Protein Secretions of the Parotid Gland: A Comparison of Stimulated and Unstimulated Flow

M.S. Candidate: Aaron M. Bailey, DMD

A Thesis submitted to the Department of Orthodontics
and
The Advanced Education Committee of the
Oregon Health & Science University
School of Dentistry
In partial fulfillment of the requirements
For the degree of
Master of Science

December 2006

Protein Secretions of the Parotid Gland:
A Comparison of
Stimulated and Unstimulated Flow

A thesis presented by Aaron M. Bailey, D.M.D.
In partial fulfillment of the requirements for the degree of Master of Science

December 2006

Larry L. David, Ph.D.
Adjunct Professor
Department of Oral Molecular Biology

David A. Covell, Jr., Ph.D., D.D.S
Associate Professor, Chair
Department of Orthodontics

Tom R. Shearer, Ph.D.
Associate Dean
Integrative Biosciences

Phillip A. Wilmarth, Ph.D.
Research Assistant Professor
Department of Oral Molecular Biology

Acknowledgements

Thank you, Dr. Larry David, for inspiring this study and allowing me to work with such a great group of individuals in your lab. You made the entire process very enjoyable through your easy going, patient personality.

Thank you, Dr. Tom Shearer, for your sound advice, in regard to both my proposal and final thesis. You have an amazing ability to get down to the truly relevant. Thanks for helping me to narrow down the focus of my thesis and not get carried away with my conclusions.

Thank you, Dr. David Covell, for guiding and directing me over the last couple of years. I appreciate your efforts to keep me always aware of the tasks ahead of me. Thanks for all of the research meetings that kept me on track, and for your feedback on this project.

Thank you, Dr. Phil Wilmarth. You have been a complete godsend. I could have never survived this project without the knowledge you were so willing to share. Thank you for teaching me everything I know about proteomics. I appreciate the many hours that you dedicated to this project. Thanks for being such a great teacher and for helping to make the many necessary revisions that made my thesis defendable.

Thank you, Mike Riviere and Lucinda Robertson, for helping with the saliva processing. I know you both often rearranged your schedules to make sure this project could be completed. Thanks, Mike, for taking the time to teach me about saliva processing.

Thank you, Dr. Larry Doyle, for supporting me at my thesis defense. You gave me strength through your presence. Thank you for all of your research follow-up conversations, which helped me to keep pressing forward.

Thank you, Dr. Chad Carver, for helping with saliva collection. You helped me to stay focused when my mind was on other things. I will always be grateful for my thesis defense practice run.

Thank you, Dr. Laura Nichols and Dr. Bart Carter, for your friendship and example. I cannot imagine making it through this process without you.

Thank you, Kimmie and kids, for your love and motivation.

Table of Contents

List of Figures

List of Tables

Abstract

The departments of Integrative Biosciences and Periodontology at OHSU School of Dentistry recently investigated the human whole saliva proteome using 2-DLC (Wilmarth et al., 2004). The study was able to identify, with high confidence, most known salivary proteins, a large number of common serum proteins, and three previously undiscovered proteins. Significantly greater numbers of identified proteins, including high molecular weight, low molecular weight, and proline-rich proteins, were found with 2-DLC compared to previously reported 2-DE studies.

In contrast to whole saliva (which contains a conglomeration of secretions from all salivary glands and contaminants from gingival fluid, bacteria, and food), parotid gland saliva can be readily isolated and collected using a Lashley cup (Lashley, 1916) or Carlson-Crittenden device (Carlson and Crittenden, 1910). This ease in collecting uncontaminated saliva as well as the ability to drastically increase parotid flow rate upon stimulation make this gland a prime candidate for study using proteomic techniques to determine the parotid proteome, measure changes in protein composition, and potentially identify protein biomarkers.

Previous investigations of the parotid proteome have used the 2-DE technique. These studies discovered 16 (Hardt et al., 2005) and 12 (Walz et al., 2006) proteins. A few of the parotid proteins (proline-rich proteins, histatin, and statherin) appeared to undergo extensive proteolytic processing (Hardt et al., 2005). The limited sensitivities of the 2-DE studies, however, did not allow for further characterization of the parotid proteome.

In this investigation, parotid gland saliva was collected from six Caucasian males (ages 28 to 34 years) during unstimulated saliva flow and during stimulated (citric acid) saliva flow. Each sample was digested with trypsin, and the peptides were separated using two-dimensional liquid chromatography (strong cation exchange/reverse phase). A quadrapole ion trap mass spectrometer was used to perform tandem mass spectrometry to identify peptides and proteins. A total of 49 parotid proteins were identified from the 504,402 MS/MS spectra with rigorous identification criteria. For positive identification, proteins were required to have least two distinct peptides, and be present in a minimum of two biological subjects. Counts of MS/MS spectra were used to provide relative protein abundance estimates, and unstimulated flow was compared to stimulated flow. The results of this study increased the number of parotid proteins by a factor of 3 to 4 over previous two-dimensional electrophoresis studies. The relative abundances of 37 proteins were compared and no statistically significant differences between saliva flow states were observed. This study establishes a parotid proteome for healthy, young males for future use in studies of parotid gland aging and dysfunction. The similar protein composition between unstimulated and stimulated flow implies that stimulated saliva flow can be used in future studies, greatly simplifying parotid saliva collection.

Background and Significance:

Saliva

Saliva origin and composition

Human saliva is a dilute aqueous solution of electrolytes, minerals, buffers, and proteins. Saliva is produced by three pairs of major salivary glands and several minor mucous and serous salivary glands located in specific areas of the oral cavity. The large major salivary glands are paired and include the parotid glands, the submandibular glands, and the sublingual glands. The minor salivary glands include the glands of the tongue (anterior and posterior lingual glands), the labial glands, the buccal glands, the molar glands, the incisive glands, and the palatine glands (Saracco and Crabill, 1993).

Salivary glands consist of myoepithelial cells surrounding serous or mucous acinar cells that produce and secrete isotonic primary saliva. As saliva first passes through the intercalated duct cells and then the striated duct cells, sodium is removed, and the excretory duct cells deliver hypotonic saliva to the oral cavity. Parotid glands produce serous secretions, minor glands produce mucous secretions, and the submandibular and sublingual glands secrete both serous and mucous saliva (Roth and Calmes, 1981).

Saliva is typically more than 99% water with a normal pH of 6 to 7. Saliva contains a variety of electrolytes (sodium, potassium, calcium, magnesium, bicarbonate, and phosphate), proteins, immunoglobulins, enzymes, mucins, and other molecules like

urea and ammonia (Humphrey and Williamson, 2001). These components act together to provide a variety of important functions discussed below.

Saliva functions

Saliva moistens the oral tissues and coats delicate surfaces to protect against a variety of irritants. Mucins provide the main source of lubrication, and aid speech, mastication, and swallowing. Saliva is an aqueous solvent necessary for taste, and acts as a masticatory wetting agent to facilitate bolus formation and swallowing. Certain salivary glands secrete enzymes, such as amylase, that initiate the digestive process.

Saliva serves as the first line of defense against bacterial and viral attack within the oral cavity, and many salivary components are involved. The highly glycosylated mucin MG1 is recruited in enamel pellicle formation to protect teeth. Other glycoproteins, such as mucin MG2 and salivary agglutinin, bind bacteria and assist oral cavity clearance. Secretory IgA is the predominant immunoglobulin in saliva, but IgM and IgG are also present. Lactoferrin, lyzozymes, peroxidase, and cystatin proteins all have antibacterial functions. Peptides produced from histatins also have potent antibacterial and anti-fungal activities (Amerongen and Veerman, 2002; Hardt et al., 2005).

Saliva has many specialized functions to protect teeth, which are the only exposed mineralized tissue in the body. Saliva neutralizes and buffers acids, contributes to enamel pellicle formation (Vitorino et al., 2006), and maintains a supersaturated solution of calcium phosphate (Pedersen et al., 2002; Amerongen and Veerman, 2002). The multiple functions of many salivary components are summarized in Figure 1.

Figure 1. Major saliva components and their functional roles in the oral cavity.

Normal and stimulated salivary flow

The average daily flow of whole saliva for a healthy individual varies between 1.0 and 1.5 L. Approximately 60 to 70% is secreted by the submandibular glands, 25 to 35% by the parotid glands, and 5% or less by the sublingual glands. The minor salivary glands contribute approximately 3 to 8% of the total daily production (Hand, 1986). These relative glandular contributions change drastically during stimulated saliva flow, with the parotid contributing more than 50% of total salivary secretions (Edgar, 1990).

Many factors cause either increased or decreased salivary flow rates. Three main stimuli that increase saliva flow are mechanical (the act of chewing), gustatory (with acid the most stimulating trigger and sweet the least stimulating), and olfactory (a relatively poor stimulus). Other factors that can affect saliva flow include psychic factors such as

pain, certain types of medication, and various local or systemic diseases affecting the glands themselves (Roth and Calmes, 1981; Shannon 1972, Grant et al., 1988).

