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Difference gel electrophoresis (DiGE) identifies differentially expressed proteins in endoscopically-collected pancreatic fluid

Joao A. Paulo, PhD,
Department of Pathology, Children's Hospital Boston and Harvard Medical School, Boston, MA

Proteomics Center at Children's Hospital Boston, Boston, MA

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Linda S. Lee, MD,
Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Peter A. Banks, MD,
Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Hanno Steen, PhD[#], and
Department of Pathology, Children's Hospital Boston and Harvard Medical School, Boston
Proteomics Center at Children's Hospital Boston, Boston, MA

Darwin L. Conwell, MD, MS[#]
Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Abstract

Alterations in the pancreatic fluid proteome of individuals with chronic pancreatitis may offer insights into the development and progression of the disease. The endoscopic pancreas function test (ePFT) can safely collect large volumes of pancreatic fluid that are potentially amenable to proteomic analyses using difference gel electrophoresis (DiGE) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Pancreatic fluid was collected endoscopically using the ePFT method following secretin stimulation from three individuals with severe chronic pancreatitis and three chronic abdominal pain controls. The fluid was processed to minimize protein degradation and the protein profiles of each cohort, as determined by DiGE and LC-MS/MS, were compared. This DiGE-LC-MS/MS analysis reveals proteins that are

Corresponding author: Darwin L. Conwell, Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, 75 Francis St., Boston, MA 02115, dconwell@partners.org, phone: (617) 732-6747, fax: (617) 566-0338.
[#]these authors contributed equally to this work

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AUTHOR CONTRIBUTIONS
JP carried out the experiments and drafted the original manuscript. JP and DC conceived of the study. JP, DC, PB and HS participated in its design and coordination. DC and LL collected the specimens and assisted in experimental design. All authors helped to draft the manuscript and approved the final manuscript.

differentially expressed in chronic pancreatitis compared to chronic abdominal pain controls. Proteins with higher abundance in pancreatic fluid from chronic pancreatitis individuals include: actin, desmoplankin, alpha-1-antitrypsin, SNC73, and serotransferrin. Those of relatively lower abundance include carboxypeptidase B, lipase, alpha-1-antichymotrypsin, alpha-2-macroglobulin, Arp2/3 subunit 4, glyceraldehyde-3-phosphate dehydrogenase, and protein disulfide isomerase. Endoscopic collection (ePFT) in tandem with DiGE-LC-MS/MS is a suitable approach for pancreatic fluid proteome analysis, however, further optimization of our protocol, as outlined herein, may improve proteome coverage in future analyses.

Keywords

pancreas; pancreas juice; pancreatic function test; biomarkers

Introduction

Chronic pancreatitis is characterized by chronic inflammation and progressive scarring leading to irreversible functional damage to the pancreas, resulting in pain, malabsorption of fat and protein, loss of endocrine function, and in some cases, pancreatic carcinoma [1; 2]. Currently diagnosis of this progressively debilitating disease is limited to moderate and advanced disease stages. A better understanding of chronic pancreatitis pathogenesis is necessary to elucidate mechanisms of early disease.

The application of proteomics to the study of pancreatic disease may accelerate the discovery of physiologically- and clinically-relevant biomarkers. Moreover, the analysis of a proximal body fluid, such as pancreatic fluid, increases the likelihood of biomarker discovery in the context of a particular diseased organ (i.e., the pancreas). Pancreatic fluid is an excellent clinical specimen for such analyses, as its protein composition is of lower complexity compared to serum and because the proteins in pancreatic fluid predominantly originate from the exocrine pancreas [3; 4].

We have developed and described an endoscopic pancreatic function test (ePFT) that collects pancreatic fluid without cannulation of the pancreatic duct and without the risk of procedure-related injury [5; 6; 7]. The ePFT collection method replaces the Dreiling tube with an upper endoscope [5]. Dreiling tubes allow for the collection of duodenal and gastric fluid during pancreatic function testing (PFT) [8], but the placement of Dreiling tubes, can be time consuming, cumbersome, and requires fluoroscopy. In addition, traditional endoscopic retrograde cholangiopancreatography (ERCP) has also been utilized to collect pancreatic fluid directly from the pancreatic duct [9]. Similarly, ERCP is highly invasive and is also associated with significant risks (5–10%) for the development of acute pancreatitis in patients [10; 11].

