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Proteomic Analysis of Saliva: 2D Gel Electrophoresis, LC-MS/MS, and Western Blotting

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Abstract

Saliva harbors a wide spectrum of proteins that may reflect the health/disease status in the human body. Profiling of the proteins in saliva from a disease population can potentially yield valuable clinical parameters to be used for diagnosis and prognosis of the disease. Advances in proteomic technologies have enabled comprehensive profiling of protein expression in cells, tissue, and body fluids. When applied to readily accessible saliva samples from disease patients for biomarker study, such a global approach allows attaining the most discriminatory protein biomarkers that can best predict the disease status. In this chapter, we describe the protocols for proteomic analysis of saliva using 2D gel electrophoresis, Western blotting, and LC-MS/MS.

Keywords

Salivary proteomics; salivary diagnostics; tandem mass spectrometry; 2D gel electrophoresis

1. Introduction

Saliva has been increasingly recognized as an acceptable alternative to blood for use in diagnostic tests because salivary testing is safe, low cost, and non-invasive (1, 2). Due to easy sample collection and processing, saliva represents a readily accessible body fluid that may be repeatedly sampled for long-term monitoring of disease progression or in vivo assessment of efficacy and toxicity of drug treatment.

Human saliva contains a large number of proteins, which play important roles in maintaining oral/general health and may serve as biomarkers to survey disease status. An in-depth analysis of the human saliva proteome as well as their posttranslational modifications can therefore provide a valuable resource for oral biology and saliva diagnostics research (3–13). To date, a total of 1,939 unique proteins have been compiled from 19,474 unique peptide sequences identified from whole and ductal saliva samples, including 740 proteins from both whole and ductal saliva. Shotgun proteomics based on multidimensional chromatography with tandem mass spectrometry (MS/MS) represent a major technology used for comprehensive identification of saliva proteins. Among the 1,939 proteins identified from human saliva, a total of 597 are also present in the human plasma proteome (14).

MS-based proteomic approaches have been used to identify saliva biomarkers for Sjögren's syndrome (SS), which is a systemic autoimmune disease characterized by dry eyes and dry mouth (15–17). It was found that the saliva proteomic profiles of SS patients are a mixture of increased inflammatory proteins and decreased acinar proteins as compared with those in non-SS controls. Promising biomarkers derived from our proteomic study included alpha-

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enolase, beta-microglobulin, cathepsin D, and carbonic anhydrase I, which have been successfully validated in a new group of SS, systemic lupus erythematosus (SLE, autoimmune disease control), and healthy control subjects (unpublished results). Diagnosing SS is complicated by the range of symptoms a patient may manifest, and the similarity between symptoms from SS and those caused by other autoimmune disorders such as SLE and rheumatoid arthritis. The availability of these unique saliva protein biomarkers may lead to a simple clinical tool for non-invasive and highly specific diagnosis of SS in the future. Meanwhile, saliva proteomics has been used in searching for biomarkers for oral and breast cancers (18, 19). By using subtractive proteomics followed by immunoassays for validation, we discovered five potential protein biomarkers (calgranulin B, Mac-2 binding protein, CD-59, catalase, and profilin) in saliva for oral cancer. The combination of these candidate biomarkers yielded a sensitivity of 90% and a specificity of 83% in detecting oral squamous cell carcinoma. Proteomics analysis of cells in whole saliva from oral cancer patients also provided an approach that may reveal protein biomarkers for oral cancer detection (20).