Individual whole salivary flow rates range from unstimulated average flow rates of 0.3 mL/min to as much as 7 mL/min upon stimulation (Edgar 1990; Grant et al., 1988). The usual, unstimulated rate of parotid secretion is about 0.04 ml/min per gland, but drastically increases to a flow rate of 0.7 ml/min per gland upon gustatory stimulation (Mandel, 1972).

The composition of saliva is dependent upon flow rate. On stimulation, the concentrations of sodium, chloride, bicarbonate, and calcium increase with increased flow rate; magnesium, phosphate, urea, ammonia, and uric acid decrease; and pH increases. Overall protein content also increases proportionally with increasing flow rate (Mandel, 1980).

Saliva as a diagnostic fluid

Various body fluids are potentially rich sources of diagnostic markers (Villanueva et al., 2004), and protein biomarkers could allow early detection of many diseases (Alaiya et al., 2005; Jacobs et al., 2005; Marko-Varga et al., 2005; Vitzthum et al., 2005). The majority of the proteomic studies looking for biomarkers have analyzed tissue biopsies and serum samples (Omenn et al., 2005). However, saliva offers several potential benefits as a diagnostic fluid (Streckfus and Bigler, 2002): less invasive collection compared to serum, low risk for health workers, low collection cost, easier storage and transport, and large population screenings in developing nations. Proteomic studies of whole saliva, similar to work characterizing serum and plasma, have been recently

reported by several researchers (Yao et al., 2003; Ghafouri et al., 2003; Vitorino et al., 2004; Hu et al., 2005; Huang, 2004; Wilmarth et al., 2004; Xie et al., 2005; and Guo et al., 2006).

The Parotid Gland

Location and size

The parotid gland is the largest of the salivary glands (see Figure 2). The gland is irregular in shape, but roughly resembles an inverted pyramid. Its weight in the adult varies from 15 to 30 g, and it is approximately 6 cm in length (superior to inferior), and 3 to 4 cm in width. It is located on the side of the face in the retromandibular fossa. More specifically, the "bed" or recess which the gland occupies is located anterior and inferior to the external acoustic meatus, inferior to the zygomatic arch, posterior to the ramus of the mandible and the masseter muscle, anterior to the mastoid process and the superior part of the sternocleidomastoid muscle, and lateral to the styloid process. The gland presents three surfaces (lateral, anteromedial, and posteromedial), four borders (superior, anterior, posterior, and medial), and an apex (Saracco and Crabill, 1993).

The parotid glands are supplied with arterial blood via glandular branches from the external carotid, posterior auricular, superficial temporal, transverse facial, and maxillary arteries. The veins, which drain the gland, empty chiefly into the retromandibular vein. The lymphatic vessels of the gland drain primarily to the deep parotid nodes embedded within the gland and to the superficial and deep cervical nodes (Saracco and Crabill, 1993).

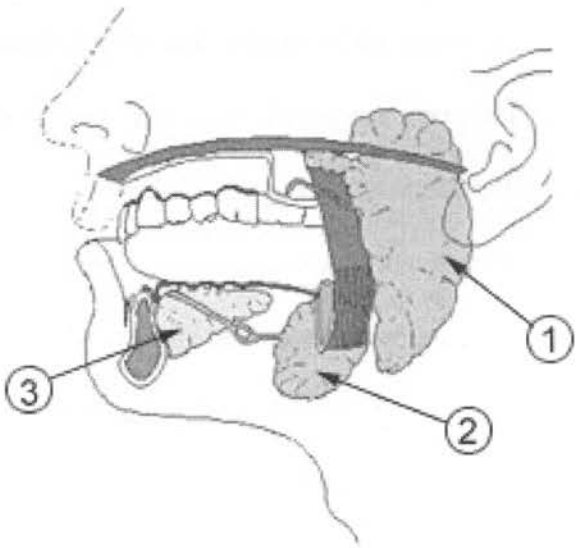

Figure 2. Location and shape of the parotid gland (1) in relation to the submandibular (2) and sublingual (3) glands. (Public domain image from http://upload.wikimedia.org/wikipedia/commons/5/51/Illu_quiz_hn_02.jpg)

Innervation of the parotid gland

The inferior salivatory nucleus of the brain stem is the parasympathetic nucleus concerned with parotid gland secretion. Visceral efferent fibers emanating from the inferior salivatory nucleus are associated with cranial nerve IX. Preganglionic parasympathetic fibers from the inferior salivatory nucleus, after joining the glossopharyngeal nerve, accompany this nerve's tympanic branch onto the medial wall of the middle ear, where they contribute to the formation of the tympanic plexus. The fibers leave the tympanic plexus as components of the lesser petrosal nerve, which passes to the otic ganglion for synapse with postganglionic parasympathetic neurons. These postganglionic neurons leave the ganglion to immediately join the undivided portion of the mandibular division of the trigeminal nerve. The postganglionic secretomotor fibers

are then distributed to the parotid gland by way of the auriculotemporal nerve (Saracco and Crabill, 1993).

The cell bodies of the preganglionic sympathetic neurons associated with the salivary glands are located in the intermediolateral cell column of the upper thoracic portion of the spinal cord. Fibers of these neurons pass to the superior cervical sympathetic ganglion where they then synapse with all the postganglionic neurons that travel to the salivary glands, both major and minor. Postganglionic sympathetic fibers to the parotid, and all salivary glands except the palatine glands, are derived from the external carotid plexus on the external carotid artery. Sympathetic fibers are then carried to the parotid gland by a secondary plexus associated with a branch of the external carotid artery which supplies the gland (Saracco and Crabill, 1993).

Formation of parotid saliva

The working parts of the parotid consist of the secretory end pieces, termed acini, and the branched ductal system. The cells in the acini of the parotid gland are arranged in a roughly spherical form surrounding a lumen, which is the start of the ductal system. Myoepithelial cells surround the acini and assist in propelling the secretion from the lumen into the intercalated ducts, which have low cuboidal epithelium and a narrow lumen. The salivary secretion continues through the striated ducts, which are lined by more columnar cells with many mitochondria, and finally passes through the excretory duct. Cuboidal cells are predominant within the excretory duct except toward the terminal part, which is lined with stratified squamous epithelium (Edgar et al., 2004).

Fluid formation in parotid glands occurs in the acini where serous cells produce a watery seromucous secretion. These secretions arise by the formation of interstitial fluid from blood in capillaries, which is then modified by the acinar cells. This isotonic plasma-like fluid is secreted into the lumen before passing through the ductal system where it is further modified. Most of the modification, through ion exchange, takes place in the striated ducts, where the fluid changes from an isotonic to a hypotonic solution. The composition is further modified (sodium and chloride reabsorbed, potassium and bicarbonate secreted) within the excretory ducts before it is finally secreted into the mouth (Edgar et al., 2004).

Parotid acinar cells are highly differentiated and polarized cells that are committed to the production of proteins for exocrine secretion. More than 90% of the protein synthetic activity of salivary acinar cells is devoted to the production of secretory proteins (Castle, 1993). The majority of polypeptides undergoing intracellular transport in salivary acinar cells are destined for storage granules that are characteristically released in response to secretory stimulation (Castle et al., 1972; Zastrow and Castle, 1987). Studies indicate that although parotid acinar cells are highly specialized for the production of storage granules for stimulus-dependent discharge, they continuously secrete the same proteins at lower levels in the absence of stimulation (Zastrow et al., 1987; Iversen et al., 1985).

Parotid proteome

Recently, parotid gland saliva has been analyzed using two-dimensional gel electrophoresis (2-DE) and mass spectrometry to characterize the parotid proteome and to

measure diurnal variations (Hardt et al., 2005). A 2-DE comparison of whole saliva to parotid and to submandibular/sublingual secretions (Walz et al., 2005) has been published. Most recently, whole saliva and parotid saliva changes in response to taste stimulation have been reported (Neyraud et al., 2006) also using 2-DE. There are advantages and disadvantages to 2-DE proteomic techniques. They have the largest protein resolving power of any available separation technique and can provide information about modifications and proteolysis. They allow semi-quantitative information to be obtained and provide an inherent visual summary of the results. However, among the limitations, are the difficulty of observing large or small molecular weight proteins, highly acidic or highly basic proteins, and insoluble hydrophobic proteins. Also, current staining methods have a limited dynamic range of about 3-4 orders of magnitude.

Protein quantification

Antibody labeling

Among the more powerful techniques for quantifying proteins are those that utilize antibody labeling. The precise antigen specificity of antibodies makes them powerful identification tools (Wilson and Matsudaira, 1993). Labeled with fluorescent dyes, they can be used to detect and quantify molecules in cell extracts and to identify specific proteins after they have been fractionated by electrophoresis in polyacrylamide gels (Harlow and Lane, 1988). The intensity of each band fluorescence or labeling is an indicator of protein abundance.

The sensitivity of antibodies as probes for detecting and assaying specific molecules is frequently enhanced by signal-amplification (Wilson and Matsudaira, 1993). A marker molecule such as a fluorescent dye can be linked directly to an antibody used for specific recognition (the primary antibody), however, a stronger signal is achieved by using an unlabeled primary antibody and then detecting it with a group of labeled secondary antibodies that bind to it.

