

Analysis of Salivary Proteome in Patients with Dental Caries employing Electrophoresis and Mass Spectrometry

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ABSTRACT

Introduction: Dental caries is a multifactorial disease; although many studies have attempted to identify salivary proteins that are associated with the disease, this is the first study to use a proteomic approach to analyze and compare the proteomic profile of whole saliva from dental caries patients and healthy controls.

Materials and methods: To analyze the saliva proteome, one-dimensional gel electrophoresis and mass spectrometry (MS) were employed.

Results: The analysis delineated the salivary protein profile of patients with dental decay. Mass spectrometry analysis revealed the presence of proteins like cytochrome c oxidase assembly protein, immunoglobulin (Ig) alpha-2 chain C region, alpha-amylase and its isomerases, serum albumin, Zymogen granule (ZG) protein, and hemoglobin subunit beta.

Conclusion: New analytical state-of-the-art technologies, such as MS have revolutionized the field of oral biology. These novel technologies open avenues for a comprehensive characterization of the salivary proteins followed by the evaluation of the physiological functions, which could make possible in a near future the development of a new series of synthetic protein for therapeutic propose able to prevent global oral diseases, such as dental caries, one of the most prevalent oral diseases in the world. This approach provides novel insight into profiles of the salivary proteome due to dental decay, which may contribute to improvements in understanding its complex etiology and prevention.

Keywords: Dental caries, Mass spectrometry, Saliva, Salivary Proteome, Zymogen granule protein.

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INTRODUCTION

One of the least understood yet important protective body fluids is saliva.¹ A drop of saliva harbors a world of diagnostic information, proteomically and genomically. Also known as the “mirror of the body” or “window on health status,” it is the ideal medium to monitor health and disease.² The general term “saliva” refers to the fluid that surrounds all hard and soft oral tissues. This oral fluid represents a mixture of individual fluids and components derived from several sources. Major and minor salivary glands make the bulk contribution to whole saliva, with minor contributions from nonglandular sources, such as crevicular fluid, oral microorganisms, host-derived cells, and cellular constituents, as well as diet-related components.³ Molecules present in saliva include a characteristic multigenic group of proteins and polypeptides grouped into single or polymorphic families that have an important biological function in the maintenance of oral health.⁴

Approximately 27% of human salivary proteins are also found in the plasma fluids. Though the degree of overlap is low, strong similarities are seen in the distribution across gene ontological categories, such as molecular function, biological processes, and cellular components. The reasons for the overlap are as follows: (1) Plasma leaks into saliva through intracellular and/or extracellular routes including outflow of gingival crevicular fluid; (2) plasma and saliva may share essential proteins needed to maintain their physiological function as body fluids, and (3) proteins derived from cell debris may be in close contact with either fluid.⁵ Traditional proteomic methods were applied to the analysis of whole-saliva peptides/protein species using two-dimensional (2D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by MS. With this approach, the most abundant salivary proteins were identified, such as several proline-rich proteins (PRPs), amylase, lipocalin-1 or calgranulin B, assigning more than 300 proteins. The presence of high amounts of mucins and other glycoproteins interferes with 2D protein separation by blocking the passage of current during the focusing step, and by forming heterotypic complexes with other salivary proteins. The use of multidimensional liquid chromatographic systems coupled to MS allowed a dramatic increase to more than 1,000 salivary proteins being identified.⁶

Dental caries is a multifactorial disease primarily dependent on biofilm development. Human saliva plays a significant role in controlling microbial adhesion since its protein aqueous components, after adsorbed to the oral surface, result in the formation of salivary protein pellicles. The acquired pellicle is a protein integument formed on the oral surface immediately after exposure of saliva to the oral environment. This protein film formed on the dental enamel is a result of specific physical bonds (i.e., hydrophobic, hydrogen bonding, ionic, and van der Waals bonds) between the substrata surface and the salivary molecules (i.e., salivary proteins, peptides, carbohydrates, and lipids).⁷ Salivary proteins act directly and indirectly through various methods on plaque and bacteria, modulating susceptibility of the tooth to dental caries.⁸ Secretions from the sublingual and submandibular glands contain a high-molecular-weight, highly glycosylated mucin (MG1) and a low-molecular-weight, single-glycosylated peptide chain mucin (MG2). The MG1 adsorbs strongly to the enamel pellicle on the tooth surface and protects from acid challenges and forms heterotypic complexes with amylase, PRPs, statherin, and histatins, contributing to bacterial attachment and provides transient bacterial nutrition. The MG2 also binds to enamel but is easily displaced and promotes the aggregation and clearance of oral bacteria inclusive of streptococci mutans. Hence, MG1 levels are higher in caries-susceptible patients and MG2 are increased in caries-resistant patients. Nonimmunologic antibacterial salivary contents, such as proteins, mucins, peptides, and enzymes (lactoferrin, lysozyme, and peroxidase), all products of acinar gland cells, help protect teeth against physical, chemical, and microbial insults. The MG2 and IgA complex bind to mucosal pathogens with higher affinity than either MG2 or IgA alone. Lactoferrin is secreted by intercalated ductal cells and binds ferric iron in saliva, which makes it unavailable as a food source for microbes, such as cariogenic streptococci, which need iron to remain viable. This process of starving bacteria of vital nutrients is called nutritional immunity. Lysozymes also promote bacterial clearance through aggregation. Peroxidase, also known as sialoperoxidase or lactoperoxidase, catalyzes bacterial metabolic by-products with thiocyanate, which is highly toxic to bacterial systems.² Acidic PRPs bind to free calcium and adsorb to hydroxyapatite surfaces to regulate hydroxyapatite crystal structures, thus inhibiting enamel crystal growth. Some PRPs like statherins also promote the attachment of bacteria to apatitic surfaces.⁹