2. Materials

2.1. 2D Gel Electrophoresis of Saliva Proteins

1. SYPRO Ruby protein stain (Invitrogen)
2. 2D-Quant total protein assay kit (Amersham)
3. Agarose sealing solution: 25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol
4. Rehydration buffer: 7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 5% glycerol, 10% isopropanol
5. Equilibration buffer: 6 M urea, 0.375 M Tris–HCl, 2% SDS, 20% glycerol, pH 8.8
6. 2% DTT in equilibration buffer
7. 2.5% iodoacetic acid in equilibration buffer
8. TGS running buffer (Bio-Rad)
9. ReadyStrip® pH 3–10 NL IPG strips (Bio-Rad)
10. MultiMark® Multi-Colored Standard (Invitrogen)
11. Protean II ready gel 8–16% Tris–HCl (Bio-Rad)
12. Protean IEF cell (Bio-Rad)
13. FX scanner (Bio-Rad)
14. ProteomeWorks spot cutter (Bio-Rad)

2.2. Protein Identification

1. Ammonium bicarbonate (Fisher Scientific)
2. Acetonitrile (ACN; Sigma-Aldrich)
3. 10 mM DTT (Bio-Rad) in 100 mM ammonium bicarbonate
4. 50 mM iodoacetic acid (Sigma-Aldrich) in 100 mM ammonium bicarbonate
5. Trifluoric acid (TFA, Sigma-Aldrich)
6. Formic acid (Sigma-Aldrich)
7. Sequencing-grade trypsin (Promega)

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8. Nano-LC system (Eksigent Technology)
9. Linear ion trap MS system (LTQ XL, Thermo-Fisher Scientific)
10. PicoTip emitter (New Objective)
11. LC mobile phase A: 95% H_2O/5% ACN/0.1% formic acid
12. LC mobile phase B: 95% ACN/0.1% formic acid

2.3. Western Blot Analysis of Saliva Proteins

1. NuPAGE Bis-Tris pre-cast minigel (Invitrogen)
2. MES SDS running buffer (Invitrogen)
3. TBS(10×): 100 mM Tris base, 1.5 M NaCl, pH 7.6
4. TBST(1×): 200 mL TBS(10×), 20 mL 10% Tween 20, 1,780 mL H_2O
5. 5% nonfat dry milk (NFDM) in freshly prepared TBST(1×)
6. See Blue Plus 2® pre-stained protein standards (Invitrogen)
7. ECL detection kit (Amersham)
8. HyBlot CL autoradiography film (Denville Scientific)
9. iBlot dry blot system and nitrocellulose transfer stack kit (Invitrogen)

2.4. Shotgun Proteomics

1. Ammonium bicarbonate (Fisher Scientific)
2. Acetonitrile (Sigma-Aldrich)
3. DTT (Bio-Rad)
4. Iodoacetic acid (Sigma-Aldrich)
5. Trifluoric acid (TFA, Sigma-Aldrich)
6. Formic acid (Sigma-Aldrich)
7. Sequencing-grade trypsin (Promega)
8. Micro-scale HPLC (HP1100, Agilent Technologies)
9. C4 reversed-phase LC column (Vydac, particle size, 5 μm; 250 × 2.1 mm I.D.)
10. Nano-LC system (Dionex)
11. QqTOF mass spectrometer (QSTAR XL, Applied Biosystems)
12. PicoTip emitter (New Objective)
13. LC Packings PepMap C18 column (75 μm × 150 mm; particle size, 5 μm)
14. LC mobile phase A for pre-fractionation: 0.1% formic acid
15. LC mobile phase B for pre-fractionation: ACN/0.1% formic acid
16. LC mobile phase A for LC-QqTOF MS: 95% H_2O/5% ACN/0.1% formic acid
17. LC mobile phase B for LC-QqTOF MS: 95% ACN/0.1% formic acid

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3. Methods

A carefully designed sample collection/pretreatment protocol is crucial to the success of a saliva proteomics project (*see* Notes 1 and 2). The proteins in saliva can be profiled first by 2D gel electrophoresis or liquid chromatography, followed by tryptic digestion, LC-MS/MS, and database search to identify the selected protein targets of interest. Western blotting can be used to quantify protein levels for validation purpose.

3.1. 2D Gel Electrophoresis of Saliva Proteins

3.1.1. Total Protein Assay—Total protein concentration of each sample was determined with 2D-Quant protein kit. 2D gel analysis can be performed on individual whole saliva samples or pooled samples (e.g., pooled cancer or control samples) prepared with equal contribution of proteins from each individual sample.

