Precursor proteins of the acquired enamel pellicle derive from glandular and non-glandular secretions, which are components of whole saliva. The purpose of this investigation was to gain further insights into the characteristics of proteins in whole saliva and in vivo formed pellicle components. To maximize separation and resolution using only micro-amounts of protein, a two-dimensional gel electrophoresis system was employed. Protein samples from parotid secretion, submandibular/sublingual secretion, whole saliva, and pellicle were subjected to isoelectric focusing followed by SDS-PAGE. Selected protein spots were excised, subjected to in-gel trypsin digestion, and examined by mass spectrometry (MS). The data generated, including peptide maps and tandem MS spectra, were analyzed using protein data base searches. Components identified in whole saliva include cystatins (SA-III, SA, and SN), statherin, albumin, amylase, and calgranulin A. Components identified in pellicle included histatins, lysozyme, statherin, cytokeratins, and calgranulin B. The results showed that whole saliva and pellicle have more complex protein patterns than those of glandular secretions. There are some similarities and also distinct differences between the patterns of proteins present in whole saliva and pellicle. MS approaches allowed identification of not only well characterized salivary proteins but also novel proteins not previously identified in pellicle.

Human teeth are exposed to whole saliva (WS), \(^1\) consisting mainly of secretions derived from three pairs of major salivary glands, which comprise parotid, submandibular, and sublingual glands. Protein components that have been identified in all of the major glandular secretions are proline-rich proteins (acidic, basic, and glycosylated families), amylase, statherin, histatins, lysozyme, lactoferrin, lactoperoxidase, and secretory IgA (1–10), whereas cystatins and mucins have been identified in submandibular/sublingual secretions (9, 11–13). However, detailed understanding of the protein composition in WS is still limited because of the lack of knowledge about proteins in other contributors to whole saliva such as secretions from minor salivary glands and gingival crevicular fluid. In addition, little is known about modifications that occur on proteins during or after secretion into the oral cavity.

The acquired enamel pellicle (EP) is a protein film thought to result from the selective adsorption of precursor proteins present in WS onto tooth surfaces. Because of its intimate contact with enamel surfaces, the EP plays an important role in maintaining tooth integrity by controlling the mineral solution dynamics of enamel. At its interface with the oral environment, the EP exerts selectivity on bacterial attachment and is involved in the initial stages of plaque formation (14). Because of the limiting amount of proteins that can be harvested from EP formed in vivo, previous investigations have utilized sensitive but indirect approaches such as enzymatic assays and immunologic detection to identify EP components (15–19).

One of the ways for direct identification of EP components is mass spectrometry (MS). MS has undergone considerable advances in the sensitive and specific analysis of biological materials (20). The development of matrix-assisted laser desorption/ionization (MALDI) MS by Hillenkamp and colleagues (21, 22) greatly increased the ability to analyze non-volatile biomolecules. Since then, improvements in MALDI-time-of-flight (MALDI-TOF) mass spectrometers and sample handling methodologies have allowed very high throughput, primarily as a result of the speed of data acquisition and greater tolerance of contaminants (e.g. salts and detergents) by MALDI when compared with other MS methods (23). The introduction of quadrupole orthogonal time-of-flight (QoTOF) MS has provided yet another level of sophisticated analysis (24, 25). The coupling of quadrupoles to the TOF analyzer initially generated electropray ionization (ESI) data with high sensitivity (<10 fmol) and mass accuracy (<20 ppm) and allowed for tandem experiments that give much more complete and reliable data to facilitate protein identification and characterization (26). The more recent addition of a MALDI source to the QoTOF mass spectrometer gave these instruments additional flexibility (27). These advances in MS have been employed in a variety of biological investigations including cataloguing bacterial proteomes (28, 29), identifying differences in protein expression in disease versus normal cells (30, 31), and characterizing post-translational modifications of specific proteins (32, 33).

Recently, our group carried out the first direct identification...
of proteins found in EP formed in vivo using a MS approach with samples separated on one-dimensional SDS gels (34). We have now addressed complications that arise from the presence of multiple components in an apparent single electrophoretic band by achieving better resolution of the protein mixture through the use of two-dimensional electrophoresis (2-DE). Application of 2-DE has only been reported in a few investigations in WS (35–37) so far so far or found in in vivo formed EP. In none of these EP studies were proteins identified using MS techniques. In the present study, we used 2-DE to resolve and compare proteins from glandular secretions, WS, and EP and identified selected components from WS and EP using mass spectrometry.