A more sensitive and versatile amplification method, termed enzyme-linked immunosorbent assays (ELISA), uses an enzyme as a marker molecule attached to the secondary antibody (Anderton and Thorpe, 1980). For example, the enzyme alkaline phosphatase, for example, in the presence of appropriate chemicals, produces inorganic phosphate and leads to the local formation of a colored precipitate. This detects the secondary antibody that is coupled to the enzyme and hence the location of the antibody-antigen complex to which the secondary antibody is bound. Since each enzyme molecule acts catalytically to generate many thousands of molecules of product, even small amounts of antigen can be identified.

However, antibody specificity and the necessity to manufacture antibodies for each candidate protein are major shortcomings of ELISA assays. Antibodies are made by injecting a sample of the antigen several times into an animal such as a rabbit or a goat and then collecting the antibody-rich serum, called antiserum (Alberts et al., 1994). Thus, this technique not only requires much time and effort in creating the labeling antibodies but also requires a fore-knowledge of which proteins to identify. This need to know candidate proteins beforehand makes discovery of new or unexpected proteins difficult

unless an inefficient and impractical shotgun approach to antibody generation is employed.

2-DE densitometry

2-DE studies provide semi-quantitative information based upon the staining intensity of separated protein spots. Optical densitometry is used for visible stains like Coomassie, and newer stains use fluorescent dyes. There are many pitfalls to 2-DE, as mentioned above. However, a major issue for salivary studies is the identification of the unusual sequences in major salivary proteins, such as the proline-rich proteins, statherin, and histatins. These proteins may not stain as efficiently as more typical protein sequences and could result in inaccurate quantification.

Spectral Counting

Recently, a new technique called spectral counting allows abundance estimates to be obtained from two-dimensional liquid chromatography/mass spectrometry (2D-LC/MS) data that was previously used only for protein identification. High abundance proteins produce a larger number of tryptic peptides, and the number of MS/MS spectra assigned to a protein is directly related to its abundance in the sample (Liu et al., 2004). This allows abundance estimates to be made directly from 2D-LC/MS data and allows samples to be compared for protein expression changes. Several recent publications have shown that spectral counting can be used to estimate relative protein abundances (Colinge et al., 2005; Gao et al., 2005; Liu et al., 2004). The method is, in some ways, analogous to flow cytometry. In this analogy, a tryptic digest and 2-DLC are used to flow the cells

(the peptides) single file past the detector (the mass spectrometer). The detector is able to identify the proteins. Counting of the cells (the peptides) from the different cell types (the proteins) gives a quantitative measure of the sample composition.

Materials and Methods:

Saliva Sample Collection

Samples were collected with informed consent from six individuals, who were required to meet a strict set of exclusion criteria. All subjects were Caucasian males between the ages of 27 and 34 years of age, currently taking no medication of any kind (prescription, over the counter, or herbal), and were to have no active periodontal disease (assessed by dental history and full periodontal probing).

Parotid gland saliva was collected using a Lashley cup (Stone Machine Company, Colton, CA), consisting of an outer suction rim and an inner collection well. The outer rim is attached via tubing to a syringe, which creates the necessary suction to the buccal mucosa and maintains the position of the Lashley cup. The collection well of the Lashley cup was positioned to completely cover the parotid duct, thus allowing the saliva to pool within the collection well and passively drain through tubing into a collection vial. In this manner, the Lashley cup directed parotid saliva outside the oral cavity and eliminated contamination from other saliva glands and gingival fluid.

Stimulated and unstimulated parotid saliva samples of approximately 1 mL and 1.5 mL, respectively, were collected from each individual at mid-morning (10 a.m.), between May 8, 2006 and May 19, 2006 to minimize diurnal variation. Volunteers were instructed to not eat, brush teeth, or drink any fluids other than water during the two hours proceeding collection. The first sample was collected without stimulation and the second with one drop of 2% citric acid solution (Walz et al., 2005) placed on the dorsum of the tongue immediately prior to collection. The first 0.5 ml of stimulated parotid

saliva was discarded in order to eliminate contamination from any unstimulated saliva remaining in the tubing. Collection times averaged an estimated 15 minutes for the stimulated flow state and 50 minutes for unstimulated flow. All collected samples were kept on ice before processing.

Sample Processing

Immediately after collection and temporary storage on ice, saliva was centrifuged at 20,000 x g for 15 min at 4° C. The supernatant was pipetted off and the pellet discarded to remove cellular debris and epithelial contaminants. The final retentate volume and protein concentration was measured by performing a Bicinchoninic Acid (BCA) protein assay (Pierce Biotechnology, Inc. Rockville, IL) in triplicate. Aliquots of 1 mg of protein were lyophilized and stored at -80° C until use.

The protein samples were dissolved in 100 µl of digestion buffer, containing: 8 M electrophoresis grade urea (in deionized water), 0.8 M Tris, 0.08 M methylamine, and 8 mM $CaCl_2$. The sample was reduced by the addition of 12.5 µl of 0.9 M DTT and incubation at 50° C for 15 minutes. Cysteine alkylation was performed by the addition of 12.5 µl 1.0 M iodoacetamide (IAA) solution and incubation in the dark for 30 min. An additional 12.5 µl of DTT solution was added to scavenge any remaining IAA before adding 210 µl of water. Ten µl of the solution was then removed and analyzed by SDS-PAGE to provide a pre-digestion assay. Forty µl of 1 µg/µl trypsin gold (1:25 ratio of enzyme to substrate), which had previously been prepared by dissolving a 100 µg vial of trypsin gold (ProMega) in 100 µl of 1mM HCl, was then added. The solution was vortexed, centrifuged, and incubated at 37 ° C for approximately 16 hours. Ten µl of the

digested solution was removed, analyzed by SDS-PAGE, and compared to the pre-digestion assay to determine the extent of digestion. 20 µl of neat 88% formic acid was added to terminate digestion.

A peptide clean up step was performed on the digest using a Sep Pak (Waters/Millipore part #20515) cartridge with a C-18 Plus column to remove the salts and reagents remaining from the previous steps.

Strong Cation Exchange (1st dimension of separation)

Strong cation exchange (SCX) separates peptides based on charge. The SCX was a 100 x 2.1 mm column of polysulfoethyl A (The Nest Group, Inc. Southborough, MA, USA). The mobile phase A was 10mM sodium phosphate (pH 3.0) and 25% acetonitrile. Mobile phase B was identical except that it contained 350 mM KCl. After 5 minutes to load and wash, the gradient was a 45 min linear gradient from 0% to 50% B, then a 20 min gradient from 50% to 100% B. One minute fractions were collected.

Fractions were then combined according to peptide quantity, as determined from the UV tracing. Fractions with low quantity of peptide were combined every 4 minutes, while the remaining fractions were combined every 2 minutes. By combining fractions, the number of samples was lowered from 80 to about 34. The samples were lyophilized and reconstituted in 100 µl of 5% formic acid.

Reverse Phase Liquid Chromatography (2nd dimension of separation)

All SCX fractions of parotid saliva digests were analyzed by LC/MS using an Agilent 1100 series capillary LC system and an LCQ Classic ion trap mass spectrometer

(ThermoFinnigan, San Jose, CA, USA). Samples (roughly one quarter of the SCX fractions) were applied at 20 µl/min to a trap cartridge (Michrom Bioresources, Inc. Auburn, CA), and then switched onto a 0.5 X 250 mm Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA) using a mobile phase containing 0.2% acetic acid. The gradient was 7-35% acetonitrile over 90 minutes at a 10 µl/min flow rate.

Mass Spectrometry

A quadrupole ion trap was configured to automatically obtain tandem mass spectra of peptides as they eluted from the reverse phase column. Data dependent collection of MS/MS spectra used the dynamic exclusion feature of the instrument control software (exclusion mass width, 3.0 Daltons; repeat count, 1; exclusion list size, 25 ions; and exclusion duration, 3 minutes) to obtain MS/MS spectra of the three most abundant ions following each centroided survey scan. The dynamic exclusion procedure limited analysis of abundant peptides, allowing a greater number of minor components to be analyzed. DTA files were generated from MS/MS spectra using extract msn software (ThermoFinnigan) with a molecular weight range of 400 - 4000 Daltons, a minimum of 25 ions and a low TIC threshold of 500. Charge state analysis (ZSA) was performed following DTA creation, and each 2-DLC run produced about 25,000 to 30,000 spectra.

Protein Identification

The half-a-million tandem mass spectra were searched with SEQUEST (Eng at al., 1994) and X! Tandem (Craig and Beavis, 2003) to identify peptides and proteins present in the parotid saliva samples. Previous mass spectrometry studies of saliva have

demonstrated that there are significant numbers of partially tryptic peptides and that faster fully-tryptic peptide searches may not identify all proteins (Wilmarth et al., 2004). "No enzyme specificity" searches of a SwissProt human-only protein database (with sequence-reversed entries and common contaminants appended to the database) were performed in both search programs. The relevant parameters used in each search program are listed in Table 1.

