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METHODS PAGE

Proteomic analysis (GeLC-MS/MS) of endoscopically (ePFT) collected pancreatic fluid

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Introduction

Mass spectrometry-based proteomics offers a means for enhancing our understanding of disease pathogenesis and progression of the exocrine pancreas. For example, the diagnosis of chronic pancreatitis can often remain elusive in mild disease due to a scarcity of predictable and reliable radiological and endoscopic abnormalities of the pancreas. Improved strategies for the diagnosis and treatment of chronic pancreatitis are necessary to reduce healthcare and patient burdens.

Pancreatic fluid is secreted by the exocrine pancreas and can be acquired safely after secretin or cholecystokinin (CCK) stimulation with an endoscope (EGD, esophagogastroduodenoscopy; EUS, endoscopic ultrasound; ERCP, endoscopic retrograde cholangiopancreatography), Dreiling tube [7, 9], or Liguory tube [11]. The ePFT collection method, which is much less invasive compared to ERCP and surgery, permits the safe collection of 10-fold larger volumes of pancreatic fluid, making it a well-suited method for comprehensive proteome analysis [3-5, 21-23].

We use GeLC-MS/MS (in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry), a powerful approach for proteomic analyses, for analysis of ePFT-collected pancreatic fluid, [17,18]. Using this technique, proteins are fractionated using one-dimensional SDS-PAGE and entire gel lanes are excised and further subdivided into smaller sections to allow for efficient processing. The proteins in these gel sections are subsequently digested in-gel with trypsin and the generated peptides are subjected to a nanoflow reversed-phase LC-MS/MS experiment to obtain peptide sequence information and hence identify the proteins present in a particular sample of pancreatic fluid.

Application of proteomics to the study of pancreatic disease may accelerate the discovery of physiologically- and clinically-relevant biomarkers. The analysis of a *proximal* body fluid, such as pancreatic fluid, will increase the likelihood of biomarker discovery in the context of particular diseased organs (i.e., pancreas) [16]. Pancreatic fluid is an excellent clinical specimen for identification of novel biomarkers as its protein composition is of lower complexity compared to serum and such proteins predominantly originate from the exocrine pancreas. Biomarkers discovered from pancreatic fluid may provide both a method for the diagnosis and/or prognosis of pancreatic diseases, and may also generate insights into the pathophysiology of the pancreas. We are able to obtain over 1000 unique proteins in the collected fluid when combining data from multiple patients (manuscript in preparation). We acknowledge that these proteins originate from an admixture of pancreatic, biliary, and gastroduodenal fluid. However, the majority of the proteins include known pancreatic enzymes, and proteins involved in inflammation and fibrosis. Our data has shown that ePFT sample collection in tandem with LC-MS/MS-based proteomics is a valuable methodology for investigation pancreatic disease [13]. The current communication details the methods and procedures for collection and analysis of these proteins.

1. Materials

1.1 Preparation of pancreatic fluid

Pancreatic fluid is collected by the secretin-stimulated endoscopic pancreatic fluid (ePFT) method that can aspirate large amounts of pancreatic fluid for laboratory analysis [4]. The ePFT collection method replaces the gastroduodenal (dual lumen) tube (Dreiling tube) with an upper endoscope [3]. We direct the reader to a recent article for further description of the secretin-stimulated ePFT [23].

The BioRAD Protein assay (cat.no.: 500-006, Hercules, CA) was used to determine the protein concentration of the pancreatic fluid prior to protein extraction.

1.2 Trichloroacetic acid (TCA) precipitation

Trichloroacetic acid (TCA) crystals (cat. no.: 641730) was purchased from CalBioChem (San Diego, CA). A 100% solution was made with water (Honeywell, AH365-4) as a solvent and stored at 4°C.

1.3 SDS-PAGE

Electrophoresis equipment, buffers, and reagents was purchased from Invitrogen (Carlsbad, CA). These items include: XCell SureLock™ Mini-Cell (cat. no.: EI0001), SeeBluePlus2 Pre-Stained standard (cat. no.: LC5925), LDS (lithium dodecyl sulfate) sample buffer (cat. no.: NP0008), NuPAGE 4-12% Bis-Tris polyacrylamide gels (cat. no.: NP0335), SimplyBlue Coomassie stain (cat. no.: LC0665), and MES-SDS (2-(N-morpholino)ethanesulfonic acid-sodium dodecyl sulfate) buffer (cat. no.: NP002). Dithiothreitol (DTT, cat. no.: 43817) and acrylamide (cat. no.: A3553) were purchased from Sigma (St. Louis, MO).

