

Biomarker of health and disease in Dentistry: Proteomics

Vamsee Krishna N^{1*}, Madhuri Nadhamuni²

¹Associate Professor, ²Clinical Practitioner, ¹Dept. of Conservative Dentistry and Endodontics, CKS Teja Institute of Dental Sciences and Research, Tirupati, Andhra Pradesh, ²Tirupati, Andhra Pradesh, India

Abstract

Every living organism contains ‘Proteins’ which are building blocks to perform various functions. Proteomics is the analysis of the whole protein in a genome which tells about the molecular mechanisms of homeostatic cell and regarding external disturbances, such as pathologic or pharmacologic treatment. Proteomics improved various biomedical fields such as medicine, dentistry, molecular biology, and genetics. This article reviews some of the currently available literature on conducting Proteomic analysis of dental tissues. A literature review is done using electronic databases, such as “PubMed,” “Google Scholar,” and “Scopus,” using keywords “Proteomics,” and “Biomarkers.” Furthermore, an advanced or refined search was carried out using the keywords “Mass Spectrometry,” “Proteins,” “Enamel,” “Dentin,” “Cementum,” “Periodontal ligament” and “pulp.” Proteomics in dentistry helps in the early diagnosis of health-disease molecular signatures with the discovery and recognition of numerous biomarkers in biological, pathological or pharmacological processes that are present in the fluids of the oral cavity. This paper explains the role of proteomics in the analysis of oral tissues.

Keywords: Proteomics, Mass Spectrometry, Biomarkers.

Key Message: Proteomics is an emerging field of analytical research that helps in the understanding of diagnosis, and pathogenesis of various diseases which helps in the prognosis and treatment plan.

Introduction

The oral cavity harbors a wide variety of 65% bacteria, 30% fungi and 5% of other organisms.¹ The human oral cavity contains hard tissues, i.e. Enamel, Dentin & Alveolar bone, soft tissues, i.e. Pulp, Periodontal ligament & Gingiva, and various biofluids like saliva and crevicular fluid. With the identification of microorganisms, disease initiation, progression, resistance, treatment plan & failures can be assessed. The culture technique has been the standard and oldest method of detecting the microorganisms involved in diseases. The disadvantages of phenotypic identification are it is impossible to culture a large number of microorganisms, time-consuming, need for transporting media and immediate processing, low specificity & sensitivity, and operator expertise. All species can't be cultured due to their specific growth factors, nutritional requirements, and also the harmful substances released by other organisms can hamper the culture technique. Hence the culture technique, which is considered as a gold standard, is not entirely justified. This has paved the way for culture-independent methods which are rapid and can directly analyze the sample with high sensitivity and specificity. A literature review is conducted using electronic databases, such as “PubMed,” “Google Scholar,” and “Scopus,” using keywords “Proteomics,” and “Biomarkers.” Furthermore, an advanced or refined search was carried out using the keywords “Mass Spectrometry,” “Proteins,” “Enamel,” “Dentin,” “Cementum,” “Periodontal ligament”

and “pulp.” This article reviews some of the currently available literature on conducting Proteomic analysis of dental tissues.

The human body contains different types of proteins which regulate the structural, catalytic, storage, transport, and transducer mechanisms of cells. Hence proteins are called “working horses” of a cell. Proteins are made up of 20 different types of amino acids that are linked with each other by peptide bonds.² Mark Wilkins first introduced the term “Proteome” in 1961.³ The complete set of proteins expressed in a given cell or biological sample is termed as proteome, and its study is called “proteomics.”⁴ Proteomics research includes the sample preparation and protein/peptide separation followed by identification, quantitative, qualitative, and functional characterization of the whole protein profile of a given cell, tissue, and/or organism by using proteomics tools such as polyacrylamide gel electrophoresis (2D-Gel), mass spectrometry (MS), real time-PCR, microarrays and chromatographical tools.⁵⁻⁷

Proteomics helps in understanding the mechanisms of bacterial resistance, virulence, and how bacteria interact with human cells and thus, of the pathogenesis of infectious diseases. Proteomic tools can analyze different hard & soft tissues samples and fluid samples of the oral cavity in both physiological and pathological conditions.⁸⁻¹⁰ Commonly used methods for separating proteins are 2D gel electrophoresis and liquid chromatography. 2D gel electrophoresis is commonly used to separate a mixture of

*Corresponding Author: Vamsee Krishna N, Dept. of Conservative Dentistry and Endodontics, CKS Teja Institute of Dental Sciences and Research, Tirupati, Andhra Pradesh, India
Email: dr.vamseekrishna@gmail.com
<http://doi.org/10.18231/j.ijce.2019.016>

proteins into single detectable protein spots. The 2D separation of proteins on the gel is usually achieved according to their isoelectric point and molecular weight. The advantages of the 2D gel electrophoresis are its consistency and high resolution. The insolubility of membrane proteins is still a main obstacle for 2D-electrophoresis. The ionic detergents used for solubilization of membrane proteins can interfere with the focusing process. Additionally, the mass range and the detection limits also represent technical limitations of 2D-electrophoresis method.