Table 1: Parameters used in SEQUEST and X! Tandem searches.

Parameter	SEQUEST[a]	X! Tandem[b]
parent ion mass tolerance	2.5 Da	-1/+3 Da
parent ion masses	average	monoisotopic
fragment ion tolerance	default	0.4 Da
fragment ion masses	monoisotopic	monoisotopic
enzymatic cleavage specificity	none	none
static modifications	+57.02@C	+57.02@C
variable modifications	none	-17@E, +80@S
no. of database entries	12077[c]	12077[c]

[a]Version 2.7
[b]Version 2006.4.1.2
[c]Number of forward entries. An equal number of sequence-reversed entries were also present

To generate a minimally redundant list of the fewest possible proteins capable of explaining all of the observed peptides, Scaffold (Proteome Software, Inc., Portland, OR) analyses of the search results were performed. Scaffold implements and extends probabilistic algorithms to identify peptides (Keller et al., 2002) and proteins (Nesvizhskii et al., 2003) in complex mixtures. It statistically combines results from multiple search programs and allows identifications across multiple samples to be aligned.

The criteria used for protein identification were peptide probability greater than 0.90, a minimum of two peptides per protein in at least two of the samples, and a protein probability of 0.95. To reduce the possibility of false positive protein assignment, reported proteins were also required to be identified in at least two different biological subjects.

Spectral Counting

The number of MS/MS spectra assigned to a protein is directly related to abundance (Liu et al., 2004). To reliably identify minor proteins (those having peptides less than 2 or 3), strict peptide and protein probabilities were necessary because the confidence of correct protein identification hinged on the quality of the peptide evidence. However, to make quantitative comparisons, only proteins having spectral counts greater than 4 or 5, and, therefore, sufficiently strong evidence of correct identification, can be used. For this reason, spectral counts were tallied with a peptide probability lowered to 0.80 rather than the 0.90 used for identification purposes, increasing the spectral counts accordingly. These slightly higher spectral counts improved the statistical testing and comparisons.

Using DTASelect, proteins with shared peptides were identified and spectral counts combined accordingly. Spectral counts for immunoglobulins; PRP1 and PRP2; and Cystatins N, T, and S were combined without double counting of the shared peptides. Only one representative pair of samples from subject 1 was used so that counts could be compared between six equivalent biological subjects.

The spectral counts for each protein identified in each of the six unstimulated and stimulated samples were exported from Scaffold into Excel (Microsoft Corp., Redmond, WA) and compared to determine changes in protein abundance. Several statistical tests were used to assess differences between the two types of parotid saliva and details are given in the Results section: Semi-quantitative abundance estimates using spectral counting.

For each 2-DLC run, the total number of peptides identified varied from 1059 to 3085. Comparisons of spectral counts between samples required normalization as do many statistical tests. The spectral counts of each protein in a given sample were adjusted to a common scale based upon the total number of peptides identified in each sample. The total peptide number did not include any peptides matched to common contaminants (trypsin, BSA, lens proteins). This normalization was designed to mimic the normalization schemes used in 2-DE studies where the sum total volume of all valid spots is used to correct gel-to-gel variation.

In each 2-DLC run, the same amount of protein was used. Therefore any change in relative abundance of amylase between unstimulated and stimulated flow could potentially influence the abundance of all other proteins accordingly. While calculations involving amylase used spectral counts normalized with all proteins present, calculations involving lower abundance proteins were based on normalization with amylase excluded from the counts. This strategy reduces the chance that differences in minor components occurred as artifacts due to changes in amylase abundance.

Data Replication

Sufficient saliva was collected to allow for four repeated mass spectrometry analyses for a single individual (both unstimulated and stimulated). These data were used to test reliability and consistency of the methods utilized. This was performed to increase confidence that any observed differences between the samples were indeed differences between glandular states and/or individuals and not due to processing or analysis errors.

Statistical Analysis

The spectral count data from the two saliva flow states of the 6 subjects were analyzed with several statistical tests to identify any relative protein abundance differences. A recent publication evaluated several statistical tests to assess sample differences using spectral counts (Zhang et al., 2006) and found the best results utilizing a Student's t-test for experiments with more than 4 replicates, or a G test for samples with fewer replicates.

Since the samples from the 6 subjects were collected pre- and post-stimulation with citric acid, a paired t-test seemed appropriate provided the spectral counts for each protein were normally distributed. Several of the proteins, however, failed standard normal distribution tests. Therefore, three non-parametric tests were also tried: a chi square test with one degree of freedom, a G test as detailed in (Zhang et al., 2006), and a Kolmogorov-Smirnov test (http://www.physics.csbsju.edu/stats/KS-test.html). The spectral counts were normalized for the t-Test using the two different methods discussed above. When testing amylase counts, the normalization included all proteins. For all of

the non-amylase proteins, the normalization factors excluded counts from amylase. Only the t-Test and Kolmogorov-Smirnov test used normalized data.

Spectral counts of the four replicate runs (of the SCX fractions from one subject) were used to evaluate the technical variance in the spectral counting technique. Spectral counts were normalized as described above, and per protein averages and sample standard deviations calculated. The coefficients of variance (100*STDEV/AVERAGE) from the technical replicates were compared to similar quantities from the biological replicates. Only stimulated parotid saliva was considered when making these comparisons. Any proteins having average spectral counts less than 10 were not included in the calculations.

Results:

In this investigation, parotid gland saliva was collected from six Caucasian males (ages 28 to 34 years) during unstimulated saliva flow and during stimulated (citric acid) saliva flow. Each sample was digested with trypsin, and the peptides were separated using two-dimensional liquid chromatography (strong cation exchange/reverse phase). A quadrapole ion trap mass spectrometer was used to perform tandem mass spectrometry to identify peptides and proteins. A total of 49 parotid proteins were identified from the 504,402 MS/MS spectra. Table 2 summarizes the samples that were collected and processed as part of this investigation.

Table 2: Summary of subjects and collected samples.

Subject[a]	Age	Parotid Saliva Flow	Protein Concentration (mg/ml)	No. of MS/MS spectra
1.1	30y 10mo	unstim	1.77	28,430
		stim	0.75	29,234
2	34y 7mo	unstim	1.5	28,239
		stim	1.15	30,416
3	31y 11mo	unstim	2.01	32,999
		stim	1.53	29,933
4	30y 2mo	unstim	6.75	31,349
		stim	1.9	28,674
5	27y 11mo	unstim	2.9	27,874
		stim	2.1	27,854
6	30y 2mo	unstim	2.2	28,631
		stim	1.4	28,723
1.2	30y 10mo	unstim	1.77	23,008
		stim	0.75	26,498
1.3	30y 10mo	unstim	1.77	22,418
		stim	0.75	24,676
1.4	30y 10mo	unstim	1.77	27,765
		stim	0.75	27,681
			grand total	504,402

[a]Four technical replicates were performed on one subject.

The final list of 49 proteins identified in the 12 samples is given in Table 3. Any matches to common contaminants, such as trypsin autolysis products or BSA, have been excluded. The proteins identified in two previous studies (Hardt et al., 2005; Walz et al., 2006) using 2-DE are denoted in the last column. All proteins reported in those studies were observed here. Immunoglobulins have several distinct protein entries in databases and can inflate the number of identified proteins; thus, the major immunoglobulin subunits are listed singly in Table 3.

Table 3. 49 parotid gland salivary proteins identified in at least two different biological subjects. 39 proteins (in bold) were observed in at least 4 of the 6 subjects in both salivas.

