed and applied to 2D-EP and separated proteins are visualized and quantified using fluorescence imaging. Fleissing and co-workers reported the salivary protein profile in patients with Sjögren's syndrome and healthy control subjects using this

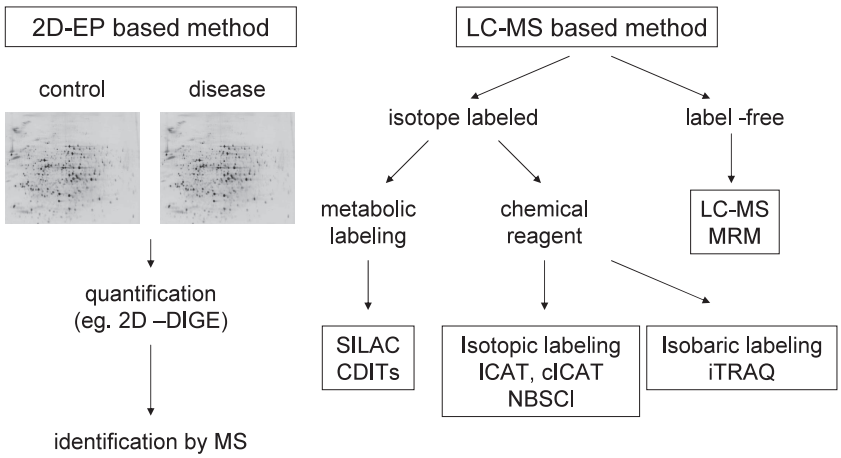

Fig. 4. Schematic Diagram of Steps Involved in Quantitative Proteome Technologies Using 2DE Based and LC-MS Based Methods Combined with Isotope Labeling

method.[14)]

The second method is a label–free direct semi-quantitation method, based on LC-MS chromatogram data. The mass peak intensity difference between two different saliva samples such as oral cancer[40)] is used to quantify the relative protein abundance. Most recently, Rao and coworkers applied this label–free method to determine salivary protein biomarkers in human type 2 diabetes.[41,42)] The 2DLC-MS/MS analyses of whole saliva from control and type 2 diabetes individuals identified 487 proteins; out of the 487, 65 proteins had a > 2.0 fold difference in relative abundance between control and type 2 diabetes. The amount of $\alpha 1$ antitrypsin, cystatin C, $\alpha 2$ macroglobulin, and transthyretin, which were increased in type 2 diabetes, were analyzed further using ELISA and western blotting to confirm the quantitative data from 2DLC-MS/MS analysis. The LC-MS based label–free semi-quantitation method was incorporated into the multiple reaction monitoring (MRM) strategy.[43)] In the first step of the MRM strategy, biological samples were analyzed to comprehensively identify proteins using LC-MS/MS technologies. The results of the first step experiments produced a lot of information, including chromatographic elution time, protein ID, peptide sequence, observed m/z and observed fragment ions of peptides. The information was used in MRM experiments, using a triple quadrupole mass spectrometer. Targeted peptides, selected from the first step, were quantified using MRM technologies. Advantages of the MRM method include sensitivity, selectivity, quantitative accuracy, and dynamic range. To perform absolute quantification of peptide/protein concentrations, isotopic labeled synthetic peptides were spiked into the sample as internal standards.

The third method is an isotope labeling strategy, based on stable isotope labeling by metabolic labeling, specific chemical probes and enzymatic transfer of the ^{18}O atom from heavy water to the C-terminus of peptides. The differential stable isotope labeling strategy quantified relative amounts of each peptide between different biological samples using the intensity values within a single LC-MS/MS analysis. Stable isotope labeling by amino acids in cell culture (SILAC) and culture derived isotope tags (CDITs)[44,45)] are widely used methods in cellular quantitative proteome analysis, using a stable isotope labeled amino acid in a culture medium. Unfortunately, the SILAC method is not applicable for use in biological fluids, including blood, plasma and saliva. In contrast, the specific chemical probes are applicable to cell culture and biological fluid and tissues. The chemical probes have either isobaric or isotopic labels. Isotopic labeling of chemical probes is performed using the Isotope–Coded Affinity Tag (ICAT) reagent, cleavable ICAT reagent and 2-nitrobenzensulfenyl Chloride (NBSCl). These reagents react with specific amino acid residues, such as cysteine and tryptophan. Light or heavy isotopic probes react with tryptic peptides from proteins of different samples. Labeled peptides can be identified and quantified by LC-MS/MS analysis, and the relative ratios of identified peptides can be calculated using mass shift analysis of heavy and light peptides. In contrast, isobaric labeling methods, such as the isobaric pep-

tide Tags for Relative and Absolute Quantification (iTRAQ) reagent,[46] are based on differential labeling of N-termini and lysine residues from digested peptides. iTRAQ labeled peptide produces specific fragment ions (reporter ions) , which have a different mass; using MS/MS, relative peptide abundance can be compared via the intensity of reporter ions. Large scale analysis of the abundance of low molecular weight peptides (<10 kDa) secreted from human parotid saliva during the day were analyzed using iTRAQ technology.[47] The peptides were categorized into up–or down–regulated groups as the concentrations oscillated throughout the day. The study also identified and measured the relative abundance of a small peptide derived from histatin. Recently, breast cancer related proteins were discovered from differential whole saliva proteome analysis between healthy women and those diagnosed with a benign breast tumor, using the iTRAQ method.[48] Forty-nine proteins were differentially expressed between healthy and cancer individuals, which could be potential candidates for breast cancer markers.

When studying human saliva samples, as mentioned previously, identification of medium and low copy salivary proteins is obscured by abundant salivary proteins and contaminant proteins from food and mouth bacteria. In order to resolve the contamination problems, an animal study can be performed where samples can be taken directly from the salivary ducts of a mouse or rat. Additionally, using this technology, it is possible to study human disease using an animal model of the disease whereby only saliva is needed. However, it is necessary to improve the highly sensitive nanoflow 2D-LC/MS based proteome technology to allow for small amounts of saliva collected from the animal models.

We aim to analyze saliva collected from rat PG, SMG and SLG ducts using nanoflow 2DLC-MS/MS combined with the iTRAQ method. Analysis of rat saliva requires only 15 microliters of PG and 150 microliters of SMG/SLG saliva. This technology, studying several diseased animal models, is expected to discover candidates for salivary markers used to identify disease in humans.

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