The proteins that are separated on 2D gels traditionally are stained by silver staining and Coomassie blue. However, these detection methods remain problematic due to low sensitivity (for Coomassie) or poor reproducibility and dynamic range (for silver). The recent development of fluorescent dyes, namely SYPRO™ Ruby, overcame these problems with its sensitive (1–2 ng) detection limits and linear dynamic range over three orders of magnitude. The resulting 2D map is then analyzed by software designed for image analysis, which allows gel-to-gel comparison. Following 2D image analysis, the protein spot of interest is excised and treated with ammonium bicarbonate and acetonitrile to remove detergents. The excised spots are then in-gel-digested with a protease (trypsin is commonly used) in an optimal buffer for its activity. The digested peptides are then easily eluted from the gel to undergo mass spectrometry analysis.

Mass spectrometry (MS) is a technique where the mass of an ion is measured for the characterization of the molecule of interest. Mass spectrometry is composed of a sample inlet, an ionization source, a mass analyzer, and a detector. Mass spectrometry helps in identification and characterization of thousands of proteins rapidly in a sample. The traditional ionization fragmentation of peptides does not provide accurate peptide mass. However, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) enabled the ionization of large biological macromolecules without fragmentation for the identification of proteins. Databases are available for theoretical digests of all known proteins. Matching the peptide-mass data obtained from a sample of interest to theoretically digested protein database can be used to identify unknown proteins successfully.¹¹ The proteomics of body fluids is conducted by two methods using MS. In top-down proteomics, intact proteins are analyzed using MALDI or ESI, and by gas-phase fragmentation method, the peptides are generated.¹² The bottom-up analysis also called “shotgun proteomics” is the more conventional approach & is used for analyzing peptides produced through chemical or enzymatic cleavage of proteins with post-translational modification as well as through liquid chromatography (LC) in conjunction with MS.¹³

Mrozik *et al.*, compared proteomes between bone marrow, periodontal ligaments (PDL), and dental pulp using 2-DE, MS/MS.¹⁴ Salmon *et al.*, identified a novel biomarker, ‘superoxide dismutase-3’ associated with

cementum cells through his proteomics study of dental cementum using LC-MS/MS and also detected 231 proteins related to cementum.¹⁵ Dame *et al.*, used a variety of proteomics analytical tools including MS, direct flow injection/liquid chromatography, inductively coupled plasma mass spectrometry, gas chromatography, nuclear magnetic resonance spectroscopy, and high-performance liquid chromatography, in his study to calculate 308 salivary metabolites.¹⁶

With the help of different proteomic tools and numerous biomarkers present in the oral fluids, a drastic change occurred in dentistry in early diagnosis, treatment with minimal interventions, and prevention of dental diseases. Dental samples for proteomics analysis are collected from dental hard tissues (enamel, dentin, cementum), soft tissues (pulp, gingiva, periodontal ligament & oral mucosa) and oral fluids such as saliva and gingival crevicular fluids (GCF). The tooth is the strongest calcified part in the human body, which is made up of three distinct highly mineralized hard tissues enamel, dentine, cementum, and soft tissue is the pulp. The Periodontal ligament is the connective tissue that surrounds, attaches, and suspends the tooth inside the alveolar socket.

Proteomic analysis of dental hard tissues must overcome many methodological hurdles. One is the need to remove calcium and other minerals from the calcified tissues. The other problem is poor solubility and low abundance of matrix proteins resulting in a very less yield of proteins during processing. Another difficulty is the substantial protein heterogeneity associated with alternative splicing and a variety of post-translational modifications.¹⁷ Conrads *et al.* were the first to make molecular biological approach in Endodontics. Kary Mullis developed Polymerase chain reaction (amplification) which helped in isolation of previously unknown species from endodontic infection- *B. forsythus* and *T. denticola*, *Dialister pneumosintes* and *Filifactor alocis*, *T. parvum* and *T. putidum*.¹⁸⁻²⁰