Protein Name[a]	SwissProt Accession Number	MW (kDa)	Distinct Peptide Count	Sequence Coverage (%)	Which Parotid Saliva	Previous Study[b]
Salivary alpha-amylase (AMYS)	P04745	58	145	88	Both	H, W
Secretory component (PIGR)	P01833	83	53	60	Both	H, W
Ig alpha-1 chain C (IGHA1)	P01876	38	34	74	Both	H
Serum albumin (ALBU)	P02768	69	48	68	Both	H, W
Lactotransferrin (TRFL)	P02788	78	35	55	Both	
Ig kappa chain C (KAC, KVxx)	P01834	12	17	87	Both	H, W
Proline-rich protein 3 (PROL3)	P02814	8	21	70	Both	
Ig lambda chain C (LAC, LVxx)	P01842	11	16	94	Both	
Basic salivary PRP 1	P04280	39	55	78	Both	W
Zinc-alpha-2-glycoprotein (ZA2G)	P25311	34	20	65	Both	H, W
Acidic salivary PRP ½ (PRP1, PRB2)	P02810	17	25	90	Both	H, W
Ig alpha-2 chain C (IGHA2)	P01877	37	23	70	Both	H, W
Lysozyme C (LYSC)	P61626	17	17	68	Both	H
Salivary agglutinin	Q9UGM3	261	22	15	Both	
Immunoglobulin J chain (IGJ)	P01591	16	12	51	Both	
Lactoperoxidase (PERL)	P22079	80	18	41	Both	W
Carbonic anhydrase VI (CAH6)	P23280	35	27	48	Both	H, W
Parotid salivary glycoprotein G1	Q04118	31	13	15	Both	H, W
Basic salivary PRP 2	P02812	37	56	64	Both	
Statherin (STAT)	P02808	7	12	53	Both	
Basic salivary PRP 4 allele S (PRB4S)	P10163	25	11	33	Both	
Prolactin-inducible protein (PIP)	P12273	17	9	67	Both	H
Cystatin D (CYTD)	P07339	16	9	69	Both	H
Antileukoproteinase 1 (ALK1)	P03973	14	7	69	Both	
WAP 4-disulfide core domain prot. 2 (WFDC2)	Q14508	13	4	53	Both	
Parotid secretory protein	Q96DR5	27	8	43	Both	
Kallikrein-1 (KLK1)	P06870	29	6	34	Both	
Histatin-1 (HIS1)	P15515	7	5	44	Both	H
Clusterin (CLUS)	P10909	52	5	19	Both	
Cystatin C (CYTC)	P01034	16	6	45	Both	
Ig mu chain C	P01871	50	14	39	Both	
Similar to common salivary protein 1	Q96DA0	19	5	45	Both	
Beta-2-microglobulin (B2MG)	P61769	14	3	30	Both	
Transcobalamin-1	P20061	48	4	16	Both	
Neutrophil gelatinase-assoc. lipocalin (NGAL)	P80188	23	3	25	Both	
Nucleobindin-2	P80303	50	3	12	Unstim.	
Hypothetical protein	Q6MZM9	23	3	25	Both	
Cystatin SA (CYTT)	P09228	16	13	66	Both	H
Salivary proline-rich protein Po (PRB4M, PRB4L)	P10161, 2	28	3	34	Both	
Cystatin B	P04080	11	5	74	Unstim.	H
Galectin-3-binding protein (LG3BP)	Q08380	65	3	12	Both	
Alpha-1-antitrypsin	P01009	47	4	17	Unstim.	
Ig heavy chain variable regions (HVxx)	HVxx_	12	2	17	Both	
Cathepsin D	P07339	45	2	9	Both	
Cystatin S (CYTS)	P01036	16	3	49	Both	
Cystatin SN (CYTN)	P01037	16	11	70	Both	W
Monocyte differentiation antigen CD14	P08571	40	2	14	Both	
Folate receptor alpha	P15328	30	2	12	Unstim.	
Uteroglobulin-related protein 2	Q96QR1	10	3	32	Both	

[a]Proteins listed in decreasing order of overall identification probability, as calculated in Scaffold

[b]H is (Hardt et al., 2005) and W is (Walz et al., 2006)

The normalized and averaged spectral counts for 37 proteins (having more than 5 spectral counts) from the 6 biological subjects during unstimulated and stimulated parotid saliva flow are shown in Figure 3.

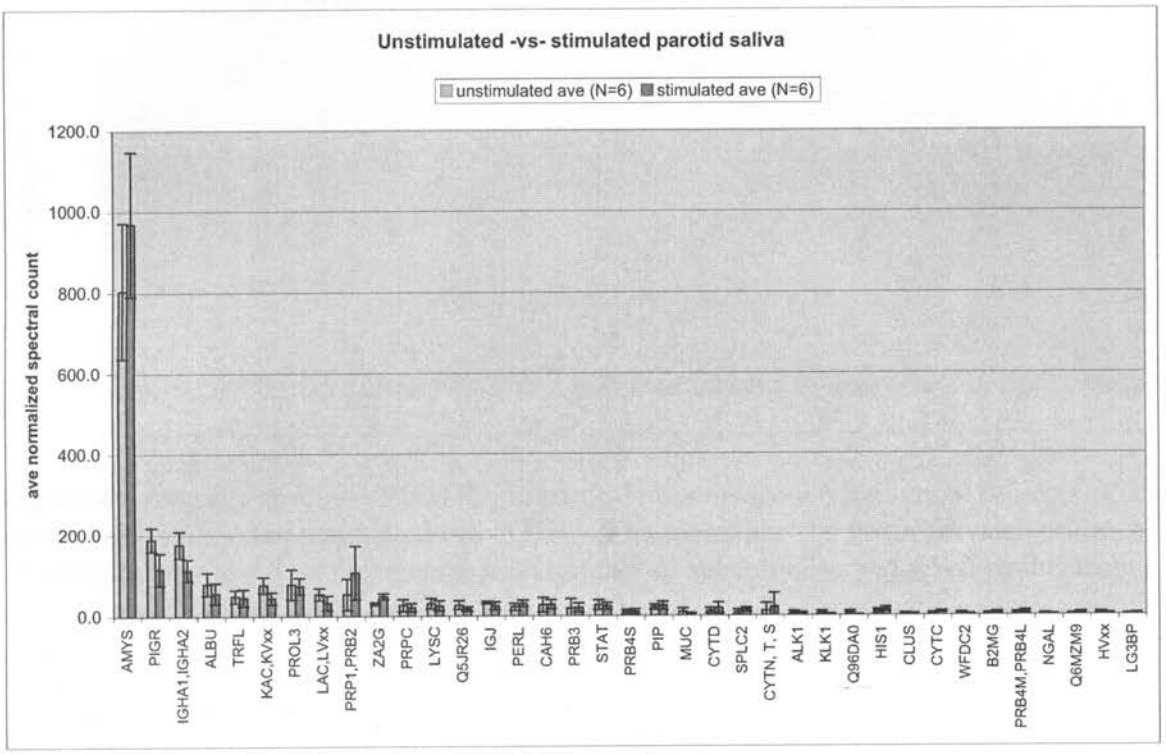

Figure 3: Average spectral counts from 6 biological subjects for 37 parotid proteins during unstimulated or stimulated saliva flow. Error bar corresponds to one standard deviation. Each individual biological sample was normalized to the average total number of spectra identified per sample, where the total included all proteins.

α-Amylase accounts for approximately 40-50% of the observed spectra, and poses normalization concerns. Therefore, Figure 4 shows the proteins with spectral counts normalized excluding amylase. There are only two proteins with averages that lie outside their uncertainties, namely, Zinc-alpha-2-glycoprotein and Cystatin C.

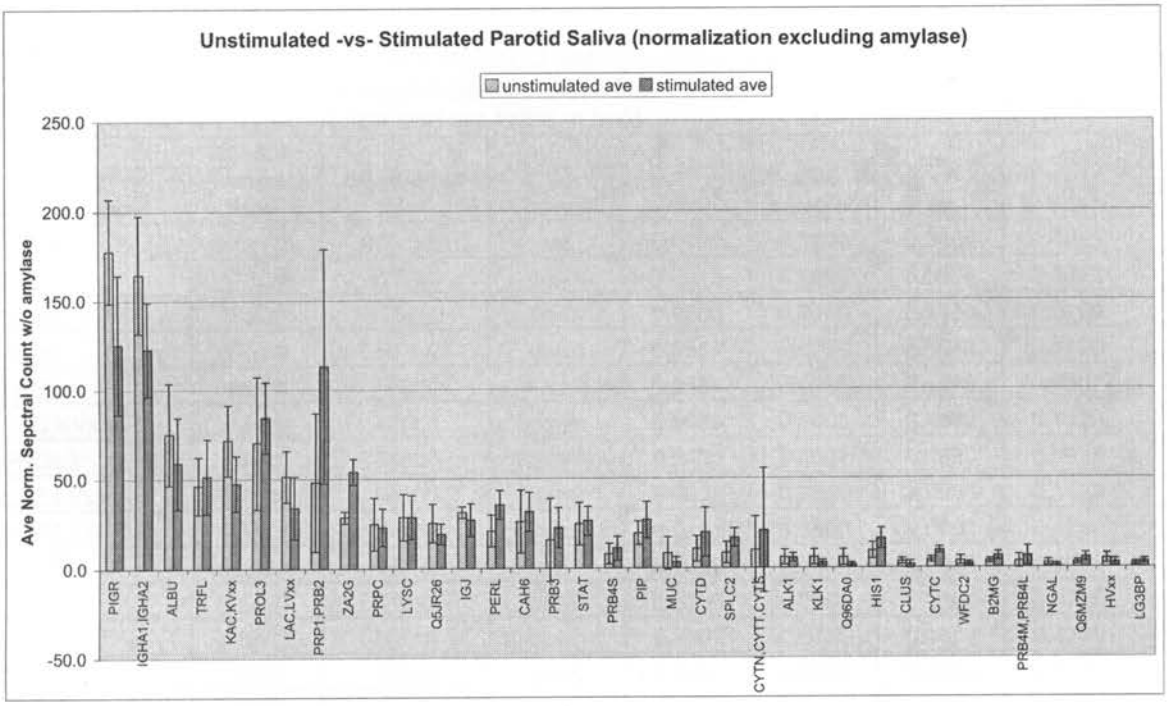

Figure 4: Average spectral counts for 36 parotid proteins from 6 biological subjects during unstimulated or stimulated saliva flow. The normalization factor for each of the 12 samples was based on the average total number of spectra observed when counts from amylase were excluded.