1.4 In-gel tryptic digestion

Trypsin (sequencing grade, V5111) was purchased from Promega (Madison, WI). The lyophilized protease was reconstituted in 200 µL of the manufacturer's provided reconstitution buffer and stored in 20 µL aliquots at -80°C until use.

1.5 LC-MS/MS

Samples were loaded into liquid chromatography polypropylene plastic vials with silicone septa (cat. no. 186002640; Waters, Milford, MA). An Eksigent NanoLC Ultra 1D plus (Dublin, CA) or equivalent high pressure liquid chromatography system or equivalent may be used for peptide fractionation prior to mass spectrometry analysis. Reverse-

phase liquid chromatography columns (15 cm x 100 µm ID) were packed in-house with Magic C18 beads (5 µm, 100 Å beads, Michrom BioResources (Auburn, CA), into 100 µm PicoTip emitter tips (cat. no. FS360-100-15-N-5-C30; New Objective, Woburn, MA). As is typical of LC-MS/MS systems, this instrument is in line with the mass spectrometer. An LTQ-FTICR (linear trap quadrupole-Fourier transform ion cyclotron resonance) Ultra from Thermo (Waltham, MA) or equivalent mass spectrometer may be used for peptide analysis.

1.6 Bioinformatics

Several computational algorithms have been developed to sequence proteins by mass spectrometry, as the sample complexity of tandem MS/MS spectra precludes a comprehensive, manual interpretation of all spectra. Software packages and associated algorithms, such as SEQUEST [8], Mascot [15], Phenyx [2], ProteinPilot [19], X!Tandem [6], and ProteinProspector [1] may be used to search a given sequence database for peptides with theoretical spectra best matching the observed spectra, and subsequently assign these peptides to the corresponding proteins. Below we describe the procedure for data analysis using ProteinPilot (AB SCIEX, Foster City, CA).

2. Methods

Figure 1 illustrates our optimized sample preparation strategy for GeLC-MS/MS of ePFT-collected pancreatic fluid.