3.1.2. Protein Precipitation—

1. In order to precipitate proteins, add 9× volume of absolute ethanol (pre-chilled at −20°C) to a saliva sample, mix briefly, and then store at −20°C for at least 2 h or preferably overnight.
2. Spin the sample down at 14,000*g* for 20 min (4°C). Remove the supernatant and collect the remaining pellet.

3.1.3. IEF – First Dimension—

1. The pellet is then re-suspended in 300 μL (for 17 cm IPG strip) of rehydration buffer and 2 μL of ampholyte (pH 3-10NL) and vortexed for 30 s.
2. The re-suspended sample is then loaded into an IEF cell, and an IPG strip is layered on the top of the sample with gel side facing down.
3. After 45 min, cover the IPG strip with mineral oil to prevent evaporation and apply a low voltage (50 V) for active rehydration overnight.
4. Next day, discard the mineral oil and place the IPG strip in the IEF cell with gel side facing down. Insert wet filter paper wicks between electrodes and IPG strips to absorb salts during electrophoresis. Afterward, cover the IPG strips with mineral oil and apply IEF voltage program. For saliva protein samples, we use the following IEF program: 250 V (rapid), 5 h; 500 V (linear), 3 h; 500 V (rapid), 5 h; 3,000 V (linear), 4 h; 3,000 V (rapid), 4 h; 10,000 V (linear), 5 h; 10,000 V (rapid), 10 h; and 250 V (rapid), 99 h.

3.1.4. SDS-PAGE – Second Dimension—

1. After IEF, the IPG strip is rinsed with fresh 2% DTT and then 2.5% iodoacetic acid solutions for 10 min each.
2. After briefly rinsing with the TGS running buffer, the strip is placed onto an 8–16% Tris–HCl Protean II ready gel.

[1] Sample preparation is critical for proteomic analysis. A consistent protocol for sample collection and processing should be used for all saliva samples. A saliva sample must be centrifuged (typically 2,600*g* for 15 min) to remove cell pellets and debris in order to collect the supernatant for proteomic analysis.

[2] To discover and validate protein biomarkers in saliva, a patient/control sample set must be well matched in terms of age, gender, ethnicity, and important risk factors such as smoking history for oral or lung cancer.

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3. A paper wick blotted with pre-stained MultiMark® MultiColored protein standards was inserted at the left side of the gel and agarose solution was then used to seal the IPG strip.
4. SDS-PAGE is performed under a separation voltage of 100–200 V.

3.1.5. Staining and Image Analysis—

1. After fixing in 7% acetic acid–10% methanol for 30 min, stain the gel with the SYPRO Ruby protein stain in the dark for overnight.
2. Next morning, the gel is de-stained in 7% acetic acid–10% methanol in the dark for at least 2 h.
3. The gel images are then acquired with the Bio-Rad FX scanner and analyzed with the PDQuest program.
4. Gel spots of interest are excised using the ProteomeWorks spot cutter.

3.2. Identification of Proteins in 2D Gel Spots

3.2.1. In-Gel Tryptic Digestion

3.2.1.1. Wash Gel Spots:

1. Gel slices are washed with 100 μL of 50 mM NH_4HCO_3 and 50% CH_3CN in a 1.5 mL micro-centrifuge tube. Wash for 10 min and then remove the supernatant.
2. 30 μL of 100% CH_3CN is added to the tube. The gel slices are washed again for 10 min and the supernatant is removed. Step 1–2 may be repeated if necessary.
3. Finally, the gel slices are dried in a Speedvac for about 5–10 min.

3.2.1.2. Reduce Disulfide Bonds and Block Free Cysteine Sulfhydryl Bonds:

1. To reduce the disulfide bonds of proteins, 20 μL of 10 mM DTT is added to gel spots and incubated at 60°C for 1 h. The supernatant is discarded.
2. 20 μL of 50 mM iodoacetic acid is added to the gel spots. The incubation should be performed in the dark at 45°C for about 45 min and finally the supernatant is discarded.