The average normalized spectra counts, their sample standard deviations, and the p-values from the 4 statistical tests are presented in Table 4. P-values less than 0.01 ($p<0.01$) were considered statistically significant. There is only one protein significantly different in all 4 tests, namely, Zinc-alpha-2-glycoprotein. Generally, there is poor consensus among the 4 tests and bolded p-values are often not very much less than 0.01. This agrees with the data in Figures 3 and 4 where only Zinc-alpha-2-glycoprotein and Cystatin C appear to have average values that differ beyond the range of the standard deviations.

Table 4: Summary of spectral count statistical testing to determine any differences in relative protein abundances during stimulated parotid saliva flow. P-values less than 0.01 were considered statistically significant and are listed in bold.

Protein Label	Unstimulated flow[a]	Stimulated flow[a]	Change[b]	t-test[c]	Chi square[d]	G test[e]	K-S test[f]
AMYS[g]	800*(170)*	970*(180)*	up	**0.0091**	**0.0000**	**0.0000**	0.1429
PIGR	178*(29)*	125*(35)*	down	0.0213	**0.0000**	**0.0000**	**0.0022**
IGHA1,IGHA2	165*(27)*	123*(15)*	down	**0.0035**	**0.0000**	**0.0000**	0.0260
ALBU	75*(28)*	59*(24)*	down	0.2847	**0.0050**	**0.0042**	0.1429
TRFL	46*(15)*	51*(23)*	up	0.5554	0.0418	0.0408	0.9307
KAC,KVxx	72*(16)*	47*(12)*	down	**0.0093**	**0.0000**	**0.0000**	0.1429
PROL3	70*(23)*	84*(35)*	up	0.5135	0.0937	0.0900	0.8928
LAC,LVxx	51*(16)*	34*(17)*	down	0.0903	**0.0000**	**0.0000**	0.1429
PRP1,PRB2	48*(33)*	113*(63)*	up	0.0163	**0.0000**	**0.0000**	0.4740
ZA2G	28*(5)*	54*(15)*	up	**0.0039**	**0.0000**	**0.0000**	**0.0022**
PRPC	25*(14)*	23*(9)*	down	0.8336	0.0392	0.0369	0.4740
LYSC	28*(12)*	28*(13)*	down	0.9992	0.6792	0.6786	0.4740
DMBT1	25*(8)*	19*(5)*	down	0.1901	**0.0065**	**0.0058**	0.0310
IGJ	31*(6)*	27*(8)*	down	0.3100	0.4511	0.4474	0.1429
PERL	21*(8)*	35*(12)*	up	**0.0098**	**0.0008**	**0.0008**	0.1389
CAH6	26*(17)*	31*(11)*	up	0.3251	0.7771	0.7767	0.9307
PRB3	16*(17)*	22*(12)*	up	0.4532	0.2151	0.2157	0.4413
STAT	25*(8)*	26*(7)*	up	0.6719	0.6738	0.6734	0.9307
PRB4S	8*(5)*	11*(7)*	up	0.1700	0.1060	0.1086	1.0000
PIP	20*(6)*	27*(9)*	up	0.0326	**0.0021**	**0.0021**	0.9307
MUC	8*(7)*	3*(3)*	down	0.1191	**0.0000**	**0.0000**	0.4413
CYTD	11*(6)*	20*(14)*	up	0.0614	**0.0000**	**0.0000**	0.4413
SPLC2	8*(7)*	17*(10)*	up	0.0211	**0.0020**	**0.0021**	0.1429
CYTN, T, S	10*(14)*	21*(31)*	up	0.1817	**0.0001**	**0.0001**	0.9307
ALK1	6*(4)*	6*(2)*	down	0.7920	0.9869	0.9867	0.8928
KLK1	6*(4)*	3*(1)*	down	0.0657	**0.0017**	**0.0011**	0.1389
Q96DA0	6*(5)*	2*(2)*	down	0.0460	**0.0000**	**0.0000**	0.1389
HIS1	9*(4)*	16*(6)*	up	**0.0089**	**0.0005**	**0.0006**	0.4740
CYTC	4*(2)*	10*(3)*	up	**0.0094**	**0.0010**	**0.0012**	0.0260
B2MG	4*(1)*	7*(2)*	up	0.0174	0.0221	0.0238	0.1429
PRB4M,PRB4L	3*(3)*	6*(5)*	up	0.0681	**0.0098**	0.0108	0.8928
Q6MZM9	3*(1)*	6*(2)*	up	0.0126	**0.0087**	**0.0096**	0.4413
HVxx	5*(2)*	3*(2)*	down	0.0153	0.0602	0.0556	0.1389

[a]Normalized average (N=6) spectral count and sample standard deviation (in parenthesis).
[b]Relative to unstimulated saliva, based on average spectral count values.
[c]p-values from a paired Student's t-Test, two-tailed. Multiple testing corrections were not applied.
[d]p-values from a Chi Square Test with one degree of freedom. Spectral counts were summed (pooled) across replicates.
[e]p-values from a G test. Spectral counts were pooled across replicates for each type of saliva.
[f]p-values from a Kolmogorov-Smirnov Test. Spectral counts were pooled across replicates for each type of saliva.
[g]Normalization and testing of amylase counts were done differently than for lesser abundant proteins.

The coefficients of variance of the technical replicates were compared to similar quantities from the biological replicates, and are shown in Figure 5. For most proteins, the coefficients of variance (CV) are smaller in the technical replicates than in the biological replicates, as would be expected. The average CV for the technical replicates was 19, considerably less than the average CV for the biological replicates, which was 42. However, the CV values for two of the proteins in the technical replicates were actually greater than the CV values in the biological replicates.

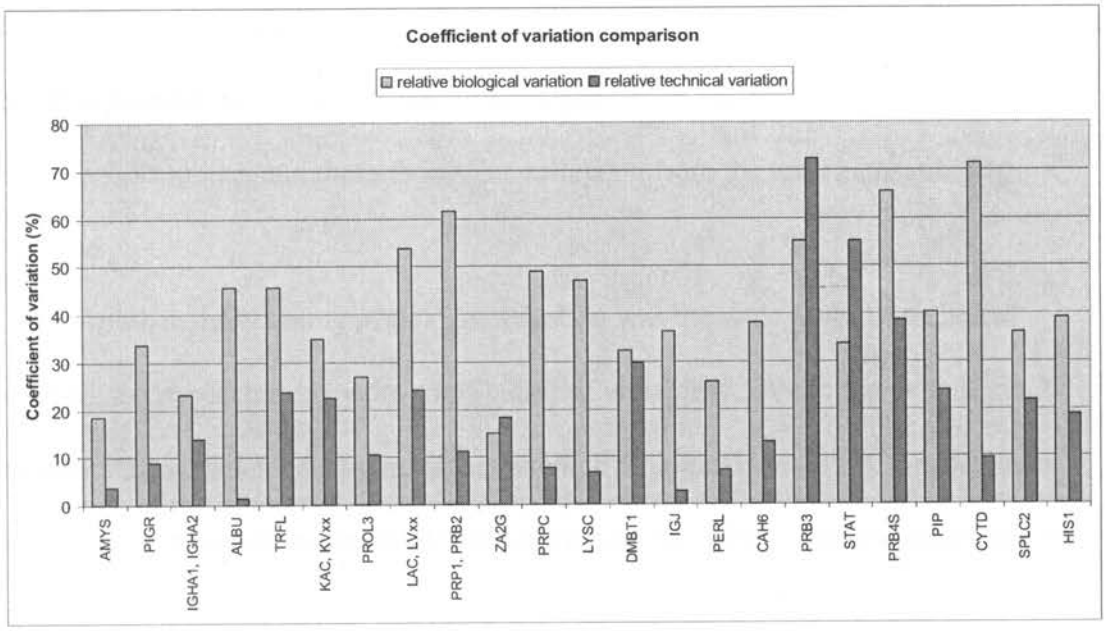

Figure 5. A comparison between the coefficients of variation of technical replicates and biological replicates. Data from stimulated parotid saliva flow for proteins with average spectral counts greater than 10 are shown.

Discussion:

Forty nine proteins were identified in parotid saliva as a result of this study. However, there were shortcomings of the investigation. Cystatins SN, SA, and S are not normally secreted by the parotid gland. While these proteins were present at relatively high counts in saliva samples from subject 1, they were not identified consistently across the biological subjects. This could be attributed to possible biological variation, however, the possibility also exists that these proteins resulted from sample contamination (either during collection or processing of samples from subject 1). Subject 1 was the first of the subjects from which saliva samples were collected using the Lashley cup. It is possible that whole saliva contamination may have occurred during collection from this first subject and that subsequent collection from the remaining subjects improved.

Another shortcoming of this investigation was the wide range of technical variation, as demonstrated by the coefficients of variability. While the overall trend demonstrates, what appears to be, higher average biological variability than technical variability, no conclusions can be drawn concerning the effect of saliva stimulation due to the large biological variability and relatively small sample size.