In contrast to the traditional pancreatic fluid collection methods, ePFT leads to significantly less morbidity for the patients, lowers cost and allows for the collection of larger volumes of fluid. Furthermore, the ePFT is now considered an acceptable alternative for the assessment of pancreas secretory physiology [12; 13]. It is possible that some of the proteins collected are from gastric or duodenal origin. However, duodenal protein secretion is minimal, and the efflux of gastric fluid is limited by placing the patient in the left lateral decubitus position during the procedure. Moreover, both fluids, (collectively known as gastroduodenal fluid [14]) are evacuated prior to ePFT and any remnants are subsequently diluted by the protein-rich secretin-stimulated pancreatic secretions. As the ePFT collection method is a valuable tool for acquiring pancreatic fluid, even from individuals without pancreas-related disease, we aim to evaluate this technique, coupled with DiGE, as a suitable approach to investigate

differential protein secretion in the pancreatic fluid of individuals with chronic pancreatitis and chronic abdominal pain.

Difference gel electrophoresis (DiGE), followed by tandem mass spectrometry (MS/MS), may be a valuable strategy to study and understand better the abnormalities in the proteomic profiles of individuals with chronic pancreatitis. In DiGE, two samples can be compared by labeling each with a specific fluorescent dye which has unique spectral properties. Following two-dimensional gel separation, gels are imaged at each dye-specific wavelength. The merged images reveal overlapping or uniquely labeled gel spots, which can be excised and in-gel digested. The resulting peptides can be analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for protein identification. We apply this mass spectrometry-based proteomics strategy to investigate protein differences in pancreatic fluid from individuals with severe chronic pancreatitis and chronic abdominal pain controls. Although our long-term goals involve the elucidation of biomarkers of early chronic pancreatitis, we have chosen to investigate two extremes of the disease so as to gain insight into what proteins to target, as no early state diagnosis is possible currently.

The primary objectives of our exploratory investigation are as follows: 1) collect pancreatic fluid with the ePFT method after secretin stimulation, 2) analyze pancreatic fluid using DiGE, 3) determine the identity of proteins that are differentially secreted in the pancreatic fluid of chronic pancreatitis and chronic abdominal pain controls using LC-MS/MS techniques, and 4) assess the utility of DiGE-LC-MS/MS analysis for the differential proteome analysis of pancreatic fluid.

The methodology established herein enables further comparative analysis of the proteins in the ePFT-collected pancreatic fluid secretome of healthy individuals and those with pancreatic disease, thus broadening our knowledge of pancreatic disease pathogenesis.

Materials and Methods

Study Design and Setting

Proteomic analysis of endoscopically collected pancreatic fluid in an academic center.

Study Population

This protocol was approved by the Institutional Review Board at Brigham and Women's Hospital (IRB # 2007-P-002480/1). The study population (Table 1) included adult patients seen in the Center for Pancreatic Diseases at Brigham and Women's Hospital for abdominal pain and dyspepsia. Subjects were referred to the *Center for Pancreatic Disease* to eliminate pancreas etiologies for their gastrointestinal symptoms. All subjects underwent the following: 1) comprehensive history and physical examination, 2) review of radiologic and endoscopic data, and 3) upper endoscopy with ePFT followed by mucosal biopsy. The diagnosis of chronic pancreatitis was deemed definitive according to the M-ANNHEIM (Multiple risk factors, Alcohol, Nicotine, Nutrition, Hereditary factors, Efferent duct factors, Immunological factors, and - Miscellaneous and metabolic factors) classification [15]. The M-ANNHEIM classification is a standardized system designed to classify chronic pancreatitis according to etiology, clinical staging, and severity of the disease [15]. This system considered clinical imaging data resulting from a wide array of laboratory test results, including ultrasound (US), endoscopic ultrasound (EUS), magnetic resonance imaging (MRI), computed tomography (CT) as well as other risk factors [15; 16].

Materials

ChiRhoStim® synthetic human secretin was from ChiRhoClin (Burtonsville MD). Other reagents and solvents were from Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Morristown, NJ), respectively.