Salivary proteins not only play a role in maintaining oral and general health but may also serve as biological markers to survey normal health and disease status. As a consequence, analysis and cataloging of the human salivary proteome will be of great interest to researchers

within the fields of saliva-based diagnostics and oral biology.⁵ Banderas-Tarabay et al⁴ studied the electrophoretic pattern of human whole saliva (HWS) in patients with dental caries and reported significant changes in MG1, MG2, and PRPs. Bhalla et al¹ also studied the electrophoretic patterns of HWS in children with early childhood caries. Hence, the present work was conducted to enhance the understanding of protein profile, and analyze electrophoretic pattern and protein composition of resting HWS in relation to dental caries, employing a confirmatory test for protein identification postelectrophoresis. Also with the current interest in the salivary proteome, it helps to identify the proteins in this complex agglomerate of oral secretions.

MATERIALS AND METHODS

One-hundred and fifty patients were randomly selected, clinically examined, written consent with a detailed case history was obtained and Decayed, Missing, Filled-teeth (DMFT) index calibrated according to the World Health Organization diagnostic criteria noted by the same examiner. Only patients free from all systemic diseases and oral diseases except dental caries, if present, were chosen. The patients on medication or indulging in any adverse habits like tobacco chewing, smoking, etc., were excluded. Those suffering from gingivitis, periodontitis, and space infection due to caries were also excluded. The patients were instructed not to eat or drink at least 2 hours prior to the collection.

The saliva collection method was adapted from Vissink et al¹⁰ and the procedure was carried out only between 9:00 and 11:00 AM. The patients were requested to rinse their mouth with water and avoid all major body movements, oral movements especially talking, and asked to keep their head relatively straight. They were asked to allow saliva pooling in the mouth for 10 minutes, which was then collected by spitting into a sterile labeled container, placed on ice.

Pretreatment of saliva was carried out on the lines of the description provided by Banderas-Tarabay et al⁴ with modifications. Saliva was then centrifuged using the Rota 4R—V/Fm, Plasto Crafts[®] centrifuge at 4°C at 4,000 rpm for 45 minutes and the pellet was discarded. Approximately 500 µL of saliva was transferred into the Eppendorf tube, sealed with punctured Parafilm[®], and dried in Labconco[®] speed evacuator. The dried saliva was then stored at -20°C and reconstituted by adding Laemmli's Buffer (1M Tris at pH 6.8—0.625 mL, 20% SDS 1 mL, glycerol 1 mL, double distilled water 0.175 mL). About 10 µL of the saliva was then subjected to protein estimation by Lowry's method and readings were taken at 750 Å using the Uvikon[®] spectrophotometer.

For each of the 150 samples, the calculated volumes for 100 µg and 10 µg of protein were taken into two separate Eppendorf tubes; half their volume of Laemmli's buffer (3× concentrations) with a 10 µL bromophenol blue dye and then boiled for 5 minutes in water bath. The samples were loaded in each gel according to increasing DMFT index of the samples and 2 µL of the Fermentas protein marker was added to the last well. Each sample was loaded in two separate gels, one for 100 µg and the other for 10 µg of protein loading respectively, after adding. The SDS-PAGE was carried out at 200 V, for approximately 50 minutes in the Biorad® apparatus. The gels were removed after electrophoresis and stored in the destainer (methanol, acetic acid, and double distilled water in the ratio of 5:1:4) for 30 minutes. The gels with 100 µg and 10 µg protein loading were subjected to periodic acid-Schiff (PAS) and silver staining respectively (Figs 1 and 2).

Seven protein bands were detected on the PAS-stained gel and labeled as B1 to B7 (Fig. 1) and 11 bands were detected in the silver-stained gel and named as S1 to S11 (Fig. 2). These gels were then scanned using the GS800 calibrated densitometer, Biorad®. Scanned images were then subjected to densitometry using the Image J Analysis Software. Data analysis was done using Statistical Package for the Social Sciences, version 16.00.