Two of the proteins, PRB3 and statherin, exhibited higher technical variation than biological variation. One possible cause for the observed technical variability is needle clogging within the source of the LCQ mass spectrometer. Future studies using the LTQ mass spectrometer, with less inherent clogging problems, may be able to overcome this

shortcoming. Future investigators should now also know to avoid this potential source of technical error.

Common contaminants, including trypsin autolysis products, BSA, and lens proteins were identified in some of the samples. The discovery of trypsin autolysis products was unsurprising as this was the enzyme used to digest the proteins. BSA was used to quality control the SCX column prior to loading the sample and was thus an expected contaminate. Lens proteins were discovered, also as a result of previous contamination of the SCX column from a prior analysis. However, the contaminating proteins, observed since the instrument was also used to study the lens, were found in very insignificant quantities, and were thus deemed insignificant.

Parotid proteome

Two previous studies (Hardt et al., 2005; Walz et al., 2006) have used 2-DE to characterize 16 and 12 proteins, respectively, in the parotid proteome. All proteins reported in those studies were observed here. Furthermore, the high sensitivity of 2-DLC resulted in 33 more identified proteins. The functions of some of these identified proteins are as follows:

Alpha-amylase starts the digestive process of carbohydrates and is by far the major protein present in parotid saliva. Zinc-alpha-2-glycoprotein has an unknown role in saliva, but may be associated with lipid degradation. Clusterin may also be involved in lipid metabolism. Several identified proteins may bind important nutrients: lactotransferrin binds Fe^{3+}, transcobalamin binds vitamin B-12, and folate receptor alpha binds folic acid.

Carbonic anhydrase VI (also know as gustin) is necessary for proper functioning of taste buds (Henkin et al., 1999). Proteins that might play a role in transport of molecules for taste are galectin-3-binding protein, which binds several sugars, neutrophil gelatinase-associated lipocalin (lipid binding and transport), and possibly serum albumin, which binds fatty acids, water, Ca^{2+}, Na^{1+}, and K^{1+}(Edgar, 1990).

Bolus formation is most likely assisted by the high water content of parotid saliva and its high flow rate during stimulation. There are no mucins, per se, secreted by the parotid gland, however, salivary agglutinin and proline-rich glycoprotein may help with lubrication and bolus formation (Amerongen and Veerman, 2002).

Acidic proline-rich proteins exhibit high affinity to hydroxyapatite, inhibit crystal growth of calcium phosphate salts from solutions supersaturated with respect to hydroxyapatite, bind calcim ions, and interact with several oral bacteria on adsorption to hydroxyapatite. Statherin acts as an inhibitor of spontaneous calcium salt precipitation and promotes the adhesion of *A. viscosus* to tooth surfaces. Statherin, Histatin, and Cystatins also exhibit affinities to mineral surfaces, inhibit calcium phosphate precipitation, and play a role in maintaining the integrity of teeth (Amerongen and Veerman, 2002).

Beta-2-microglobulin is a salivary agglutinin which has the capacity to clump bacteria into large aggregates which are more easily cleared by swallowing. Lysozyme C destroys bacterial cell walls through muramidase activity (hydrolyzing bonds in the peptidoglycan layer of the wall), and through activation of bacterial autolysins. Lactotransferrin has a bacteriostatic effect by depriving iron from pathogenic microorganisms. In its iron free state, lactotransferrin has a bactericidal effect by directly

binding to a number of bacteria. Antimicrobial domains (called lactoferricins) and fragments inhibiting adherence of *S. mutans* to saliva-coated hydroxyapatite have also been identified. Lactoperoxidase catalyzes the oxidation of salivary thiocyanate ions (SCN^-) to the antimicrobial component hypothiocyanite (OSCN) and protects host proteins and cells from the toxicity of H_20_2. Cystatins C, B, and D inhibit proteases and demonstrate antiviral and antibacterial activity. Proline-rich proteins have the ability to bind tannins, present in such beverages as tea and red wine, and reduce their toxicity (Edgar, 1990).

Ig alpha-1 chain C, Ig kappa chain C, Ig lambda chain C, Ig alpha-2 chain C, Ig mu chain C, Ig heavy chain variable regions are the contributing pieces of the heavy and light chains that make up salivary IgA antibodies, which provide immunological defense against specific organisms. Ig J chain is a carbohydrate portion of the sIgA complex which links the dimeric molecules constituting sIgA antibodies. Secretory component is a small glycoprotein portion of the salivary IgA complex that makes these antibodies more resistant to proteases in the oral environment (Edgar, 1990).

The proteins described above are those with known functions in saliva. However, many of the identified proteins have yet unknown functions. The identification and characterization of the parotid proteome is important, if for no other reason than to expand the current understanding of salivary components and their origins. In addition, an understanding of the healthy parotid proteome is essential in order to make comparison to the proteome of individuals in disease states or parotid dysfunction. A recent investigation identified 10 parotid salivary biomarkers for Sjögren's syndrome (Ryu et al., 2006), all of which were identified in this study. As protein research

continues, and the understanding of low abundance protein function broadens, the proteins identified in this investigation may prove useful as biomarkers for other diseases as well.

Unstimulated versus stimulated flow

The relative protein abundances shown in Figures 3 and 4 indicate that parotid saliva protein composition is essentially the same during stimulated saliva flow. There seems to be an apparent trend that acinar cell products (such as amylase, PRPs, ZA2G, etc.) are slightly increased during stimulated flow, and that secretory IgA components are slightly decreased. However, within the biological and technical variations, there were few statistically significant overall differences that could be determined between unstimulated and stimulated parotid flow, when performing multiple statistical tests. Since protein synthesis does not occur instantaneously, the similar protein composition of stimulated parotid saliva is not unexpected. Perhaps saliva collected after prolonged stimulation would have a protein composition that differs from unstimulated saliva to a greater extent.

Conclusion

The parotid saliva proteome was greatly expanded as a result of this thesis. A list of 49 parotid saliva proteins identified in at east two different biological subjects was compiled. These results reveal the relative simplicity of the parotid proteome when compared with that of whole saliva. Among the 49 identified parotid saliva proteins, 39 were observed in at least 4 of the 6 subjects in both stimulated and unstimulated saliva, demonstrating the consistency of the parotid proteome across biological replicates. The results presented here establish a baseline young, healthy parotid proteome. This reference parotid proteome could make detection of parotid gland dysfunction markers in future studies much easier.

It was originally hypothesized that proteins playing roles in taste, digestion, or bolus formation might be secreted in greater abundance in response to conditions that stimulate saliva flow. This hypothesis was not validated by this investigation, the data revealing few differences between unstimulated and stimulated parotid saliva. This suggests that the major glands may not be very dynamic in response to taste, and that minor gland secretions may play a more important role in this regard. This agrees with Neyraud et al. (2006) where no differences in parotid secretions could be measured with 2-DE during 4 different taste stimulations. However, technical variation, as described above, may have also been a factor in our inability to detect any protein abundance differences between stimulated and unstimulated parotid saliva.

References

Alaiya, A.; Al-Mohanna, M.; Linder, S., Clinical cancer proteomics: promises and pitfalls. *J Proteome Res* **2005,** 4, (4), 1213-22.

Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J.D., Molecular Biology of THE CELL, 3rd ed., NY and London: Garland Publishing, Inc., **1994**, 186-187.

Amerongen, A.V.; Veerman, E. C., Saliva – the defender of the oral cavity. *Oral Dis.* **2002**, *8*, 12-22.

Anderton, B.H.; Thorpe, R.C. New methods of analyzing for antigens and glycoproteins in complex mixtures. *Immunol. Today*. 1:122-127, 1980.

Carlson, A. J.; Crittenden, A. L., The relation of ptyalin concentration to the diet and to the rate of secretion of saliva. *Am. J. Physiol.* **1910**, 26, 169-177.

Castle, J. D., Cell Biology of Salivary Protein Secretion, in Biology of the Salivary Glands, Dobrosielski-Vergona, K., CRC Press **1993**, 81-98.

Castle, J. D., Jamieson, J.D., Palade, G. E., Radioautographic analysis of the secretory process in the parotid acinar cell of the rabbit, *J. Cell Biol.* **1972,** 53, 290.

Colinge, J.; Chiappe, D.; Lagache, S.; Moniatte, M.; Bougueleret, L., Differential Proteomics via probabilistic peptide identification scores. *Anal Chem* **2005,** 77, (2), 596-606.

Craig, R.; Beavis, R. C., A method for reducing the time required to match protein sequences with tandem mass spectra. *Rapid Commun Mass Spectrom* **2003,** 17, (20), 2310-6.

Edgar, M.; Dawes, C.; O'Mullane, D., editors, Saliva and oral health, 3rd ed., London: British Dental Association, **2004**, 6-7.

Edgar, W.M., Saliva and dental health. Clinical implications of saliva: report of a consensus meeting. *Br Dent J* **1990**, 169, 96-8.

Eng, J. K.; McCormack, A. L.; Yates, J. R., III, An Approach to Correlate Tandem Mass Sectral Data of Peptides with Amino Acid Sequences in a Protein Database. *Journal of the American Society for Mass Spectrometry* **1994,** 5, 976-989.