EXPERIMENTAL PROCEDURES

Human Subjects—Healthy non-medicated male and female volunteers, ranging in age from 20 to 60 years, were selected. The subjects exhibited no overt signs of gingivitis or caries. Saliva and pellicle collection protocols were approved by the Institutional Review Board of Boston University Medical Center, and informed consent was obtained from each subject.

Collections of Glandular Secretions and WS—Parotid secretion (PS) from both glands was collected by means of a Carlson-Crittenden device (38). Sublingual secretion (SUBL) was collected using a custom-fitted device consisting of a plastic core covered with Impregum F Impression material (3M ESPE, Seefeld, Germany). The flow of glandular secretion was provoked by gustatory stimulation using sugar-free lemon-flavored candies. Collection of WS was carried out under masticatory stimulation using a uniform bolus of Paraffilm™ (Fisher Scientific, Pittsburgh, PA). The samples were kept on ice during the collection procedure. Immediately after the collection, WS samples were centrifuged at 14,000 g for 20 min in a microcentrifuge at 4 °C to remove undissolved materials. Samples of glandular secretions and WS supernatant collected from two subjects were pooled and stored in 200-µl aliquots at −20 °C. Protein concentrations were determined using a micro-BCA protein assay (Pierce). Harvesting of Human EP—The collection procedure for the in vivo EP was carried out as described (34). Briefly, each donor was subjected to a thorough dental prophylaxis employing a coarse pumice containing sucrose, followed by a second equilibration with a tris-buffer (Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, 6 M urea with 1% dithiothreitol) for 10 min using the same buffer except that diithiothreitol was replaced by 4% iodoacetamide (Sigma). Subsequently, the IPG strip was applied horizontally on top of a 10% SDS-polyacrylamide gel (20 × 20 cm), and proteins/peptides were separated vertically for 16 h at a constant voltage of 105 V. The resulting two-dimensional gel was stained either with silver (Owl Separation System, Portsmouth, NH) or with Sypro-Ruby (Molecular Probes, Eugene, OR). Gels with the Sypro-Ruby staining were visualized under ultraviolet light using a Gel Doc 1000 Imager (Bio-Rad). Mass Spectrometry—Protein spots were excised from two-dimensional gels using a sterile, cut pipette tip. Proteins contained in the gel were digested with sequencing-grade trypsin (Promega, Madison, WI) as previously described (41). Tryptic peptides were extracted from gel spots using 1% trifluoroacetic acid in 50% acetonitrile and then dried under a Speed-Vac™ (Thermo Savant, Holbrook, NY). The resulting samples were resuspended in 0.1% trifluoroacetic acid and desalted using ZipTip™ (Millipore) as per the instructions from the manufacturer. Samples were then dried and resuspended in 50% methanol with 1% formic acid and were analyzed using both the Finnigan MAT Vision 2000 MALDI-TOF reflectron mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an ultraviolet laser (nitrogen, 337 nm) and the Applied Biosystems/MDS-Sciex QStar Pulsar, quadrupole/orthogonal acceleration TOF mass spectrometer (QoTOF) with nanospray and MALDI (UV laser; nitrogen, 337 nm) sources (Applied Biosystems Inc., Framingham, MA). The MALDI-TOF MS was used initially to both screen samples and to identify and analyze samples at higher mass ranges. The QoTOF was then used to obtain data with high mass accuracy as well as to obtain tandem MS data. The MALDI matrix was 2,5-dihydroxybenzoic acid (DHB), and typically 50–200 laser shots were summed for each spectrum. The laser power used was between 50 and 60% when obtaining the Vision spectra and 30–35 μJ when obtaining the QoTOF spectra. When obtaining QoTOF nanospray data, 1-μm nanospray tips, pulled with a Sutter model P-87 micropipette puller, were used with an ion source voltage of 1000–1300 V. For tandem data, nitrogen was used as the collision gas and a range of operator-controlled collision voltages (12–50 V for electrospray; 35–90 V for MALDI) were employed. Spectra were analyzed manually by manually searching masses/proteins (through the use of two-dimensional electrophoresis (2-DE) showed that sonication of PS under these conditions provoked undetectable fragmentation of proteins/peptides. To separate the extraction liquid from the membrane, a small needle-size (16-gauge) hole was placed on the bottom of the tube followed by centrifugation in a microcentrifuge and the pellet extract was collected into a separate tube. Pellicle samples were then desalted using sequential dialysis–centrifugation steps in an Amicon microcentrifuge device (Millipore) with a molecular mass cut-off of 3000 Da. Desalted pellicle samples were then analyzed using a micro-BCA protein assay to determine protein concentration.