Proteins in the PAS stained of single representative spots were identified by matrix-assisted laser desorption/ionization time-of-flight (TOF)-TOF analysis according to Schevchenko described by Fulzele et al¹¹ (Brucker®, Germany). The PAS-stained gel plugs were not destained after thorough rinsing with water, and the gels were dehydrated in 100% acetonitrile (ACN) which was removed by drying in a speed-vac. Proteins in the resolved gel plugs were reduced using 10 mM dithiothreitol and then

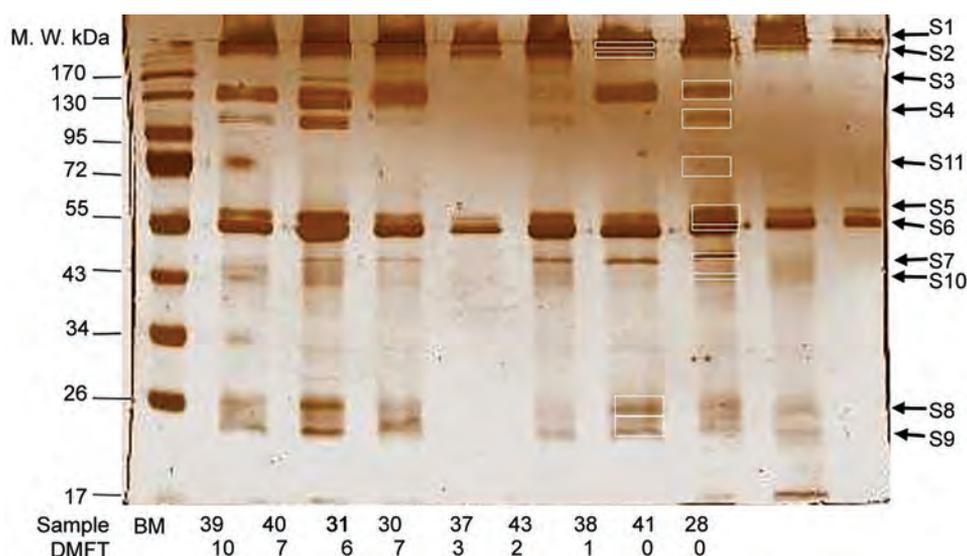


Fig. 1: PAS-stained gel with each lane marked with sample number and DMFT index. Bands B1–B9 are marked by arrows

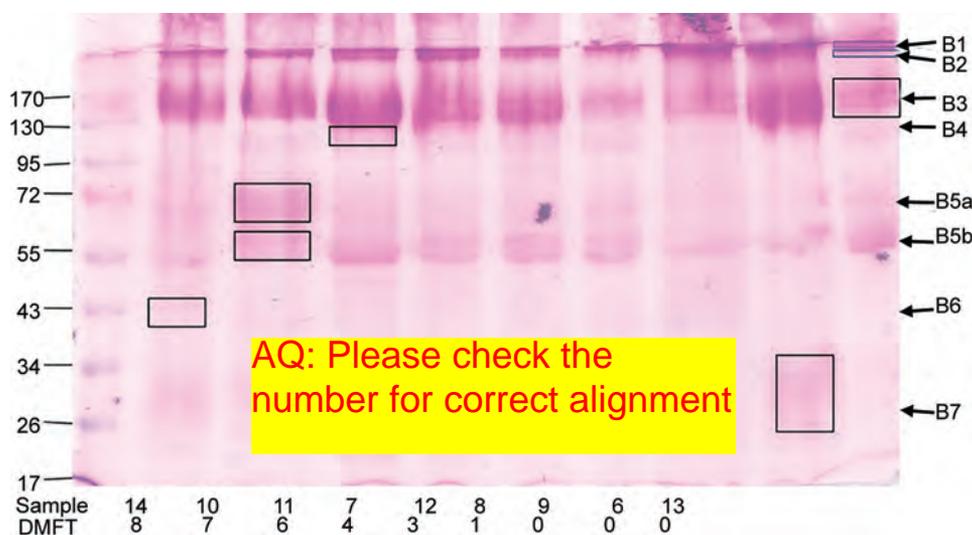


Fig. 2: Silver-stained gel with each lane marked with sample number and DMFT index. Bands S1–S11 are highlighted by arrows