3.2.1.3. Wash Gel Spots:

1. The gel spots are washed with 50 μL of 100 mM NH_4HCO_3 buffer for 10 min.
2. After the supernatant is removed, 50 μL of 100% CH_3CN is added for another 10-min washing and the supernatant is discarded.
3. Repeat steps 1 and 2 and dry the gel spots in a vacuum concentrator (e.g., Speedvac) for 5–10 min.

3.2.1.4. Digest Proteins in Gel and Extract Peptides from Gel Spots:

1. The dried gel spots are swelled in 10 μL of 20 ng/μL trysin on ice and incubated for 30–45 min.
2. Next, 10 μL of 100 mM NH_4HCO_3 is added and the digestion is allowed at 37°C overnight.
3. Next day, 20 μL of H_2O is added to each tube and the supernatant is transferred to a new 1.5 mL micro-centrifuge tube.

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4. 30 μL of 50% CH_3CN/0.1% TFA is added to the gel spots. After shaking at 150 rpm for 30 min, the supernatant is removed and added to the previous supernatant collected in step 3. This step is repeated twice.

5. Finally, the extracted peptides are dried in a Speedvac and redissolved in 10 μL of LC mobile phase A for LC-MS/MS analysis.

3.2.2. LC-MS/MS—The peptide samples are analyzed using nano-LC (Eksigent Technology) with linear ion trap MS (LTQ XL, Thermo-Fisher Scientific). The LC separation is performed with a PepMap C18 column (75 μm × 150 mm; particle size 3 μm, Dionex, Sunnyvale, CA) at a flow rate of 400 nL/min. The LC gradient elution started from 15% mobile phase B to 95% mobile phase B within 30 min and then held at 95% B for 20 min and finally put back immediately to 5% mobile phase B for a 15 min column equilibration. The eluent is introduced directly to the LTQ mass spectrometer via electrospray using a PicoTip emitter (tip inner diameter, 10 μm). Each full MS scan is followed by 5 data-dependent MS/MS scans on the most intense ions at a 35% normalized collision energy.

3.2.3. SEQUEST Database Searching—The acquired MS/MS data are searched against the human IPI (International Protein Index) database version 3.32 using the SEQUEST algorithm (Thermo-Fisher Scientific).

1. The search parameters are as follows: enzyme limit: partially enzymatic cleaves at either end; missed cleavage sites: 2; precursor peptide tolerance: 2.000 AMU; fragment ion tolerance: 1.000 AMU; modifications: carbamidomethylated cysteine (+57) and oxidized methionines (+16).

2. The filter parameters are as follows: delta CN = 0.100; RSp = 1; Xcorr vs. charge state = 2.0, 2.5, 3.0; peptide probability = 0.001; number of different peptides = 2.

3.3. Western Blot Analysis of Saliva Proteins

3.3.1. SDS-PAGE—First the total protein concentration of each individual sample is determined (*see* **Section 3.1.1**). Samples with same amount of proteins (for example, 20 μg) as well as See Blue Plus2 pre-stained protein standards are then loaded into gel wells. SDS-PAGE is run on 12% NuPAGE minigels (1 mm × 12 wells) in MES SDS running buffer at 100 V for 1–1.5 h (*see* Note 3).

3.3.2. Western Blotting—PAGE gels are removed from gel cassettes and rinsed gently in water. Then the gel is placed on the nitrocellulose membrane of an iBlot anode stack, followed by a pre-wetted filter paper and the cathode stack. The blot transfer is completed at 23 V within 6 min and the membrane is kept in a container for the following blotting steps.

1. *Blocking*: 5% NFDM/TBST(1×), 2 h at room temperature with gentle shaking.

2. *Washing*: TBST(1×), 30 min × 3 times with gentle shaking.

3. *Primary antibody incubation*: Primary antibody at the recommended concentration is prepared with 5% NFDM/TBST(1×). Incubate for 2 h at room temperature with gentle shaking.