Two-dimensional Gel Electrophoresis—2-DE (28, 39, 40) was carried out by isoelectric focusing (IEF) using pre-made immobilized pH gradient (IPG) strips in the Protean IEF cell (Bio-Rad) followed by SDS-PAGE using the Protean II device (Bio-Rad). Approximately 50 µl of PS, SMLS, WS, or pooled EP samples containing 100 µg of proteins was mixed with 300 µl of IEF rehydration buffer in a focusing tube upon which a 17-cm-long, pre-made IPG strip was added. Rehydration was carried out in the tray under a constant voltage of 50 V for 12 h. The voltage was gradually increased to 300 V, and samples were focused for an additional 6 h. To prepare the IEP strip for the second dimension, the strip was first equilibrated in a buffer containing 50 mM Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, 6 M urea with 1% dithiothreitol (Sigma) for 10 min at room temperature, followed by a second equili-
tributed into zones I–III in the vertical dimension, they were focused horizontally into wider pI ranges in zone I and II with major spots shifted to a more basic pI region than was observed with EP. To our knowledge, this is the first time that well resolved EP and WS samples are shown in 2-DE gels with a full view (pI range 3–10 and molecular mass range 0–200 kDa).

However, the relatively similar pI values among some components in both biological samples clearly pointed to the necessity of further resolving these proteins/peptides. Because most of the major components were contained in the pI 5–8 range, narrow range IPG strips (pH 5–8) were subsequently applied in the IEF phase of the 2-DE.

The pattern of spots visualized by 2-DE of proteins in WS and EP (Fig. 1) was next compared with those in PS and SMSL because the latter secretions are thought to contain primarily intact proteins which can serve as precursors to those in WS and the EP. The electrophoretogram of protein (100 μg) from pooled PS showed a profile of proteins with well defined separation (Fig. 2, panel A). Using the same zoning criteria, zone I contained ~12 spots that were clearly discernible. A similar, but not identical, electrophoretic pattern was observed with SMSL (Fig. 2, panel B). The differences are likely caused by the presence of some proteins in SMSL such as mucins and cystatins, which are absent in PS. The tailing effect in the region above zone I (Fig. 2, panel B) may be caused by migration of MUC-7 glycoforms (42). Additional spots seen in the basic region of zone III (Fig. 2, panel B) likely represent cystatin molecules. Although the data in Fig. 2 were obtained using pooled samples from two subjects, very similar electrophoretic patterns were obtained with samples from several other subjects.

Given the relatively simple electrophoretograms from both PS and SMSL, one may imagine that WS, representing mostly a mixture of glandular secretions with only minor contributions from gingival crevicular fluid, would show an electrophoretic pattern that would nearly be a summation of the two protein patterns (Fig. 2, A and B). Therefore, it was surprising to find that there were significant differences between the protein patterns observed for glandular secretions (Fig. 2, A and B) and WS (Fig. 3) in the pI range 5–8. First, the number of detectable protein spots in WS was dramatically greater than those in either PS (Fig. 2A) or SMSL (Fig. 2B). There were approximately 65 spots in zone I, 43 spots in zone II, and 30 in zone III. Second, proteins/peptides in WS seemed to be more distributed evenly throughout the pI range 5–8, in contrast to the clustering of spots in specific pI ranges that was observed in glandular secretions. Some proteins in glandular secretions such as the 5-protein spot series in the 40–45-kDa region (zone

Fig. 1. Two-dimensional gel electrophoresis using isoelectric focusing with pH range 3–10 in the horizontal dimension and SDS-PAGE (10%) in the vertical dimension. Panel A, 100 μg of EP, stained with Sypro-Ruby and shown in inverted form for better contrast. Panel B, 100 μg of WS, stained with silver. Gel spots that were cut out for in-gel trypsin digestion and analyzed by MS were labeled P1–P13 and S1–S3 (P denotes EP, and S denotes WS). Horizontal lines divide the gels into zones I, II, and III, representing high, middle, and low molecular mass regions, respectively. *: zone I 5-protein spot series.