alkylated with 55 mM iodoacetamide. The proteins in the plugs were then trypsinized overnight with 10 mg/ μ L trypsin in 25 mM ammonium bicarbonate (10 μ L/plug) and the peptides were recovered by extraction with 40 μ L of 50% ACN and 5% trifluoroacetic acid (TFA). Tryptic protein digests were reconstituted in 10% ACN with 0.1% TFA solvent before subjecting them to MS analysis. Mass calibration was carried out using peptide mixture of five known peptides spanning a mass range of 757 to 3147 m/z and error was kept to less than 10 ppm. Accelerating voltage of 25 kV was applied to the first TOF tube. The MS data were acquired in an automated manner using a solid state neodymium-doped yttrium aluminum garnet laser at 337 nm. The resulting MS data were analyzed using Flex analysis 3.0 (Bruker Daltonik, Germany) software and were acquired using Biotoools software (Bruker Daltonik, Germany). The chosen peptides were searched against SwissProt database version 2012-08 onward using MASCOT search engine for protein ID with precursor tolerance of 100 ppm for MS and fragment tolerance of 0.7 to 1 Da for MS/MS analysis.

RESULTS

The samples were divided into four groups according to their DMFT score, i.e., groups I, II, III, and IV, with 0, 1 to 3, 4 to 7, and above 8 DMFT scores respectively. Mean

protein concentrations in whole human saliva calculated for groups I to IV were 33.84, 34.62, 36.06, and 40.53 μ g/10 μ L respectively. An ascending pattern of protein levels with caries was noted; however, the p-value obtained was 0.09 and hence, the increase was insignificant. Higher values of total salivary proteins were observed in the male against female subjects, the mean concentrations being 34.22 and 34.71 μ g/10 μ L respectively. The p-value being 0.18, no significant correlation could be concluded. No significant correlation was proven between the age of the subjects and total salivary protein concentration. Total protein concentrations and its correlation to several parameters is highlighted in Table 1.

The mean protein densities of each of the bands were calculated and compared among the groups. Table 2 shows the mean densities of the protein bands, B1 to B7 isolated by PAS stain in 100 μ g loaded gels and a negative trend was noted in band B5a which can be better appreciated in Graph 1. Similarly, the mean densities of the protein bands S1 to S11 identified by silver staining with 10 μ g loading are illustrated in Table 3. A negative trend was noted in band no. S5 and can be appreciated in Graph 2. However, p-values were above 0.05 for both the bands in question and hence, no correlation could be proved statistically.

Mass spectrometry analysis revealed the protein IDs of the PAS-stained bands and results are summarized

Table 1: Total salivary protein levels in relation to various parameters

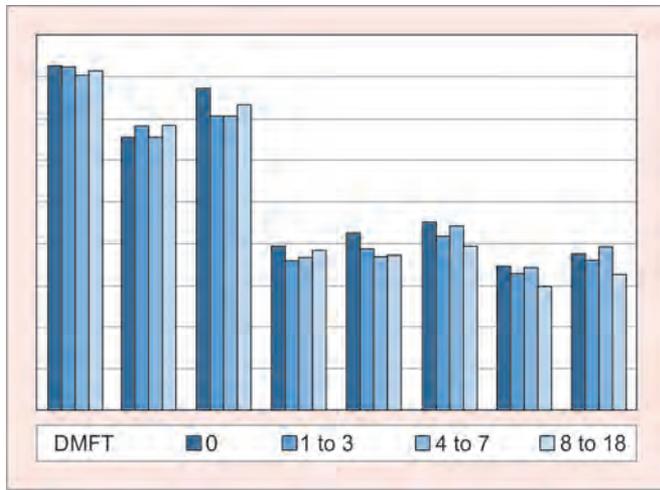
| | DMFT index score | | | | Sex | | Age (years) | |
|----------------------------------------------------------|----------------------|-------------------------|--------------------------|--------------------------|-------------------|-------------------|-------------------|-------------------|
| | Group I (0 score) | Group II (1–3 score) | Group III (4–7 score) | Group IV (8–18 score) | Male | Female | 15–24 | >25 |
| Mean total salivary protein levels in μ g/10 μ L | 33.84 \pm 9.72 | 34.62 \pm 10.39 | 36.06 \pm 11.23 | 40.53 \pm 13.19 | 37.22 \pm 11.34 | 34.71 \pm 11.03 | 35.27 \pm 10.99 | 37.37 \pm 11.62 |
| N (sample size) | 34 | 44 | 43 | 29 | 77 | 73 | 98 | 52 |
| p-values (<0.05 significant) | 0.09 | | | | 0.18 | | 0.16 | |

Statistical tests employed include one-way analysis of variance, *post hoc* Scheffe test

