Proteomic Analysis Of Muc7 12-Mer Induced Protein Expression Changes In Streptococcus Mutans

A. Specific Aims:

MUC7 peptides (derived from the N-terminal region of the parent human salivary mucin, MUC7) possess strong antifungal and antibacterial activities. MUC7 20-mer has been shown to internalize in the fungal cell cytoplasm, suggesting that MUC7-derived peptides involve an intracellular mechanism of action. Amongst bacteria, MUC7 peptides have been shown to be most effective in killing the cariogenic bacteria \textit{S. mutans}. The overall goal is to determine the MUC7 peptides’ mechanism of action. The objective of this proposal is to investigate the protein profile changes in \textit{S. mutans} as a result of MUC7 12-mer peptide exposure, utilizing proteomic techniques.

My hypothesis is that low concentration doses below the LD50 level of MUC7 12-mer peptide (truncated 20-mer) against the \textit{S. mutans} cells, will cause changes in the expression pattern of proteins when compared to the untreated \textit{S. mutans} cells. By utilizing the same strain of bacteria, and creating an environment where only the presence of the peptide is variable, I believe the changes seen in the protein expression pattern will be a direct consequence of the presence of the peptide. These changes will become apparent when protein samples are run on a 2-D gel, analyzed using statistical software and peptide mass fingerprint data is collected. The compilations of proteins that are either up or down-regulated will indicate the biochemical pathways that are affected by the presence of the antimicrobial peptide.

Along with Dr. Guoxian Wei and Dr. Maciek Lis of Dr. Bobek’s lab, I am working on the overall goal of explicating the MUC7-derived peptides’ mechanism of action. Dr. Lis is currently analyzing data obtained over the past year comparing the
effect of the peptides on two strains of *C. albicans* (a wild-type strain and a MUC7 12-mer resistant strain). Dr. Wei is planning to investigate nucleic acid transcription variation between *S. mutans* treated and untreated cells utilizing microarray technology. My proteome studies will investigate protein profiles of the two cell populations (treated and untreated). These studies aim to determine the differentially expressed proteins (up-regulated and/or down-regulated), thus, hopefully give some clues regarding the mechanism of MUC7 peptide antibacterial action.

**B. Background and Significance:**

*Streptococcus mutans* is a gram positive bacterium that grows on teeth, especially when exposed to sucrose. *S. mutans* adheres to the surface of teeth and is most active in an acidic pH environment. This bacterium is the prominent cause of dental caries (in combination with the right growth environment and nutrient supply). Dental decay is caused by the irreversible solubilization of tooth mineral by acid produced by bacteria such as *S. mutans* that adhere to the tooth surface in bacterial communities known as dental plaque. As indicated above, our lab has determined that MUC7 12-mer peptide is very effective in killing *S. mutans* (with the minimum bactericidal concentration of 6.25μM).ii

Due to the emergence of antibiotic-resistant pathogens, and the toxicities associated with common antibiotic drugs such as amphotericin B, the need for new antimicrobial agents is becoming more urgent every day. The discovery of naturally occurring cationic antimicrobial peptides looks promising for future treatment of infection. These compounds show little toxicity towards mammalian cells and have a low tendency to elicit resistance. These peptides are small, amphipathic, and have a net
positive charge. One family of these peptides is the MUC7 derived peptides. They peptides have both antifungal and antimicrobial properties, and so the long range goal is to develop them for clinical use after gaining a better understanding of their mechanism of action.

The low-molecular-weight human salivary mucin encoded by the human MUC7 gene is composed of 357 residues. Its N-terminal domain (domain 1), comprised of 51 amino acid residues, was found to have antifungal activity. Seven out of the eight positive charges on this MUC7 domain 1 peptide, thought to be important for the activity, were found to be at the C-terminal 20 residues, so the MUC7 20-mer peptide was developed. This peptide showed comparable or better activity compared to the 51 residue parent peptide. The 20-mer spans the residues 32-51 of the parent MUC7 with a net positive charge of 7.