Fig. 2. Two-dimensional gel electrophoresis of salivary glandular secretions by isoelectric focusing with pH range 5–8 in the horizontal dimension and SDS-PAGE (10%) in the vertical dimension. Panel A, proteins from PS (100 μg); panel B, proteins from SMSL (100 μg). Both gels were stained with Sypro-Ruby. Zones I, II, and III correspond to high, middle, and low molecular mass regions, respectively. *: zone I 5-protein spot series.
I, indicated by * in Fig. 2, A and B) were absent in WS, whereas more proteins/peptides were detectable in zone II of WS. These observations lend support to the finding that WS represents a mixture of proteins not only derived from different sources but, more importantly, proteins that have undergone significant processing and modification upon entering the oral cavity.

EP was also analyzed using the narrow focusing range pH 5–8 (Fig. 4). A more refined pattern of spots was observed when EP was separated by this range in comparison to the pattern shown in Fig. 1. This is particularly pronounced in the high (zone I) and middle (zone II) molecular mass zones because of greater resolution of the proteins/peptides separated in a narrower pH range exhibiting very close pI values. When components had greater pI variance, adequate separation could be obtained in both pH ranges as seen in zone III of Figs. 1 and 4. More than 40 spots were observed in each of the three zones, showing an overall pattern that was essentially the same as that observed with the broad pH range gels (Fig. 1). The majority of the spots in zone I (Fig. 4) were focused between pI values of 5 and 6. This range expanded to pI 5–7 for zone II and to 5–8 for zone III. Spots with high staining intensity appeared in the same location for both gels and represent proteins/peptides of relatively high abundance. Despite of the fact that the EP analyzed over the pH range 5–10 was derived from a pool of pellicle proteins obtained from different subjects than the EP analyzed over pH range 5–8, there are considerable similarities in the overall EP patterns. These similarities point toward a consistency in EP composition and suggest that the generation of its constituents is dictated by a common mechanism.

The difference between the electrophoretic patterns of EP and WS (Fig. 1, panels A and B) became more obvious when these proteins were separated in the focusing range of pH 5–8 (Figs. 3 and 4). In zone I, few of the spots observed in the pI range of 5–6 in EP were observed in WS, whereas the staining intensity for the protein series between pI 6 and 7 was significantly reduced in EP compared with WS. In zone II, very few of the spots observed in EP seemed to relate to those in WS. Although a few EP proteins in zone III were comparable with those in WS with respect to spot location, they varied in staining intensity. To identify specific protein/peptide components in EP and WS, MS methods were employed. Selected protein

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**Table I**

| Spot no. | Protein assigned | Resource data base code | Mass pI | Mass pI | Sequence coverage (MS) |
|----------|------------------|-------------------------|---------|---------|-----------------------|
| S1       | Cystatin SA      | P09228                  | 14,000  | 4.6     | 14,381 4.67 74        |
| S2       | Cystatin SA-III  | UDHHUP 1                | 14,000  | 4.5     | 14,271 4.76 43        |
| S3       | Statherin        | SBHUP                   | 6000    | 4.0     | 5380 4.22 30          |
| S4       | Cystatin SN      | UDHHUP 2                | 17,000  | 7.64    | 13,288 7.84 81        |
| S6       | Calgranulin A    | BCHUICF                 | 10,000  | 7.44    | 10,834 6.51 68        |
| S8       | Cystatin SN      | UDHHUP 2                | 15,000  | 6.99    | 13,288 7.84 34        |
| S13      | Amylase          | ALHUS                   | 56,000  | 6.97    | 55,938 6.34 48        |
| S14      | Amylase          | ALHUS                   | 56,000  | 6.32    | 55,938 6.34 48        |
| S15      | Amylase          | ALHUS                   | 56,000  | 6.25    | 55,938 6.34 48        |
| S16      | Serum albumin    | ABHUS                   | 66,000  | 6.34    | 66,433 5.90 17        |
| P1       | Lysozyme         | LZHU                    | 14,000  | 7.93    | 14,700 9.28 62        |
| P2       | Histatin 3       | B32541                  | 6000    | 7.8     | 4062 10.0 84          |
| P3       | Histatin 1       | A32541                  | 6000    | 7.2     | 4928 7.00 71          |
| P6       | Statherin        | SBHUP                   | 6000    | 4.0     | 5380 4.22 25          |
| P11      | Cytokeratin 13   | P13646                  | 55,000  | 5.3     | 49,586 4.9 27         |
| P12      | Cytokeratin 15   | P19012                  | 50,000  | 5.4     | 49,167 4.7 25         |
| P17      | Calgranulin B    | B31848                  | 10,000  | 6.84    | 13,111 5.71 95        |
| P18      | Calgranulin B    | B31848                  | 10,000  | 6.0     | 13,111 5.71 48        |
| P19      | Phosphodiesterase| Q0HC9                   | 6000    | 6.0     | 6439 6.73 43          |