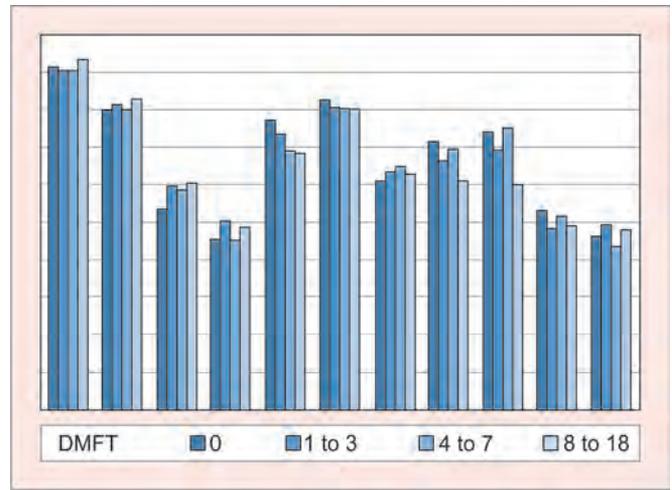
Table 2: Mean densitometric values of PAS-stained protein bands in relation to DMFT index score

| DMFT index score | PAS-stained protein bands | | | | | | | |
|------------------------------|---------------------------|--------------------|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | B1 | B2 | B3 | B4 | B5A | B5B | B6 | B7 |
| Group I (0 score) | 165.04 \pm 47.27 | 131.03 \pm 46.97 | 154.14 \pm 45.58 | 78.37 \pm 27.07 | 84.95 \pm 35.49 | 89.9 \pm 34.14 | 69.44 \pm 28.29 | 75.21 \pm 29.15 |
| Group II (1–3 score) | 164.76 \pm 48.93 | 136.11 \pm 52.23 | 141.27 \pm 49.27 | 71.6 \pm 24.10 | 77.35 \pm 35.51 | 83.47 \pm 35.58 | 66.08 \pm 27.47 | 71.89 \pm 32.10 |
| Group III (4–7 score) | 160.29 \pm 47.22 | 131.2 \pm 55.04 | 141.18 \pm 52.93 | 73.31 \pm 26.04 | 74.17 \pm 26.46 | 87.95 \pm 36.35 | 68.93 \pm 26.76 | 78.63 \pm 27.20 |
| Group IV (8–18 score) | 162.74 \pm 50.79 | 136.39 \pm 54.84 | 146.35 \pm 55.66 | 76.5 \pm 31.65 | 74.29 \pm 39.89 | 78.96 \pm 38.55 | 59.73 \pm 27.66 | 65.67 \pm 29.36 |
| N (Sample size) | 150 | 150 | 133 | 133 | 133 | 133 | 133 | 133 |
| p-values (<0.05 significant) | 0.98 | 0.98 | 0.92 | 0.92 | 0.73 | 0.73 | 0.58 | 0.40 |

Statistical tests employed include one-way analysis of variance, *post hoc* Scheffe test



Graph 1: Silver-stained gel: Relation of mean band intensities with DMFT levels



Graph 2: Variation in the PAS-stained band intensities with increasing DMFT score

Table 3: Mean densitometric values of silver-stained protein bands in relation to DMFT index score

| DMFT index score | Silver-stained protein bands | | | | | | | | | | |
|------------------------------|------------------------------|----------------|----------------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|---------------|
| | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 |
| Group I (0 score) | 183.13 ± 27.44 | 159.6 ± 40.43 | 106.99 ± 42.00 | 90.50 ± 31.45 | 154.5 ± 38.52 | 165.55 ± 37.43 | 122.28 ± 42.50 | 143.21 ± 44.27 | 148.07 ± 56.80 | 105.97 ± 40.98 | 92.37 ± 30.87 |
| Group II (1–3 score) | 180.92 ± 31.93 | 162.84 ± 38.73 | 119.71 ± 45.64 | 99.75 ± 38.91 | 147.13 ± 44.13 | 160.74 ± 44.31 | 126.48 ± 35.80 | 133.01 ± 40.62 | 138.37 ± 47.40 | 96.51 ± 30.21 | 92.97 ± 28.74 |
| Group III (4–7 score) | 180.71 ± 26.36 | 159.8 ± 40.88 | 117.4 ± 46.23 | 90.45 ± 31.88 | 137.97 ± 39.72 | 159.44 ± 38.93 | 130.17 ± 44.07 | 138.66 ± 42.29 | 149.54 ± 43.70 | 102.94 ± 32.84 | 86.36 ± 23.08 |
| Group IV (8–18 score) | 187.41 ± 25.47 | 165.48 ± 32.35 | 122.19 ± 45.35 | 97.30 ± 32.71 | 137.11 ± 43.37 | 160.95 ± 40.61 | 125.46 ± 49.17 | 122.41 ± 45.91 | 121.00 ± 53.36 | 97.84 ± 42.00 | 95.87 ± 31.29 |
| N (Sample size) | 150 | 141 | 141 | 141 | 150 | 150 | 141 | 141 | 27 | 141 | 141 |
| p-values (<0.05 significant) | 0.80 | 0.99 | 0.76 | 0.88 | 0.37 | 0.94 | 0.90 | 0.42 | 0.92 | 0.81 | 0.87 |