Experimental Workflow

The overall analysis is shown in Figure 1: 1) ePFT sample collection, 2) trichloroacetic acid precipitation, 3) DiGE analysis, 4) in-gel tryptic digestion followed by liquid chromatography-coupled tandem mass spectrometry (GeLC-MS/MS), and 5) bioinformatic analysis.

Pancreatic Fluid Collection (ePFT method)

The ePFT procedure was performed in three major stages, as follows: 1) pre-procedural assessment, 2) endoscopic procedure and 3) post-procedural assessment/recovery.

A. Pre-procedural assessment—Prior to upper endoscopy, all study subjects underwent a history and physical examination including list of allergies, medications, substance use/abuse, vital signs, and physical examination. 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.

B. Procedure—Endoscopic collection was performed at Brigham and Women's Hospital endoscopy unit as follows: 1) the patient was placed in the left lateral decubitus position with slight head elevation. 2) The posterior pharynx was sprayed with topical cetacaine spray. 3) A sedation and analgesia bolus was administered. 4) Further sedation doses were given if necessary for patient comfort. 5) After the sedation bolus, a bite-block was placed. 6) Esophagogastroduodenoscopy (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). 7) Gastroduodenal fluid [14] was aspirated (approximately 1 minute) as completely as possible through the gastroscope. 8) A test dose of synthetic human 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. 9) Pancreatic fluid was aspirated from the descending duodenum at specific timed intervals (0 to 60 minutes) following hormonal stimulation and stored on ice. Only the 30-minute time point for each patient was used for the ensuing analysis. Samples were divided and sent to the Brigham and Women's Hospital Biochemistry Laboratory for measurement of electrolyte profiles and the Proteomics Center at Children's Hospital Boston for proteomic analysis. Approximately 5–10 mL of pancreatic fluid was collected from each patient at the 30 minute time point. Biopsies of the stomach and duodenum were obtained to eliminate microscopic gastrointestinal disease, such as *Helicobacter pylori* or celiac sprue, as a cause of dyspepsia/abdominal pain.

C. Post-procedural Assessment / Recovery—Study participants recovered and were discharged from the endoscopy unit based on hospital procedural sedation guidelines assessing level of consciousness, vital signs, oxygen saturation, alertness, gag reflex, degree of nausea, and ability to ambulate.

Pancreatic Fluid Biochemical Analysis

Pancreatic fluid samples were frozen at −80°C and stored until analysis; all measurements were conducted within two weeks of sample collection in the CLIA-certified Brigham and

Women's Hospital Clinical Chemistry Laboratory with an AU640 (Olympus America, Center Valley, PA) automated chemistry analyzer. Total bicarbonate was measured by the two-step phosphoenolpyruvate carboxylase-malate dehydrogenase enzymatic-photometric method [17]. The mean peak bicarbonate concentration from previously published studies in secretin-stimulated pancreatic fluid is 103 ± 11 meq/l [18]. The cut-off point of 80 meq/L was two standard deviations below the mean and considered abnormal [19].

Pancreatic Fluid Sample Preparation for DiGE and Mass Spectrometry

Pancreatic fluid was processed as described previously [20]. In brief, pancreatic fluid samples were collected on ice, centrifuged at 4°C at 14,000 rpm to remove cellular debris, and aliquoted (500 μL) prior to storage at −80°C. Protein concentration was determined using the BioRAD protein assay (BioRAD, Hercules, CA) according to the manufacturer's instructions. The protein concentration of pancreatic fluid typically ranges from 0.6–1.2 mg/mL. The proteins were extracted from an aliquot of pancreatic fluid via precipitation with the addition of 12.5% trichloroacetic acid (TCA) [21]. This process limits protein degradation by instantaneously deactivating enzymes and removing salts that will interfere with the subsequent electrophoresis. Approximately 50 μg of pancreatic fluid protein from three different subjects were pooled, so that a total of 150 μg of protein could be labeled with the appropriate Cy dye.

Difference Gel Electrophoresis (DiGE) Analysis

The lyophilized samples were transported on dry ice to the W. M. Keck facility at Yale University (New Haven, CT). The samples were dissolved in buffer (7M urea, 2M thiourea, 4% CHAPS (w/v), 25 mM Tris, pH 8.6 at 4°C). The pancreatic fluid from the chronic abdominal pain (CAP) controls was labeled with Cy-3 dye and the fluid from the chronic pancreatitis (CP) subjects was labeled with Cy-5 dye.