**Fig. 3.** Two-dimensional gel electrophoresis of WS (100 µg) by isoelectric focusing with pH range 5–8 in the horizontal dimension and SDS-PAGE (10%) in the vertical dimension. The gel was stained with Sypro-Ruby. Gel spots that were cut out for in-gel trypsin digestion and analyzed by MS were labeled S4–S17. Zones I, II, and III indicate high, middle, and low molecular mass regions, respectively. Framed area contains eight protein doublets representing amylase isoforms.

**Fig. 4.** Two-dimensional gel electrophoresis of EP (100 µg) by isoelectric focusing with pH range 5–8 in the horizontal dimension and SDS-PAGE (10%) in the vertical dimension. The gel was stained with Sypro-Ruby. Gel spots that were cut out for in-gel trypsin digestion and analyzed by MS were labeled P14–P23. Zones I, II, and III indicate high, middle, and low molecular mass regions, respectively.
Superscript \(^{\text{CAM}}\) indicates that the peptide contains carboxamidomethylation of a cysteine residue. Superscript \(^{\text{O}}\) indicates that the peptide contains an oxidized methionine residue. Superscript \(^{\text{P}}\) indicates that the peptide contains a phosphorylated residue. Asterisk denotes the tryptic fragments from which tandem MS data were obtained.