Gao, J.; Friedrichs, M. S.; Dongre, A. R.; Opiteck, G. J., Guidelines for the routine application of the Peptide hits technique. *J Am Soc Mass Spectrom* **2005,** 16, (8), 1231-8.

Ghafouri, B.; Tagesson, C.; Lindahl, M., Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics* **2003**, *3*, 1003-15.

Grant D.A., Stern I.B., Listgarten M.A., editors, Saliva, In: Periodontics. 6th ed. St Louis: CV Mosby, **1988**, 135-46.

Guo, T.; Rudnick, P. A.; Wang, W.; Lee, C. S.; Devoe, D. L.; Balgley, B. M., Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. *J Proteome Res* **2006**, 5, (6), 1469-78.

Hand, A.H., Salivary glands, in Orban's Oral Histology and Embryology, S.N. Bohaskar, Ed., C.V. Mosby, St. Louis, **1986**, 354.

Hardt, M.; Thomas, L. R.; Dixon, S. E.; Newport, G.; Agabian, N.; Prakobphol, A.; Hall, S. C.; Witkowska, H. E.; Fisher, S. J., Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry* **2005,** 44, (8), 2885-99.

Hardt, M.; Witkowska, H. E.; Webb, S.; Thomas, L. R.; Dixon, S. E.; Hall, S. C.; Fisher, S. J., Assessing the effects of diurnal variation on the composition of human parotid

saliva: quantitative analysis of native peptides using iTRAQ reagents. *Anal Chem* **2005,** 77, (15), 4947-54.

Harlow, E.; Lane, D. Antibodies. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, **1988**.

Henkin, R. I.; Martin, B. M.; Agarwal, R. P., Decreased parotid saliva gustin/carbonic anhydrase VI secretion: an enzyme disorder manifested by gustatory and olfactory dysfunction. *Am. J. Med. Sci.* **1999**, 318, (6), 380-91.

Hu, S.; Xie, Y.; Ramachandran, P.; Ogorzalek Loo, R. R.; Li, Y.; Loo, J. A.; Wong, D. T., Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and gel electrophoresis-mass spectrometry. *Proteomics* **2005**, 5, (6), 1714-28.

Huang, C. M., Comparative proteomic analysis of human whole saliva. *Arch Oral Biol* **2004**, 49, (12), 951-62.

Humphrey, S. P.; Williamson, R. T., A review of saliva: Normal composition, flow, and function. J of Prosthet Dent 2001, 85, 162-9.

Iverson, J. M., Kauffman, D. L., Keller, P. J., and Robinovitch, M., Isolation and partial characterization of two populations of secretory granules from rat parotid glands, *Cell Tissue Res.* **1985**, 240, 441.

Jacobs, J. M.; Adkins, J. N.; Qian, W. J.; Liu, T.; Shen, Y.; Camp, D. G., 2nd; Smith, R. D., Utilizing human blood plasma for proteomic biomarker discovery. *J Proteome Res* **2005,** 4, (4), 1073-85.

Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R., Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search, *Anal. Chem.* **2002**, 74, (20), 5383-5392.

Lashley, K. S., The human salivary reflex and its use in psychology. *Psychol. Rev.* **1916**, 23, 446.

Liu, H.; Sadygov, R. G.; Yates, J. R., 3rd, A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem* **2004,** 76, (14), 4193-201.

Mandel, I.D., Saliva, In: Orban's Periodontics, Grant D., Stern I., Everett F., Eds C.V., Mosby, St Louis, **1972**, 99.

Mandel, I.D., Sialochemistry in diseases and clinical situations affecting salivary glands. *Crit Rev Clin Lab Sci* **1980,** 12, (4), 321-66.

Marko-Varga, G.; Lindberg, H.; Lofdahl, C. G.; Jonsson, P.; Hansson, L.; Dahlback, M.; Lindquist, E.; Johansson, L.; Foster, M.; Fehniger, T. E., Discovery of biomarker candidates within disease by protein profiling: principles and concepts. *J Proteome Res* **2005,** 4, (4), 1200-12.

Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R., A statistical model for identifying proteins by tandem mass spectrometry, *Anal. Chem.* **2003**, 75, (17), 4646-4658.

Neyraud, E.; Sayd, T.; Morzel, M.; Dransfield, E., Proteomic analysis of human whole and parotid saliva following stimulation by different tastes. *J Poteome Res* **2006**, 5, (9), 2474-80.

Omenn, G. S.; States, D. J.; Adamski, M.; Blackwell, T. W.; Menon, R.; Hermjakob, H.; Apweiler, R.; Haab, B. B.; Simpson, R. J.; Eddes, J. S.; Kapp, E. A.; Moritz, R. L.; Chan, D. W.; Rai, A. J.; Admon, A.; Aebersold, R.; Eng, J.; Hancock, W. S.; Hefta, S. A.; Meyer, H.; Paik, Y. K.; Yoo, J. S.; Ping, P.; Pounds, J.; Adkins, J.; Qian, X.; Wang, R.; Wasinger, V.; Wu, C. Y.; Zhao, X.; Zeng, R.; Archakov, A.; Tsugita, A.; Beer, I.; Pandey, A.; Pisano, M.; Andrews, P.; Tammen, H.; Speicher, D. W.; Hanash, S. M., Overview of the HUPO Plasma Proteome Project: results from the pilot phase

with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* **2005,** 5, (13), 3226-45.

Pedersen, A. M.; Bardow, A.; Jensen, S. B.; Nauntofte, B., Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. *Oral Dis.* **2002**, *8*, 117-129.

Roth G., Calmes R., editors, Salivary glands and saliva. In: Oral biology. St Louis: CV Mosby, **1981**,196-236.

Ryu O. H., Atkinson J. C., Hoehn G. T., Illei G. G., Hart T.C., Identification of parotid salivary biomarkers in Sjögren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. *Rheumatology*, **2006**, 45, 1077-1086.

Saracco, C.G., Crabill, E.V., Anatomy of the human Salivary Glands, in Biology of the Salivary Glands, Dobrosielski-Vergona, K., CRC Press, **1993**, 1-14.

Shannon I.L., The biochemistry of human saliva in health and disease. In: Rowe WH, editor. Salivary glands and their secretion. Ann Arbor: University of Michigan Press; **1972**, 92-121.

Streckfus, C. F.; Bigler, L. R., Saliva as a diagnostic fluid, *Oral Diseases* **2002**, 8, 69-76.

Villanueva, J.; Philip, J.; Entenberg, D.; Chaparro, C.A.; Tanwar, M.K.; Holland, E. C.; Tempst, P., Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. *Anal. Chem.* **2004**, 76, 1560-1570

Vitorino, R.; de Morais Guedes, S.; Ferreira, R.; Lobo, M.J.C.; Duarte, J.; Ferrer-Correia, A.J.; Tomer, K.B.; Dominguez, P.M.; Amado, F.M.L., Two-dimensional electrophoresis study of in vitro pellicle formation and dental caries susceptibility, *Eur J Oral Sci* **2006**, 114, 147-153.

Vitorino, R.; Lobo, M. J.; Ferrer-Correira, A. J.; Dubin, J. R.; Tomer, K. B.; Domingues, P. M.; Amado, F. M., Identification of human whole saliva protein components using proteomics. *Proteomics* **2004**, *4*, 1109-1115.

Vitzthum, F.; Behrens, F.; Anderson, N. L.; Shaw, J. H., Proteomics: from basic research to diagnostic application. A review of requirements & needs. *J Proteome Res* **2005,** 4, (4), 1086-97.

Walz, A.; Stuhler, K.; Wattenberg, A.; Hawranke, E.; Meyer, H. E.; Schmalz, G.; Bluggel, M.; Ruhl, S., Proteome analysis of glangular parotid and submandibular-

sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. *Proteoemics* **2005**.

Wilmarth, P. A.; Riviere, M. A.; Rustvold, D. L.; Lauten, J. D.; Madden, T. E.; David, L. L., Two-dimensional liquid chromatography study of the human whole saliva proteome. *J Proteome Res* **2004,** 3, (5), 1017-23.

Wilson, L.; Matsudaira, P., eds. Antibodies in Cell Biology (Methods in Cell Biology, Vol. 37). New York: Academic Press, **1993**.

Xie, H.; Rhodus, N. L.; Griffin, R. J.; Carlis, J. V.; Griffin, T. J., A Catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. *Mol Cell Proteomics* **2005**.

Yao, Y.; Berg, E. A.; Costello, C. E.; Troxler, R. F.; Oppenheim, F. G., Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. *J. Biol. Chem.* **2003**, *278*, 5300-5308.

Zastrow, M. V., Tritton, T. R., and Castle, J.D., Exocrine secretion granules contain peptide amidation activity, *Proc. Natl. Acad. Sci. U.S.A.* **1986**, 83, 3297.

Zhang, B.; VerBerkmoes, N. C.; Langston, M. A.; Uberbacher, E.; Hettich, R. L.; Samatova, N. F., Detecting differential and correlated protein expression in label-free shotgun proteomics. *J. Proteome Res.* **2006**, 5, (11), 2909-2918.