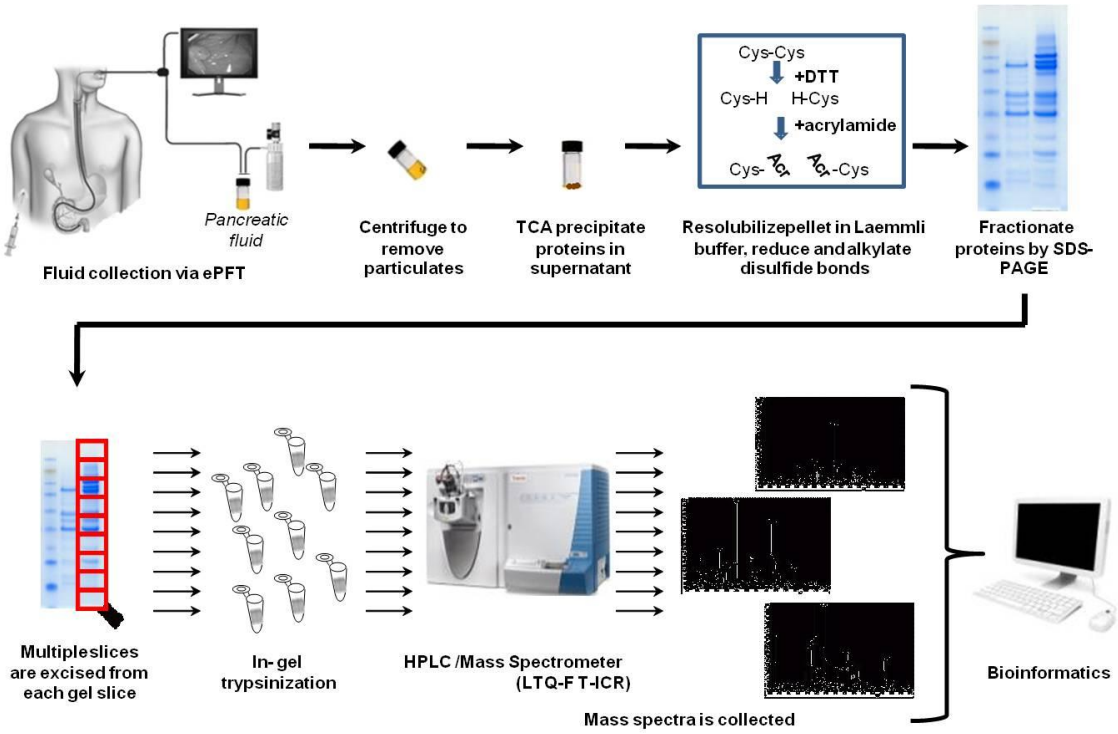

Figure 1: Workflow for proteomic analysis of pancreatic fluid. Pancreatic fluid is collected via ePFT, particulates are removed with centrifugation, proteins are extracted from the remaining supernatant with trichloroacetic acid (TCA), the protein pellet is reconstituted, reduced with dithiothreitol (DTT) and alkylated with acrylamide (Acr) prior to analysis by SDS-PAGE. GeLC-MS/MS analysis is performed, in which gel lanes are divided into smaller segments that are individually in-gel tryptically digested. Digested peptides are eluted from a reversed-phased high performance liquid chromatography (HPLC) column into a mass spectrometer for accurate mass analysis. The resulting mass spectra are processed to identify corresponding peptides and determine the proteins from which these peptides originate.

2.1 Pancreatic Fluid Collection (ePFT method)

1. *Pre-procedural assessment:* Prior to upper endoscopy, all study subjects underwent a history and physical examination including list of allergies, medications, substance use/abuse, and vital signs. Pre-procedural sedation review included airway assessment based on Mallampati airway scale and American Society of Anesthesiologists Physical Status Classification (ASA Class). All study subjects in this protocol had a Mallampati score of B, Class 2 and ASA Class II or better.
2. *Procedure:* Endoscopic collection was performed in a stepwise manner as follows:
    a. The patient was placed in the left lateral decubitus position with slight head elevation.
    b. The posterior pharynx is sprayed with topical cetacaine spray.
    c. A sedation and analgesia bolus was administered.
    d. Further sedation doses were administered if necessary for patient comfort.
    e. After the sedation bolus, a bite-block was placed.
    f. fEsophagogastroduodenoscopy (EGD) was performed using a standard (10 mm) or thin (6 mm) gastroscope for visualization of the esophagus, stomach, and duodenum (2 to 5 minutes).
    g. Gastroduodenal fluid was aspirated (approximately 1 minute) as completely as possible through the gastroscope.
    h. A test dose of synthetic human hormone secretin (ChiRhoStim®) was administered and patients were monitored for anaphylaxis or adverse reaction, followed by a standard weight-based intravenous bolus (0.2 µg/kg) given over 1 minute.
    i. Pancreatic fluid was aspirated from the descending duodenum at specific timed intervals following hormonal stimulation and stored on ice.
    j. The duodenal aspirates were collected at 0, 5, 10, 15, 20, 30, 45 and 60 minutes after stimulation. Only the 30-minute time point was used for the ensuing proteomic analysis.
    k. Biopsies of the stomach and duodenum were obtained to rule out microscopic gastrointestinal disease, such as *Helicobacter pylori* or celiac sprue, as a cause of dyspepsia.
3. *Post-procedural Assessment / Recovery:* Study participants were discharged from the endoscopy unit based on hospital procedural sedation guidelines assessing levels of consciousness, vital signs, oxygen saturation, alertness, gag reflex, degree of nausea, and ability to ambulate.

2.2 Preparation of pancreatic fluid

1. Pancreatic fluid samples for proteomic analysis were collected on ice, centrifuged at 4°C at 14,000 rpm for 15 minutes to remove cellular debris, and aliquoted (500 µL) prior to storage at -80°C.
2. Protein concentration was determined using the BioRAD protein assay according to the manufacturer's instructions. The microtiter assay was used and readings of optical density at 570 nm were obtained on a BioRAD model 450 plate reader.