Proteomics of dental hard tissues

The only dental hard tissue of ectodermal origin is Enamel. It is formed by ameloblasts before the eruption of teeth with an acellular structure, exposed to calcification in the absence of blood vessels and nerves. It is composed of 96% inorganic, 1% organic, and 3% water by weight, in which the organic substance consists of proteins, carbohydrates, and lipids. The protein content in the enamel is only a small fraction of the entire mass ranging from 0.5% and 3%–4%. The enamel proteins are 0.17% acid-soluble, 0.18% acid-insoluble peptides, and 0.15% free amino acids, 0.6% lipids, and 0.1% citrates.²¹ In developing teeth, the secretory-stage enamel contains >30 % protein. By evaluating the changes in protein content of enamel through proteomics, physiological & pathological changes in the enamel can be studied and predicted in an early stage.⁸

Amelogenin, ameloblastin, enamelin, and tuftelin are the major enamel proteins.²² Amelogenin is the main

structural protein for enamel formation, and it regulates the initiation and growth of hydroxyapatite crystals during the mineralization of enamel. Ameloblastin is an enamel-specific extracellular matrix glycoprotein secreted by ameloblasts. It is believed to be controlling the elongation of enamel crystals and helps in enamel mineralization process during tooth development thereby maintaining the rod integrity.²³⁻²⁴ Vymetal *et al.*, observed the expression of ameloblastin in developing mesenchymal dental hard tissues, and in trauma-induced reparative dentin during early craniofacial bone formation²⁵ Enamelin is first formed as a soft, protein-rich extracellular matrix, which is essential for enamel formation. The studies conducted by Brookes, Hu, and Yamakoshi proved that enamel is critical for proper enamel formation as mutations in the enamel gene cause amelogenesis imperfecta.²⁶⁻²⁸ Tuftelin stimulates the initiation of the mineralization during tooth development by acting as a nucleator for hydroxyapatite crystal formation. In enamel hypomineralization, the enamel defects appear as opaque, yellow or brown discoloration and these changes are accompanied by an increased protein content when compared to sound enamel. The elevated levels of serum albumin and presence of antithrombin & antitrypsin were observed that inhibit apatite crystal growth.²⁹

Dentin forms the main bulk of the tooth. It protects the pulp as well as brace the overlying enamel and cementum. It is composed of 70% minerals, 20% organic, and 10% Water by weight.² Many studies were done using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by tandem mass spectrometry for identification of the human dentin proteins.³⁰⁻³² Odontoblasts secrete the proteins which play a key role in dentin extracellular matrix organization and the regulation of mineralization during dentinogenesis.³³⁻³⁴ The proteins by producing enzymes modify the functions and act as nucleators by regulating ion transport in the extracellular matrix formation or prevent the mineral formation in unwanted sites.³⁵ Proteomics helps in identification of collagenous and non-collagenous proteins in dentine. The principal collagenous proteins identified in the dentin are matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), and matrix metalloproteinase 20 (MMP-20).³⁶⁻³⁸ The dentin sialophosphoprotein (DSPP) is the major noncollagenous protein identified in human dentin. Lee *et al.* stated that dentin dysplasia type II, and dentinogenesis imperfecta types II and III are caused due to genetic defects in dentin sialophosphoprotein.^{39,40} Sialic acid-rich proteins are another group of non-collagenous protein which includes osteonectin, osteocalcin, osteopontin, bone sialoprotein, bone acidic glycoprotein, dentin matrix protein I, and integrin-binding sialoprotein.⁴¹ These proteins also play a role in the process of dentin mineralization. Proteoglycans and hydroxyapatite crystals have been identified in pre-dentin and dentin.⁴²⁻⁴⁴

Dental cementum is a bone-like avascular and non-innervated mineralized tissue that covers the root and anchors the periodontal ligament which binds the tooth to

the alveolar bone. It is produced by specialized cells called cementoblasts and is slightly softer than dentin and consists of about 45–50% inorganic (apatite crystals), 50–55% organic (collagen and glycoproteins) and water by weight.^{45,46} The predominant extracellular matrix component of cementum is type I collagen. The noncollagenous proteins like glycoproteins (e.g., osteonectin and arginine-glycine-aspartic acid integrin-binding proteins), proteoglycans (e.g., versican, decorin, and biglycan), and other proteins (e.g., osteocalcin, matrix Gla protein, and protein S) constitute the remaining organic matrix of dental cementum. All these proteins participate in the matrix deposition and mineralization, regulation of cell metabolism, and thus contributes to determining the structure and biomechanical properties of the tissue.^{8,15}