For the first dimension isoelectric focusing gel, the labeled samples were pooled and mixed with 400 μL rehydration buffer containing 7 M urea, 2M thiourea, 4% CHAPS (w/v), 1% DTT (w/v), 2% (v/v) Pharmalytes pH 3–10 and a trace amount of bromophenyl blue, and loaded onto 24 cm pH 3–10 linear IPG strips (G.E. Healthcare) by reswelling for 3 hours at 20°C. Isoelectric focusing was performed on an Ettan IPGphor 3 (GE Healthcare) for approximately 60 kVh at 20°C, 50 μA/strip using the following voltage gradient: (i) 1 h at 30 V, (ii) 1 h at 500V, (iii) 1 h at 1000V, then approximately an 8 h linear gradient to 8000V continuing until reaching 60kVh. After focusing, IPG strips were incubated with an equilibration buffer containing 6M urea, 10mM Tris (pH 6.8), 30% glycerol (w/v), 1% SDS (w/v), and 2% DTT for 15 minutes at room temperature. This solution was replaced with equilibration buffer containing 5% iodoacetamide for another 10 minutes. For the second dimension gel, IPG strips were applied to 22 × 24 cm SDS-PAGE gels (12% T, 2.6% C) (Jule, Inc., Milford, CT), which were run overnight at 125V (constant) and 15 °C in an Ettan DALT twelve electrophoresis chamber (GE Healthcare, Fairfield, CT).

For image acquisition, gels were scanned using a Typhoon 9410 Imager (GE Healthcare, Fairfield, CT). Cy3 images were scanned using a 532 nm laser and an emission filter of 580 nm. Cy5 images were scanned using a 633 nm laser and a 670 nm Band Pass 30 Hz emission filter. Photomultiplier voltage was adjusted for each channel to minimize any signal saturation. All gels were scanned at 100μm resolution, and images were further processed using ImageQuant V5.0 (GE Healthcare, Fairfield, CT) prior to analyses on DeCyder (GE Healthcare, Fairfield, CT) software. Gel image analysis was performed using DeCyder v6.5 (GE Healthcare, Fairfield, CT). Spot detection was conducted on image pairs consisting of each sample from the same gel. These two images overlay and allow direct measurement (if applicable) of volume ratios of spots between the standard and the sample.

Selected spots were excised using an Ettan Spot Picker instrument (GE Healthcare, Fairfield, CT).

Mass spectrometry

Mass spectrometry analysis of excised gel plugs was performed at the Proteomics Center at Children's Hospital Boston. Proteins in each gel plug were digested in-gel with trypsin [22; 23]. The extracted peptides from each gel plug were subjected to peptide fractionation using reversed-phase high performance liquid chromatography (HPLC; Thermo Scientific) and the gradient-eluted peptides were analyzed by an in-line LTQ FT mass spectrometer (Thermo Scientific). The liquid chromatography columns (15 cm × 100 μm ID) were packed in-house (Magic C18, 5 μm, 100 Å, Michrom BioResources, Auburn, CA, into PicoTips, New Objective, Woburn, MA). Samples were analyzed with a 30 minute linear gradient (0–35% acetonitrile with 0.2% formic acid) with a constant flow rate of 400 nL/min. Data were acquired in a data-dependent manner, in which MS/MS fragmentation was performed on the six most intense peaks of every full MS scan.