2.3 Trichloroacetic acid (TCA) precipitation

1. In preparation for SDS-PAGE analysis, the proteins from pancreatic fluid specimens were isolated by precipitation with the addition of 12.5% trichloroacetic acid (TCA), as previously described [13, 14] (see Note 1).
2. Ice-cold 100% trichloroacetic acid (25 µL, TCA) was added to 200 µL of pancreatic fluid, vortexed and incubated at 4°C for 2 hours.
3. The sample was centrifuged at 20,000×*g* at 4°C for 30 min and the supernatant was carefully aspirated.
4. One milliliter of 100% ice-cold acetone was added to the pellets which were briefly vortexed and incubated at -20°C for 1 hour.
5. The sample was centrifuged at 20,000×*g* at 4°C for 30 min and the pellet was washed twice with 100% ice-cold acetone. The final pellets were allowed to air dry at 23°C.

2.4 SDS-PAGE

1. The precipitated protein pellets were re-dissolved in 50 µL of reducing Laemmli buffer [21] (with 10 mM DTT for 1 hr at 56°C and alkylated with 1% acrylamide at room temperature for 30 minutes for subsequent GeLC-MS/MS analysis (see Note 2).
2. The proteins were fractionated using 4-12% NuPAGE pre-cast SDS-PAGE gels at 175V for 45 min using MES-SDS running buffer (see Note 3).
3. Gels were rinsed with water for 5 min, fixed in 45% methanol, 10% acetic acid for 30 min and stained with SimplyBlue Coomassie stain for 1.5 hr. Gels were destained in water overnight. Figure 2 illustrates a representative gel.

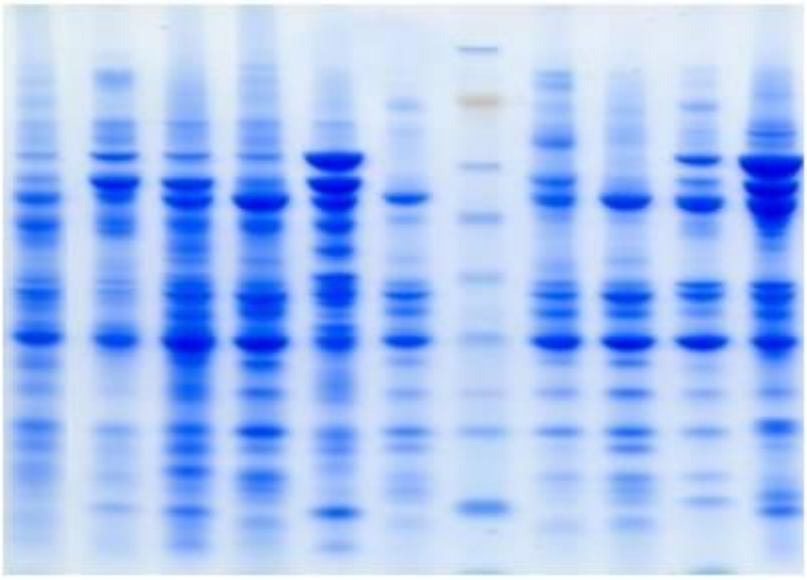

Figure 2: SDS-PAGE image of pancreatic fluid processed using our optimized sample preparation protocol. Pancreatic fluid from 10 patients were TCA precipitated (**Section 2.3**) and equal amounts (~100 µg) were analyzed via SDS-PAGE (**Section 2.4**)

2.5 In-gel tryptic digestion

1. Subsequently, entire gel lanes were divided into 15 sections. Proteins in each gel section were digested in-gel with trypsin [12, 20].
2. Gel slices were excised from the partially-destained Coomassie-stained polyacrylamide gel and washed four times with 200 µl of 50% acetonitrile, 50% 50 mM ammonium bicarbonate solution for 30 min at 37°C. Some

protocols include an in-gel reduction and alkylation step; however, as these two steps have been performed during SDS-PAGE sample preparation, they may be omitted.