| Spot ID | Proteins assigned | Neutral monoisotopic mass \(^{a,b}\) | Matching peptides |
|---------|-------------------|--------------------------------------|------------------|
| S1'     | Cystatin SA       | 530.4                               | 1153.2 (46–54), 1969.8 (96–110\(^{\text{CAM}}\)), 2170.0 (53–71), 4973.4 (9–49) |
| S2'     | Cystatin SA-III   | 1275.6                              | 807 (111–117), 882 (46–52), 969 (111–117\(^{\text{CAM}}\)), 1309.8 (111–121\(^{\text{CAM}}\)), 1969.7 (96–110), 2197 (95–110\(^{\text{CAM}}\)), 2322.4 (27–45) |
| S3'     | Statherin         | 1420.65* (37–47\(^{\text{CAM}}\)), 1433.69 (24–35), 1561.80 (24–36) | 917 (7–13), 1271.81 (1–9\(^{\text{P}}\)), 1428.4 (1–10\(^{\text{P}}\)) |
| S4      | Cystatin SN       | M\(^{\text{O}}\)                        | 1568.8 (19–31), 1805.1 (78–93), 2821.2 (8–31) |
| S5      | Cystatin SN       | M\(^{\text{O}}\)                        | 615 (42–46), 843 (103–109), 885 (33–38), 1921.6 (30–44), 2823.2 (87–107) |
| S13     | Amylase           | M\(^{\text{O}}\)                        | 795.34 (393–398\(^{\text{O}}\)), 952.12 (73–80), 1001.37 (244–252), 1195.62 (344–352), 1196.53 (338–346), 1286.63 (11–20), 1288.59 (21–30\(^{\text{CAM}}\)), 1426.68* (292–303), 1537.65 (279–291), 1553.61 (73–85), 279–291\(^{\text{O}}\), 1569.72 (73–85\(^{\text{O}}\)), 1614.76 (304–319), 1631.92* (162–176), 1680.77 (253–267), 1696.66 (323–337), 1712.2 (323–337\(^{\text{O}}\)), 1728.72 (323–337\(^{\text{O}}\)), 1738.81* (143–158), 1934.02 (179–195\(^{\text{O}}\)), 2299.97 (369–387\(^{\text{CAM}}\)), 2584.17 (399–421), 2989.48 (36–61) |
| S14     | Amylase           | M\(^{\text{O}}\)                        | 840.38 (459–465), 926.47 (137–143), 1107.56 (128–136), 1478.73 (397–409), 1638.99 (413–427), 1793.83 (363–377\(^{\text{CAM}}\)), 1939.93 (257–273\(^{\text{CAM}}\)), 2044.00 (144–159) |
| S15     | Amylase           | M\(^{\text{O}}\)                        | 947 (116–122\(^{\text{CAM}}\)), 1400.9 (51–62), 1939.7 (6–21\(^{\text{CAM}}\)), 2078.8 (99–115), 3340.5 (22–50\(^{\text{CAM}}\)), 3645.7 (11–41) |
| P1      | Lysozyme          | E\(^{\text{O}}\)                        | 844 (12–17), 952 (6–12), 1265.0 (23–32), 1719.9 (13–25) |
| P2      | Histatin 3        | E\(^{\text{O}}\)                        | 844 (12–17), 952 (7–13), 1964.8 (23–38), 1272.0 (1–9\(^{\text{P}}\)), 1428.6 (1–10\(^{\text{P}}\)) |
| P3      | Histatin 1        | E\(^{\text{O}}\)                        | 1454.65 (26–38), 1805.93 (11–25) |
| P4      | Statherin         | E\(^{\text{O}}\)                        | 877.2 (44–50), 1004.6 (43–50), 1133.8 (43–51), 1456.8 (26–38), 1633.3 (73–85\(^{\text{O}}\)), 1649.1 (73–85\(^{\text{O}}\)), 1802.2 (11–25), 1954.4 (26–42), 2081.3 (26–43), 2970.3 (1–25\(^{\text{CAM}}\)), 3179.9 (86–114\(^{\text{CAM}}\)) |
| P11     | Cytokeratin 13    | E\(^{\text{O}}\)                        | 502.27 (50–53), 673.33 (3–7), 876.48 (49–55), 1454.72 (6–20) |
| P12     | Cytokeratin 15    | E\(^{\text{O}}\)                        | 1004.57 (43–50, 44–51), 1544.63* (26–38), 1628.8 (73–85\(^{\text{O}}\)), 1645.76 (73–85\(^{\text{O}}\)), 1757.79* (56–72\(^{\text{O}}\)), 1805.91* (11–25), 1953.00 (26–42) |
| P17     | Calgranulin B     | E\(^{\text{O}}\)                        | 1454.65 (26–38), 1805.93 (11–25) |
| P18     | Calgranulin B     | E\(^{\text{O}}\)                        | 877.0 (44–50), 1004.5 (44–51), 1133.0 (43–51), 1456.6 (26–38), 1649.3 (73–85\(^{\text{O}}\)), 1954.1 (26–42) |

\(^{a}\) Data were obtained from either MALDI-QoTOF (M) or ESI-QoTOF (E).

\(^{b}\) Data in parentheses indicate the starting and ending amino acid residue of the sequence matched to the observed m/z value.

\(^{c}\) Data were obtained from MALDI-TOF.

spots from WS and EP 2-DE gels were subjected to in-gel trypsin digestion (43) and analyzed using a MALDI-TOF and ESI- and MALDI-QoTOF mass spectrometers. Data were acquired with several types of mass spectrometers to ensure more complete coverage of identified spots. Analyzed protein spots are labeled numerically in Figs. 1, 3, and 4 (S for WS and P for EP). Results for individual proteins identified are summarized in Table I. In general, the experimentally observed pI values were in good agreement with values reported in protein data bases. Exceptions are lysozyme, histatin 3, and calgranulin B. The discrepancy could be related in part to covariant modifications occurring during the EP formation and in part to steric influences and structural/conformational contributions. Details with respect to matching peptide masses as well as observed modifications (oxidation, pyroglutamidation, phosphorylation, and alklylation) for each protein are given in Table II.
Proteins identified in WS include cystatin SA, cystatin SA-III, statherin, cystatin SN, calgranulin B, and phosphodiesterase. These proteins include lysozyme, histatin 3, histatin 1, statherin, cytokeratins, calgranulin B, and phosphodiesterase. The corresponding gel and spot number as well as relevant electrophoresis data (apparent pI and molecular mass) are also listed. Not surprisingly, the samples analyzed from the narrow range gels tended to contain less mixed protein spectra that the broad range gels. Quality results were, however, still obtained from the broad range gels. For example, Fig. 6 shows representative ESI-QqTOF MS (panel A) and ESI-QqTOF MS/MS (panel B) spectra from P12 (shown in Fig. 1). Resultant data base searches allowed for the identification of this protein as cytokeratin 15, an oral epithelial specific type 1 keratin. Although a number of ions corresponding to cytokeratin 15 are detectable, there are many peaks present that do not correspond to this protein, indicating that an unidentified protein is also present in the spectrum.