Proteomics of dental soft tissue

The dental pulp is the uncalcified soft connective tissue located within the pulp cavity, surrounded by hard dental tissues. It forms the dentin, aids in sensory function and provides tooth nourishment and immune defense reactions, and demonstrates strong repair & regenerative potential. It is composed of cells (odontoblasts, fibroblasts, mesenchymal nerve fibers, blood vessels, and lymphatics).^{47,48} Paakkonen *et al.* reported the first proteomic study of dental pulp using Two-dimensional gel electrophoresis followed by Tandem mass spectrometry.⁴⁹ In an *in vitro* proteomic study of the dental pulp, 23 proteins related to early odontogenetic differentiation were identified by difference gel electrophoresis followed by MS.⁵⁰ The dental pulp contains unique tissue-specific proteins and small leucine-rich proteoglycans like biglycan, lumican, and mimecan.⁵¹ Paakkonen *et al.*, analyzed for the first time the gene and protein expression in healthy and carious dental pulp organs using cDNA microarray and 2D-gel electrophoresis and identified 96 proteins.⁴⁹ McLachlan *et al.* studied dental pulp tissues for detailed characterization, and molecular changes in dental caries and a total of 445 genes were identified out of which 85 genes were reported abundant in health and 360 abundant in disease.⁵²

Periodontal ligament (PDL) is the connective tissue which helps in maintaining PDL space, anchorage and provides regeneration or repair of the periodontium in response to disease and mechanical trauma. Reichenberg *et al.* reported a first study on periodontal ligament (PDL) fibroblast proteome for understanding its physiology and identifying disease-related protein markers.⁵³ On exploring the early osteogenic differential protein-profile in human PDL cells, 29 proteins were identified which have been primarily linked to the cell membrane-binding, cytoskeleton, nuclear regulations, matrix synthesis signal conduction, and metabolic enzymes that participate in the intra- and inter-cellular processes.⁵⁴⁻⁵⁶

Oral fluid proteomics

Collection of Oral Fluids (saliva and GCF) for proteomic analysis is a noninvasive technique and causes minimal

patient discomfort and anxiety.⁵⁷ Saliva which is produced by the major and minor salivary glands is composed of water, proteins, peptides, hormones, lipids, sugars, and ions.⁵⁸ The proteins in saliva are differentially expressed in secretions, depending on the salivary gland that secretes them. The predominant proteins and peptides in the whole saliva include amylase, carbonic anhydrase, mucins, cystatins, histatins, statherin, and proline-rich proteins.^{59,60} Cystatins and mucins are the salivary defense proteins.⁶¹

The acquired enamel pellicle is a thin layer (0.5-1 µm) of mucins, glycoproteins, and proteins formed on the enamel surface by selective adsorption of salivary compounds.⁶² It plays an important role in the crystal growth homeostasis and the physicochemical defense of tooth surfaces.⁶³ Siqueira et al. by analyzing a global proteome of the human pellicle identified 130 proteins and grouped into three types. The first group proteins bind to calcium ions (18%), the second group (15%) bind to phosphate ions, and the third group (28%) interacts with the other proteins.⁶⁴ Disease-related salivary biomarkers were summarized (i.e., for hereditary and autoimmune diseases, malignancies, dental caries, periodontal diseases) and identified by various studies of salivary proteomics.^{65,66}

The protein composition and content of human gingival crevicular fluid (GCF) varies based on periodontal health and disease. GCF contains serum transudate in the gingival sulcus, subgingival microbial plaque, extracellular proteins, host inflammatory mediators, and cells.⁶⁷ Many noninvasive methods are available for the collection of GCF such as gingival wash, capillary tubes, paper strips, and cones. The paper strip is mostly used for the collection of GCF due to easy insertion into the gingival crevice up to 1 mm of sulcular depth without bleeding from periodontal pockets.⁶⁸ GCF volume for proteomic analysis is limited due to the severity of tissue inflammation.⁶⁹

Conclusion

The role of microorganisms in causing the dental infections is well established, and identification of microbial taxonomy serves as a rationale for setting the clinical protocols. Culture technique has been the common diagnostics method for infectious diseases for many years. Due to factors like time consumption, low sensitivity and specificity of culture techniques led to the era of molecular analysis of sample. The proteomic research of human teeth has a high impact on the understanding of various diseases, not only on dental caries. The overall analysis on proteomics in dentistry shows that more studies are directed toward the structural formation, diagnosis, and pathogenesis and limited studies have been done on evaluation of treatment, the prognosis of interventions, and prevention of diseases.

Source of Funding

None.

Conflict of Interest

None.

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How to cite this article: Vamsee KN, Madhuri N. Biomarker of health and disease in Dentistry: Proteomics. *Indian J Conserv Endod* 2019;4(3):69-74.