3. Gel slices were then dehydrated by adding 200 µl of neat acetonitrile and incubated at room temperature for 15 min.
4. After removing the acetonitrile and drying for 30 min at 37°C, the gel slices were rehydrated with the addition of 10 µl of 25 mM ammonium bicarbonate, pH 8.0 containing 200 ng of trypsin.
5. Following a 30 min room temperature incubation, an additional 50 µl of 25 mM ammonium bicarbonate was added to completely immerse the gel slices in buffer.
6. After an overnight incubation at 37°C, the peptides were extracted with 40 µl of 1% formic acid in increasing concentrations of acetonitrile (0%, 50%, and 100%).
7. These fractions were combined in a single low-retention microcentrifuge tube and vacuum centrifuged to dryness.

2.6 LC-MS/MS

1. The extracted peptides from each gel section were reconstituted in 20 µL of MS-loading buffer (5% formic acid, 5% acetonitrile in water).
2. A 5 µL aliquot of sample was placed in an LC vial and subjected to peptide fractionation using reversed-phase high performance liquid chromatography (HPLC) prior to analysis using an in-line LTQ-FTICR Ultra mass spectrometer.
3. Peptides were fractionated using a 60 or 90 min linear gradient (0 to 35% acetonitrile with 0.2% formic acid), followed by a steeper 5 min gradient (from 35% to 100% acetonitrile with 0.2% formic acid). The flow rate of the HPLC system was set to 750 nL/min for sample loading and 500 nL/min for the gradient elution. HPLC solvents for LC-MS/MS were as follows: buffer A: HPLC grade water (Honeywell, cat. no.: AH365-4) with 0.2% formic acid (Sigma, cat. no.: 27001), buffer B: HPLC grade acetonitrile (Honeywell, cat. no.: AH015-04) with 0.2% formic acid.
4. Data were obtained using data dependent acquisition mode, in which MS/MS fragmentation is performed on the 6 most intense peaks of every full MS scan. MS/MS parameter settings for the LTQ-FTultra were as follows: the activation type was CID, the isolation width was 3.2 Th, the normalized collision energy was 32, activation Q was 0.25 and the activation time was 30 msec. In addition, charge screening was enabled and singly charged ions were rejected for MS/MS fragmentation.

2.7 Bioinformatics

1. RAW files were converted to the mascot generic format (mgf) using in-house software. Alternatively, Mascot distiller and/or Proteome Discoverer may also be used for the RAW to mgf file conversion.
2. Mgf files of data collected for entire gels lanes were searched together against the IPI-human database (v3.61) using the Paragon Algorithm [19] integrated into the ProteinPilot search engine (v.3).
3. Search parameters were set as follows: sample type, identification; Cys alkylation, acrylamide; Instrument, Orbitrap/FT (1-3 ppm); special factors, gel-based ID; ID focus, none; database, international protein index (IPI) human (v.3.61); detection protein threshold, 99.0%; and search effort, thorough ID. Thus, using our stringent criteria, we defined an identified protein with $\geq$99% confidence, as determined by the Paragon Algorithm.

4. Protein lists were exported into Microsoft Excel, or analogous spreadsheet program for ease of comparison and manipulation.

3. Notes

1. TCA precipitation limits protein degradation by instantaneously deactivating enzymes and removing salts that will interfere with the subsequent electrophoretic mobility-based fractionation by SDS-PAGE, as described below. We have determined that protease inhibitors at the recommended concentration (e.g. Roche Complete, cat # 11 836 153 001) do not effectively inhibit protease activity, likely due to the high concentrations of these proteases. Higher concentrations of inhibitors may be effective; however, to date we are unaware of such studies. Rapid freezing, handling on ice, and TCA precipitation has produced robust results.
2. DTT reduces disulfide bonds permitting protein unfolding/denaturing prior to electrophoresis. The 10 mM final concentration of DTT was diluted from a 1 M aqueous stock solution which is stored at -20°C in small, single-use aliquots. The alkylation step is essential to prevent reformation of disulfide bonds during SDS-PAGE. The 1% acrylamide was diluted from a 40% aqueous stock solution which is stored at 4°C.
3. MOPS (3-(N-morpholino)propanesulfonic acid) buffer was tested in place of MES, however bands were more distorted and less resolved when using MOPS instead of MES. Other buffer systems may also produce comparable results.

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