Fig. 7 contains representative MALDI-QqTOF MS (panel A), ESI-QqTOF MS (panel B), and ESI-QqTOF MS/MS (panel C) spectra from P17. The data from the MALDI-QqTOF MS (Fig. 7A) and the ESI-QqTOF MS (Fig. 7B) are comparable, with the exception that ions are detected at higher m/z values in the MALDI-QqTOF mass spectrum. This is a result of the difference between MALDI ionization, which produces singly charged species (\(z = 1\)), as compared with a nano- or electrospray source, which produces primarily multiply charged ions (\(z > 1\)). Additionally, the use of different ionization methods yielded different distributions of peptide ions with some only present in the MALDI spectrum (e.g. m/z 3180.3), whereas other species are only visible in the ESI spectrum. These may represent peptides for which analysis reveals the presence of additional components in the mixture, the signals of which are suppressed in the MALDI or nano-ESI mass spectrum.

The triply charged ion at m/z 602.98 (M + 3H)\(^{3+}\) shown in...
Fig. 7 was subjected to a range of collision voltages (18–50 V), and the resultant data were summed (Fig. 7C). In this ESI-QqTOF tandem MS spectra the parent ion was m/z 602.97 (M+H)$_3$ corresponding to residues 125–136 of calgranulin B. This confirmed the identification of P12 as calgranulin B. Spectra were accumulated for 30–60 s.

As lower collision energy was required to fragment this multiply charged peptide, not as many immonium and internal ions, and amine and water losses, were observed, making the spectrum less complex than the CID spectrum of a singly charged peptide (Fig. 5B). However, it should be noted that, when running nano-ESI CID experiments, one also must be mindful of multiply charged fragment ions. For example, in the spectrum shown in Fig. 7C, both doubly and triply charged fragment ions are observed (m/z 790.38; $y_{13}^{3+}$ and 564.96; $y_{14}^{3+}$). The resolution of the QqTOF makes determination of peptide charge states straightforward (see insets). Reexamination of the MS spectra (Fig. 7, A and B) yielded other matching peptides confirming the protein identification with 61% sequence coverage. Again, several ions are observed that do not appear to correspond to the theoretical digest of calgranulin B. Some of these ions can be accounted for when the oxidation of methionine residues is taken into account. Furthermore, pyroglutamination is also observed. The inset of Fig. 7B shows tryptic peptide 73–85 with a methionine oxidized (m/z 823.89) and with pyroglutamination (m/z 815.38).

**DISCUSSION**

Data generated in these experiments using 2-DE provided qualitative comparison of the proteins present in EP, WS, and major glandular secretions, as well as definitive identification of selected proteins/peptides. It demonstrates that the combination of 2-DE coupled with MS techniques is a powerful tool to resolve individual components from complex protein/
peptide mixtures and to characterize them with subpicomolar sensitivity.

Both 2-DE and mass spectrometry have unique characteristics and advantages for studies on complex mixtures such as EP and WS. The utilization of 2-DE allows for separation of not only different molecules with similar molecular masses, but also different modification patterns or isoforms of the same molecule. This resolution is exemplified by α-amylase, of which eight isoforms were detected with identical molecular mass ranging in pI from 5.9 to 7.2 (Fig. 3). Eight additional isoforms in the same pI range were found, each exhibiting a mobility change of 3 kDa in apparent molecular mass. Although this 3-kDa difference between glycosylated and non-glycosylated amylase was originally reported using a one-dimensional electrophoresis system (48, 49), the resolution into eight different isoforms was made possible through the use of this 2-DE approach.