Bioinformatics and Data Analysis

All data generated from the gel spots were searched against the IPI-human database (v3.36) using the Mascot search engine (v.2.204; Matrix Science). One miscleavage per peptide was allowed and mass tolerances of ± 10 ppm (monoisotopic) for precursor and of ± 0.8 Da for fragment ions were used, as was default for LTQ FT-ICR data analysis. Variable amino acid modifications: deamidation (Asn/Gln), pyro-glutamate (N-terminal Glu/Gln), CyDye-Cy5 (Cys), CyDye-Cy3 (Cys) and oxidation (Met). Mascot search results were combined using in-house-developed software. In strict compliance with a set of recommendations [24; 25; 26] proposed by the major proteomic journals, we present the following protein identification validation method that minimizes false positives and reports only high confidence identifications. Our false discovery rate (FDR) was 1% at the peptide level as determined by searching the same dataset against the target database and a decoy database; the latter featured the concatenated forward and reversed amino acid sequences of all the entries in the IPI-human database (v3.36) [27; 28]. We applied the following stringent identification criteria for protein identifications to ensure a false positive rate of ≤ 0.1% at the *protein* level: 1) a minimum of 2 unique peptides was required for protein identification, 2) each peptide had a score equal or greater than the 1% FDR cut-off (see above), and 3) each matched peptide corresponds to the highest scoring peptide for that MS/MS spectra. Gene ontology (GO) classification of subcellular localization and biological function were extracted from the UniProt database [29].

Results

Demographics of study population

Pancreatic fluid was safely collected via secretin-stimulated ePFT from the duodenum of all six (6) subjects (Table 1). Of these individuals, three were diagnosed with definite chronic pancreatitis (CP) and the remaining three served as chronic abdominal pain (CAP) controls, without evidence of pancreatic dysfunction and structural abnormalities. The three individuals with chronic pancreatitis were classified as such according to the M-ANNHEIM classification [15]. Conversely, the M-ANNHEIM criteria for chronic pancreatitis were not met for any of the three chronic abdominal pain controls. The mean peak bicarbonate levels of the individuals with chronic pancreatitis and chronic abdominal pain controls were 43 and 97 meq/L, respectively (p-value=0.0070).

Difference gel electrophoresis (DiGE) analysis revealed differentially-imaged gel spots

The image of the Cy3-conjugated chronic abdominal pain sample (Figure 2A) differed substantially from that of the Cy5-conjugated chronic pancreatitis sample (Figure 2B). When the two images were superimposed, 65 spots were identified as being differently abundant in the two samples (Figure 3). Of these 65 protein spots, 30 were more intense in the samples originating from the chronic abdominal pain controls, while 35 were more intense for chronic pancreatitis fluid samples.

Using the intensity measurement at the appropriate wavelength, the DeCyder software (GE Healthcare, Fairfield, CT) calculated the ratios (Cy5/Cy3 and −Cy3/Cy5) of the proteins in each spot with respect to the sample of origin. Of the 35 spots that were of higher intensity in the chronic pancreatitis subjects, 23 had greater than 1.5-fold increase over the controls, of which 9 had greater than a 3-fold difference. Similarly, when examining the 30 spots that showed relatively lower abundance in chronic pancreatitis subject samples, 29 had greater than a 1.5-fold decrease over the controls, of which 9 had greater than a 3-fold decrease (Table 2). Mass spectrometry-based proteomics techniques were used to identify these differentially expressed proteins.

Mass spectrometry analysis identified different proteins in chronic pancreatitis and control pancreatic fluid

All 65 spots determined to be differentially-imaged were excised and subjected to LC-MS/MS for protein identification. In Table 2, we listed the proteins with the most significant Mascot score corresponding to each spot (as numbered in Figure 3). In addition, we included the international protein index (IPI) number, the Mascot score, and the number of peptides for each protein identified using Mascot in Table 2. Likewise, we noted the theoretical isoelectric point (pI) and molecular weight (MW) of each protein, as well as the corresponding values that were estimated from the two-dimensional gel. Finally, the “dye ratio” was listed, which represents either the Cy5/Cy3 or −Cy3/C5 ratios for proteins of higher or lower abundance, respectively, in the pancreatic fluid of chronic pancreatitis individuals compared to chronic abdominal pain controls.

The proteins of higher abundance in pancreatic fluid from chronic pancreatitis individuals with respect to chronic abdominal pain controls included cytoskeletal proteins: actin and desmoplankin, and extracellular proteins: alpha-1-antitrypsin, SNC73, and serotransferrin. Those proteins that were of relatively lower abundance included known secreted pancreatic enzymes: carboxypeptidase B and lipase; and secreted protease inhibitors: alpha-1-antichymotrypsin and alpha-2-macroglobulin. In addition, the cytoplasmic proteins actin-related protein (Arp) 2/3 complex subunit 4 and glyceraldehyde-3-phosphate dehydrogenase, as well as endoplasmin and protein disulfide isomerase, which were localized to the endoplasmic reticulum, were also found to be of lower abundance in the pancreatic fluid of chronic pancreatitis individuals with respect to chronic abdominal pain controls.