Statistical tests employed include one-way analysis of variance, *post hoc* Scheffe test

Table 4: MS analysis of PAS-stained spots

| Spot | Protein name | Protein ID | Accession no. | Peptide mass fingerprinting | | | | |
|------|---------------------------------------|-------------|---------------|-----------------------------|-----------|--------|--------|-----|
| | | | | Total score | Mass (Da) | SC (%) | IC (%) | pl |
| P1 | Cytochrome c oxidase assembly protein | COX19_HUMAN | Q49B96 | 59.60 | 10615.180 | 55.6% | 22.8 | 9.9 |
| P2 | Ig alpha-2 chain C region | IGHA2_HUMAN | P01877 | 68.6 | 37301.310 | 22.6 | 32.3 | 5.7 |
| P3 | Alpha-amylase | AMY1_HUMAN | P04745 | 38.9 | 58415.170 | 13.1 | 14.8 | 6.5 |
| P4 | Alpha-amylase | AMY1_HUMAN | P04745 | 154.0 | 58415.170 | 34.8 | 71.9 | 6.5 |
| P5 | Alpha-amylase | AMY1_HUMAN | P04745 | 103.0 | 58415.170 | 25.0 | 39.1 | 6.5 |
| P6 | Serum albumin | ALBU_HUMAN | P02768-1 | 57.6 | 71317.250 | 23.5 | 34.2 | 5.9 |
| P7 | Serum albumin | ALBU_HUMAN | P02768-1 | 173.0 | 71317.250 | 36.0 | 63.7 | 5.9 |
| P8 | ZG protein | ZG16B_HUMAN | Q96DA0 | 89.90 | 22724.570 | 26.4 | 62.5 | 7.6 |
| P9a | ZG protein | ZG16B_HUMAN | Q96DA0 | 89.90 | 22724.570 | 30.8 | 55.3 | 7.6 |
| P9b | Hemoglobin subunit beta | HBB_HUMAN | P68871 | 67.6 | 16102.330 | 46.3 | 19.4 | 6.9 |
| P10 | Alpha-amylase | AMY1_HUMAN | P04745 | 68.2 | 58415.170 | 15.7 | 22.0 | 6.5 |

IC: Intensity coverage; SC: Sequence coverage; pl: Isoelectric point (no. of peptides with $p < 0.05$)

in Table 4. The band P1 was identified as cytochrome c oxidase assembly protein; P2 as Ig alpha-2 chain C region; P3, P4, P5, and P10 were recognized as alpha-amylase; P6 and P7 were noted to be serum albumin; P8 and P9a as ZG protein; and P9b as ZG protein and hemoglobin subunit beta (Fig. 3).

DISCUSSION

Salivary defense systems including salivary proteins play a significant role in maintaining the health of the oral cavity. Salivary constituents and their role in the etiology of dental caries has long been a topic of great interest and research among the dental researchers. This study was



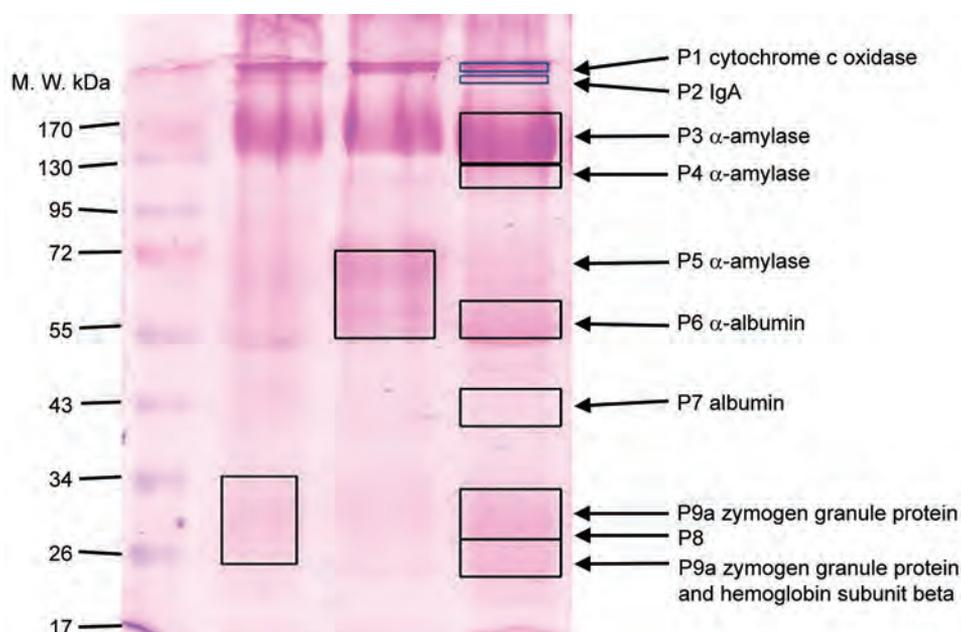


Fig. 3: Schematic representation of the protein identification obtained using MS

undertaken to analyze the protein content of saliva and explore its interrelationship with caries.

de Farias and Bezerra¹² have studied the mean protein levels between caries-free and early childhood caries patients and found no significant difference. Similar results have been obtained by Bhalla et al¹ and Roa et al.¹³ This study also reports and corroborates with the above research work.