The MUC7 20-mer possesses a wide array of antimicrobial activity in vitro. The 12-mer L (natural form) and 12-mer D isomers derived from the C-terminus end of the 20-mer and retaining 6 of the seven positive charges were determined to retain antimicrobial activity that is comparable to the 20-mer. The amino acid sequence for the MUC7 12-mer is RKSYKCLHKRCR.

According to a review of cationic antimicrobial peptides, the common feature is that they all have an amphipathic structure allowing them to bind to the interface of the cytoplasmic membrane. However, although disturbance of cytoplasmic membranes is a prominent mechanism of cytotoxicity, the authors discuss alternate modes of killing, involving intracellular mode of action, including inhibition of nucleic acid synthesis and protein synthesis.
Our previous experiments indicated that the MUC7 20-mer has some membranolytic activity on fungal cells, but is also internalized by these cells. Confocal fluorescence microscopy analysis of double-labeled cells stained with a mitochondrion-specific dye suggested that the mitochondria are not the target of the 20-mer.⁴

Another study has indicated that basic (positively charged) polyamino acids inhibit translational elongation factor-3 (EF-3) in yeast and fungi by inhibiting the ribosomal-activated ATPase.⁵ The positively charged MUC7 12-mer-L and 12-mer-D peptides (6 positive charges) may interact with the translational functions of cells in this manner.

C. Preliminary Data:

Utilizing two different strains of C. albicans, Dr. Maciek Lis conducted proteomic experiments in the summer of 2005 in a parallel manner as my proposed project. A wild type strain of C. albicans was compared to a MUC7 12-mer resistant mutant. The DIGE method was followed by MALDI-TOF mass spectrometry analysis, and the differences in protein expression are currently being studied. A series of enzymes associated with metabolism have been shown to be either up or down-regulated in the mutant variant of C. albicans.

D. Materials and Methods:

The first step of my experiments is to grow the S. mutans cells both treated and untreated with the MUC7 12-mer peptide. Harvesting the cells at a similar point in their growth phase will necessary, so consideration of the peptide’s effects on the growth of the treated S. mutans cells will be established prior to proceeding. Once the cells are harvested and proteins have been extracted (as described below), I will perform 2-
Dimensional gel electrophoresis. This will be followed by extraction of individual proteins from the gel, digestion of the proteins, and analysis by mass spectrometry (MALDI-TOF). Using the data collected from the mass spectrometer, I will use the online database MASCOT to identify the proteins extracted from the gel.

The first step in the 2-D gel electrophoresis will be isoelectric separation, followed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Electrophoresis is the process by which molecules that contain a net charge can move in an electric field. For biomolecular techniques, gels are utilized to perform the electrophoretic separations of proteins. These proteins separate from one another based on their molecular size with smaller proteins moving faster than the larger proteins. Prior to the electrophoresis on the polyacrylamide gel, I must perform the 1st-dimensional aspect of the experiment: isoelectric separation of my protein sample. The isoelectric point is the pH where there is no net charge on a protein. By creating a pH gradient on a thin gel (DryStrip), it is possible to separate the individual proteins of a protein sample by their isoelectric point. Isoelectric separation is run horizontally. Once the proteins have finished migrating according to their isoelectric point, the 2nd-dimensional part of the experiment is running the SDS-PAGE. The SDS-PAGE is run vertically. By this method, hundreds of proteins can be separated on a single polyacrylamide gel.

In order to run the variety of protein samples from the treated and untreated S. mutans on gels, the Ettan DIGE method will be used. In this method, proteins are pre-labeled before 2-D electrophoresis. The main differences between Ettan DIGE and other 2-D differential analysis techniques is Ettan DIGE incorporates the same internal
standard on every gel, and each gel can have up to three samples run simultaneously. This eliminates any problems of inter-gel variation. Another benefit of the Ettan DIGE technique is their use of three distinct CyeDye DIGE fluors that all have the same mass and charge but differ in how they are resolved spectrally. This eliminates intra-gel variation due to the dyes.