The utilization of MS allows for unambiguous identification of proteins/peptides whether they already exist in the current data base or are hitherto unidentified molecules. This aspect is extremely important for studies of complex biological samples such as WS and EP. These samples comprise both known and novel proteins subject to modifications in the oral environment. The advantage of the technique employed is well demonstrated in the current study for the identification of calgranulin and cytokeratin family members, which represent novel EP constituents originating from non-salivary glandular sources. These discoveries were made feasible by our initial approach of obtaining tandem MS for selected tryptic fragments observed in the MS spectra to generate sequence-related product ions, which were then matched to theoretical data from proteins in a data base. Once a protein match with reasonable probability was obtained, the initial MS data were reevaluated to determine whether other tryptic peptides and known modifications from the proposed protein could be observed.

The combined utilization of 2-DE and MS provides complementary information on proteins/peptides. In MS experiments, we obtained a low coverage from some of the selected spots after trypsin digestion. This difficulty could be the result of resistance to proteolytic digestion of some proteins, incomplete recovery of digested peptides from the gel as a result of large size/hydrophobicity of some fragments, and/or low efficiency in peptide ionization. If only a minimal amount of data was acquired and no corroborating data (tandem MS or detection of a known modification) was obtained, protein identification was not reported. Whether an identified protein is intact or is a proteolytic fragment was determined by examining both MS and electrophoretic data. If an identified protein migrates to a similar pI and molecular mass as the reported intact form, it is likely that this protein is intact, even if not all peptides were observed in the MS spectrum. Using these criteria, we found evidence for the presence of intact statherin, amylase, albumin, cytastins SA-III, SA, and SN in WS, and intact histatin 1, statherin, and lysozyme in EP. The detection of intact cytastins, well known to be cysteine protease inhibitors, is consistent with previous studies where initial complete sequences were obtained on cytastin SN and SA purified from WS (50, 51). Contrary to expectation, these data reveal that other proteins can resist proteolysis and other modifications and a portion of such molecules can survive unaltered in the oral cavity. The two-dimensional electrophoretic positioning of amylase and albumin in our study were similar to those described in PS (52), suggesting that little, if any, modification occurred on these proteins after exposure to the oral environment. Although statherin and histatin 1 are known to be susceptible to bacterial degradation in the oral cavity, the mechanism by which a portion of these proteins remain intact in EP is unknown.

Mechanistic possibilities for this finding include formation of covalent/non-covalent complexes with other proteins making enzyme cleavage sites inaccessible or conformational changes occurring after adsorption to enamel crystallites, rendering them resistant to proteolysis (53).

The present investigation showed that the electrophoretic patterns of proteins in glandular secretions were significantly different from those of WS and EP. These results suggested that proteins originating from non-glandular sources may contribute more significantly to WS and EP than previously recognized or that proteins may have undergone extensive proteolysis, cross-linking, and other modifications (54, 55). Some interesting contributors to EP are members of the cytokeratin family, cytokeratin 13 and 15 (56), pointing to oral epithelium as one of the sources of proteins deposited on the tooth surface. The cytokeratins identified in EP were distinct from those normally found in skin keratinocytes or hair (e.g., cytokeratin 9 or cuticular keratin), excluding the possibility that the proteins identified stem from sample contamination. Another novel component found in EP was calgranulin B, which has been previously identified in WS and was shown to be a component of gingival crevicular fluid (37). The calgranulin family contains a calcium-binding domain possibly involved in enamel deposition.

This study also revealed that the protein/peptide components of WS differed markedly from those of EP. This suggests that some EP constituents may derive directly from glandular secretions and other oral sources and that, contrary to expectations, the formation of EP is not totally dependent on protein modification occurring in WS. Comparison of WS and EP confirms the previously held notion that pellicle formation is dictated by a selective protein/peptide absorption process (5) and that the presence of phosphoproteins histatin 1 and statherin (2, 57) is consistent with the ionic interaction between proteins and enamel surfaces.

It is obvious that the identifications made in this study represent only a fraction of major pellicle components. Nevertheless, the current investigation showed the variety of novel as well as expected components in WS and EP, which are both extremely important to oral homeostasis. The use of proteomic technology overcame a number of limitations imposed by class proteins isolation and characterization methods. The results obtained open up a new avenue to directly characterizing EP, which should ultimately lead to an understanding of its three-dimensional structure and true functions.

Acknowledgments—We thank ThermoBioanalysis Corp. and Applied Biosystems, Inc. for the loan of the Vision and QStar™, respectively.

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