Age and total protein in this study show a positive correlation, which is statistically not significant. Deshpande et al¹⁴ have shown a significant correlation between total protein and age in patients between 3 and 16 years. Morzel et al¹⁵ stated that salivary profiles were modified substantially between the ages of 3 and 6 months due to the eruption of teeth and change in diet. This could be attributed to the vast difference in ages of the samples in each of the studies. In our study, the ages of the patients ranged between 16 and 35 years, while the above studies have considered a younger and developing age group.

Taking into account sex and total protein content of saliva, Dodds et al¹⁶ studied the protein profile of parotid fluids in caries-free and caries-active individuals and found women had significantly higher proteins than men. Roa et al¹³ too reported similar results in the Colombian population. This study is in congruence with the findings of Banderas-Tarabay et al⁴ that females show greater mean salivary total protein levels than the male patients; however, this difference is statistically insignificant.

Electrophoretic separation revealed a considerable variation in patterns of different individuals as shown by substantial differences in number, intensity, and size of bands observed. The presence of genetic polymorphism

in salivary proteins of HWS can be appreciated in this study in correspondence to findings reported by Schwartz et al,¹⁷ Banderas-Tarabay et al,⁴ and Bhalla et al.¹

Banderas-Tarabay et al⁴ identified high-molecular-weight mucin (MG1) and low-molecular-weight mucin (MG2) above the 97.4 kDa molecular weight marker band on their PAS-stained gel and PRP band at 35 kDa level on the Coomassie brilliant blue (CBB)-stained gel.⁴ Bhalla et al¹ have identified PRP between the 30 and 50 kDa marker and alpha-amylase at approximately 75 kDa level in both CBB and silver-stained gels. In these studies, a significant correlation was identified in all the above proteins except alpha-amylase in relation to dental caries. While these two are the only studies conducted on saliva samples of patients with dental caries according to our knowledge, it important to note that there were no confirmatory tests conducted for protein identification. In this study, on MS MG1, MG2, and PRPs were not detected on the PAS-stained gel and three bands of alpha-amylase were found between 55 and 170 kDa.

Amylase levels and incidence of dental caries have been studied by several authors. Bergeim and Barnfield,¹⁸ Hess and Smith,¹⁹ Sullivan and Storvick²⁰ reported lack of correlation between dental caries and salivary amylase. They used techniques, such as starch hydrolysis in their experiments. Banderas-Tarabay et al⁴ and Bhalla et al¹ both used electrophoresis to determine the relationship between amylase and caries but found no significant results. The results of this study are in line with the above studies and also report a lack of correlation between salivary amylase and dental caries.

The whole saliva presents a large array of proteins and peptides participating in a multitude of functional roles in

the oral cavity, such as microbiological protection, lubrication, digestion, and general maintenance of teeth and mucosal surface health. Traditional proteomic methods were applied to the analysis of whole-saliva peptides/protein species using 2D SDS-PAGE followed by MS. With this approach, the most abundant salivary proteins were identified, such as several PRPs, amylase, lipocalin-1 or calgranulin B, assigning more than 300 proteins.⁶

Zymogen granule protein 16 homolog B was identified in the sample in both B7a and B7b bands. Gonçalves et al²¹ reported the presence of this protein in patients with gingivitis. This was attributed to its low abundance or unknown proteolysis. Again, it must be noted that gingivitis was an exclusion criterion for our study. The acinar cells of the exocrine pancreas are among the cells with the highest rate of protein synthesis in higher organisms and target more than 90% of newly synthesized proteins to the secretory pathway. They are “specialists” in the synthesis, mass packaging/sorting, storage, and regulated secretion of a complex mixture of different digestive enzymes and isoenzymes. These digestive enzymes are packaged and condensed in a predominantly inactive form into large secretory organelles called ZGs. Some proteins present in the parotid secretory granules have been identified as ZGs in recent proteomic studies, such as Rac26, Noc2, Rab27B, Rap1, and Rab3D. The constituents of saliva are mainly produced by acinar cells and are conveyed to the oral cavity by a cell-lined duct system in which the fluid and electrolyte components are subject to secondary modifications. Secreted salivary proteins are predominantly stored at high concentrations in dense-core secretory granules. Despite a large number of studies devoted to the characterization of the dense-core granules, information about their composition remains limited.⁶