The first step will be to prepare a protein lysate from the *S. mutans* cells. Washing and lysing the cells prepares them for CyeDye DIGE fluor labeling. Adequate cell washing will be necessary in order to minimize any contamination of proteins associated with the culture material. Appropriate cell concentration between 5mg/ml and 10mg/ml are determined using a protein assay, and the pH of the protein sample is adjusted to between 8.0-9.0. Each protein sample is labeled utilizing a different CyeDye (at least one for the treated sample and one for the untreated sample). At this point, a pooled internal standard is made utilizing small aliquots from each protein sample.

The Ettan DIGE system allows for up to three different protein samples to be run on a single 1\textsuperscript{st} and 2\textsuperscript{nd} dimension gel by mixing the sample prior the 1\textsuperscript{st} dimension gel run. Samples are focused utilizing Immobiline DryStrip before being run on the 2-D gel. Specially sized gels are utilized for the 2-D run, and an ‘electrophoresis tank’ that is designed to hold twelve of these gels is required. The gels, scanners and mass spectrophotometer that will be utilized are found in the Proteomic Facility in the Department of Biochemistry.

Once the images of the gels are scanned, computer software is utilized to help detect, quantitate and match differential protein expression from the gels. This software is equipped with a series of algorithms designed to allow accurate differential expression
analysis. It is important to be able to differentiate between protein spots on the gel, and non-proteinaceous background material. The DeCyder software allows for multiple gels to be matched, followed by statistical analysis to determine which spots on the gels (corresponding to proteins) are significantly up or down-regulated.\textsuperscript{vii}

In a manner similar to that described by Kusch, protein spots will be cut out of the stained gels and digested.\textsuperscript{viii} Digestion of the protein of each sport by the protease trypsin will generate many small peptides. The enzyme trypsin always cuts downstream to the two basic amino acids: arginine and lysine.

Trypsin-digested spots will then be analyzed by MALDI-TOF (matrix-assisted laser desorption ionization, time of flight). The laser ionizes the sample into an aerosol that enters the mass spectrometer. The mass to charge ratio of the aerosol fragments are measured and correspond to the mass of the fragment.\textsuperscript{ix}

The data collected by the mass spectrometer is then submitted to the online sequencing database, Mascot. This is accomplished by adding the list of monoisotopic masses on the search page, and indicating that trypsin was the digestion protease. The generated masses of the tryptic peptides are searched against theoretical masses of \textit{S. mutans} proteins.

The database will produce a list of proteins based on the probability of the experimental data matching theoretical protein data. From this point, the names of the proteins are compiled, and investigation into their function is researched. The final goal will be to ascertain the relationship between these up or down-regulated proteins, and the action of the MUC7 12-mer peptide.
E. Literature Cited:


ix. Jefferies, James R. Protein Identification by Peptide Mass Fingerprinting Tutorial. Institute of Biological Sciences, Univeristy of Wales at Aberystwyth, Ceredigion, Wales, UK. http://www.aber.ac.uk/parasitology/Proteome/MS_Tut.html#Section%201

F. Resource Information:
Research will be conducted primarily in the Oral Biology Department of the State University of New York at Buffalo: 109 Foster Hall, 3435 Main Street, Buffalo, New York. Cell incubation, experimental set-up and data analysis generated from the gels will take place in this lab. The MALDI-TOF facility is located in Room 116 Farber Hall and houses a Bruker Biflex IV MALDI-TOF spectrometer. A Bruker Scout spotting robot is also available in this lab.

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H. Human Subjects
Not applicable.

I. Vertebrate Animals
Not applicable.

J. Recombinant DNA, Recombinant DNA Molecules
Not applicable.