4. *Washing*: TBST(1×), 30 min × 3 times with gentle shaking.

[3]The viscosity of saliva is high and may be different between samples. Prior to Western blot analysis, saliva samples must be denatured well in order to minimize the background. For validation of protein biomarkers using Western blotting, each individual patient sample should be tested side by side with its healthy control on the same gel.

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5. *Secondary antibody incubation*: Secondary antibody is prepared with 5% NFDM/TBST(1×). Incubation for 1 h at room temperature with gentle shaking.
6. *Washing*: Washed with 1× TBST, 30 min × 3 times with gentle shaking.
7. *Detection*: The A and B solutions of the ECL detection kit are mixed at a ratio of 1:1. The mixture solution is applied to the membrane blots carefully and the film is immediately developed in a dark room. The film is then scanned and analyzed using the Scion Image software.

3.4. Shotgun Proteomics

3.4.1. LC Pre-fractionation of Whole Saliva Proteins—Whole saliva samples (100 μg proteins in total each) are separated by HP1100 LC system (Agilent Technologies) using a Vydac C4 reversed-phase column (particle size, 5 μm; 250 × 2.1 mm inner diameter; The Nest Group, Inc.) at a flow rate of 250 μL/min (*see* Note 4). The LC gradient elution started from 5% mobile phase B to 85% mobile phase B within 40 min and then held at 85% B for 25 min. In total, 35 LC fractions were collected for each whole saliva sample (1 fraction per min).

3.4.2. In-Solution Digestion—

1. The proteins in each LC fraction are reduced with DTT (10 mM, 1 h), alkylated with iodoacetamide (55 mM, 1 h), and digested by trypsin (60 ng trypsin for each fraction) at 37°C for overnight.
2. The resulting peptide digests are dried, reconstituted in 0.1% formic acid, and then analyzed by capillary LC-quadruple time-of-flight (QqTOF) MS.

3.4.3. Capillary LC-QqTOF MS for Peptide Analysis—LC-MS/MS analysis is performed using a LC Packings nano-LC system (Dionex) with a nanoelectrospray interface (Protana) and QqTOF mass spectrometer (QSTAR XL, Applied Biosystems). The samples are first loaded onto a home-packed C18 precolumn (300 μm × 1 mm; particle size, 5 μm) and then injected onto a LC Packings PepMap C18 column (75 μm × 150 mm; particle size, 5 μm) for nano-LC separation at a flow rate of 250 nL/min. The LC gradient elution started from 5% mobile phase B to 60% mobile phase B within 55 min and then held at 95% B for 15 min and finally back immediately to 5% mobile phase A for a 15 min column equilibration. A New Objective PicoTip (tip inner diameter, 8 μm) is used for electrospraying with the voltage at 1,850 V for online MS and MS/MS analyses (*see* Note 5).

3.4.4. MASCOT Database Searching—MASCOT (version 1.9; Matrix Science) is used for database searches with search parameters containing the following modifications: carbamidomethylated cysteine (+57) and oxidized methionines (+16). All searches are performed against the human IPI database with one missed cleavage allowed and a mass tolerance of 0.3 Da for both precursor and product ions. A Mascot score with $P < 0.05$ is considered a significant match of a peptide (*see* Note 6).

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[4]C4 reversed-phase column is often used for LC separation of proteins whereas C18 column is often used for separation of peptides. Besides reversed-phase LC, other separation techniques including ZOOM IEF, size-exclusion LC, free-flow electrophoresis, ultracentrifugation, and ion-exchange LC can be used to pre-fractionate whole saliva proteins.

[5]Large-scale identification of whole saliva proteins can be accomplished using MudPIT (Multidimensional Protein Identification Technology), which is a powerful technique for the separation and identification of complex protein and peptide mixtures (21). MudPIT is based on direction insolution digestion of proteins followed by 2D liquid chromatography (strong cation exchange and reversed-phase LC) and MS/MS for identification of the resulting peptides.

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