To compare the MS analysis of HWS, the study by Morzel et al¹⁵ analyzed samples of 3- and 6-month-old infants and isolated 21 bands up to 11 kDa on silver staining, while we have identified 9 bands on PAS staining up to 25 kDa. They noted two serum albumin bands at 38.9 and 48.9 kDa, similarly we located two serum albumin bands at approximately 55 and 43 kDa. Albumin is derived from serum and therefore, enters the oral cavity through mucosal permeability or through gingival crevices. It is most likely that the increase of serum albumin rather results either from the increased permeability of inflamed epithelia or from the formation of gingival crevices and the concomitant higher proportion of crevicular fluid in whole saliva.²² The same explanation can also be compounded by the presence of hemoglobin in band B7b in our samples. It is important to note that patients with gingivitis and periodontitis were excluded from the study group.

The alpha-amylase was identified by Morzel et al¹⁵ at 43.3 kDa and in our study, three bands of alpha-amylase

were found between 55 and 170 kDa. The fact that alpha-amylase coexists in saliva under multiple forms, especially at molecular weights lower than that of the theoretical full-length form of the protein, has been previously reported. The authors described electrophoretic differences to the carbohydrate moiety of the enzyme, although a thorough study of 140 isoforms of alpha-amylase in human saliva concluded that glycosylation alone cannot account for the multiplicity of amylase spots covering the 18 to 59 kDa range.¹⁵

The Ig alpha-2 chain C region was detected as band B2. The IgG, IgM, IgA, and secretory IgA (slgA) form the basis of the specific salivary defense against oral microbial flora, including mutans streptococci. The most abundant Ig in saliva, as in all other human secretions, is dimeric slgA, which is produced by plasma cells located in the salivary glands. Two IgA subclasses are present in saliva; IgA1 forms the major component of Igs, although the relative amount of IgA2 is higher in saliva than in other secretions.⁹ The constant domain is IgA2 heavy chain, which is most probably correlated to the proportion of the whole IgA2 in saliva.²¹

The presence of high amounts of mucins and other glycoproteins interferes with 2D protein separation by blocking the passage of current during the focusing step, and by forming heterotypic complexes with other salivary proteins.⁶ A similar factor may be postulated to cause the discrepancies seen in the molecular weights of the proteins identified by MS. Current efforts to elucidate the proteome for the whole saliva have progressed rapidly with advances in MS and protein separation tools. Nevertheless, the characterization of the salivary proteome is far from complete as salivary proteins are often present in polymorphic isoforms and as unique splice variants with deletions, truncations, and posttranslational modifications as additional sources of complexity.²³

CONCLUSION

An initial key step for saliva to be of practical use for disease diagnosis and health monitoring is the classification of its protein components. Recently, several reports aiming to comprehensively catalog the salivary proteome have been published with numbers of proteins identified ranging from hundreds to over thousands. Translating scientific findings of nucleic acids, proteins, and metabolites in body fluids to clinical applications is a cumbersome and challenging journey.⁵ It would be very interesting if a potential treatment for oral diseases could be currently in our oral cavity in the form of salivary proteins. Therefore, biofilm-dependent oral diseases can be controlled at the pellicle level—the interface between pathogenic microbes and the solid oral surfaces. By controlling, or

perhaps altering, the composition of the pellicle, we could potentially interfere with the adhesion process of the oral microbiota to oral surfaces. Therefore, the future of oral therapeutics should focus on the interaction between salivary proteins and microorganisms. In conclusion, assessing and understating salivary composition can be applied as a feasible and reliable tool for predicting and treating several oral infections, diagnosing systemic diseases, and determining the state of patient's immune systems. Therefore, collecting and analyzing saliva would not only help to better monitor and maintain the oral health of patients, but could also significantly improve the health care system.⁷ Identification of significant changes in salivary protein composition that are associated with disease processes could be used as potential diagnostic or prognostic indicators that could be used in clinical context for noninvasive detection and monitoring of human diseases. One the most common oral diseases, i.e., dental caries and its association with salivary factors, has long been acknowledged; salivary proteins and their role could help enlighten the multifactorial etiology of the disease and in the future open new avenues for early diagnosis and prevention of the same.

The role of ZG proteins in pancreatic diseases is currently a topic of interest among researchers. Other secretions, such as saliva are far less explored avenues for the same. However, few have demonstrated these proteins in patients suffering from periodontitis. In the current study, these proteins were demonstrated in most samples with dental caries and controls. There is a critical need for validation experiments and functional studies to provide a better understanding of the process of ZG formation, sorting/packaging of cargo, exocytosis and membrane fusion, and to generate a complete picture of the ZG architecture. The role of ZG in oral cavity homeostasis also needs to be delineated and better understood. This study also provides novel insight into profiles of the salivary proteome during dental caries, which may contribute to improvements in prevention, diagnosis, and treatment.

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