Proteolytic fragments of amylase and serum albumin were identified in both samples

Several proteins listed in Table 2, such as amylase and serum albumin, were determined to be of both relatively higher and lower abundance in the pancreatic fluid of chronic pancreatitis patients as a result of the specific gel spot from which the protein was identified. Although apparently contradictory, these proteins were identified in multiple spots on the gel typically below their nominal molecular weight, and were likely to have undergone differential proteolysis. Our current aim, however, was to identify proteins which were differentially abundant in the pancreatic fluid of chronic pancreatitis individuals compared with chronic abdominal pain controls, however future studies may investigate this

differential proteolysis or optimize further the 2DGE protocol to prevent or lessen the degree of proteolysis. Table 3 listed those proteins which were consistently either relatively higher or lower in abundance in fluid specimens from the chronic pancreatitis subjects. In addition, we included in this table the subcellular localization and biological function of these proteins according to their gene ontology classifications in the UniProt database [29].

Discussion

We successfully use the ePFT collection method and DiGE coupled with LC-MS/MS to identify proteins from pancreatic fluid. DiGE analysis reveals differentially imaged gel spots when pancreatic fluid from chronic pancreatitis and chronic abdominal pain individuals are compared. Differential imaging analysis reveals 65 spots to be more intense in either the Cy5 (CP) or Cy3 (CAP) image. Of these protein spots, 30 are more intense in the samples originating from the chronic abdominal pain controls and 35 are more intense from the chronic pancreatitis subject samples. Using tandem mass spectrometry analysis, we successfully identified differentially-abundant proteins in pancreatic fluid of individuals with chronic pancreatitis and chronic abdominal pain controls.

Proteins that were consistently identified with higher abundance in the pancreatic fluid of chronic pancreatitis subjects, with respect to the control group, are listed at the top of Table 3. Using gene ontology analysis, we determine these proteins to be classified as either secreted or have roles in cellular structure and movement. Among the secreted proteins is serotransferrin, which is a protein responsible for the transport of iron from sites of absorption and heme degradation to sites of storage and utilization [30]. A proteomic analysis of serotransferrin in blood has shown association with pancreatic cancer [31]. Also of higher abundance is SNC73, a protein that shares homology with immunoglobulins. Although SN73 has not been previously associated with pancreatitis, it has been found to be a potential biomarker of colorectal cancer and other human epithelial carcinomas [32]. Another intracellularly secreted protein, alpha-1-antitrypsin (serpin A1), is a serine protease inhibitor that can bind covalently and inactivate trypsin, and is often associated with pancreatic dysfunction [33]. In addition to these secreted proteins, we also identify desmoplakin, a major cytoskeletal component of desmosomes that are part of intercellular junctions that tightly link adjacent cells [34]. Finally, actin, which is a ubiquitous protein involved in cell motility and structure [35], is also identified via LC-MS/MS analysis. Actin and cell-to-cell interaction proteins (e.g., desmoplakin) have been shown to have a role in the development of chronic pancreatitis via stellate cell activation [36; 37; 38].

Similarly, proteins that were consistently identified with lower abundance in the pancreatic fluid of chronic pancreatitis subjects, with respect to our control group, are listed in the lower portion of Table 3. These proteins include both those localized intracellularly and those which are secreted. Many of these proteins have been shown to have implications in pancreatic function. Intracellular proteins include metabolic enzymes protein disulfide isomerase and glyceraldehyde-3-phosphate dehydrogenase, as well as an Arp 2/3 complex subunit and endoplasmin. Protein disulfide-isomerase (PDI) generally is localized to the endoplasmic reticulum and catalyzes the formation, breakage, and rearrangement of disulfide bonds between cysteine residues [39; 40]. Along with its function in the transport and processing of secreted proteins, endoplasmin has been identified as a molecular chaperone, and is also a heat shock protein [41]. Both PDI and endoplasmin have been previously identified as being exported from the endoplasmic reticulum in the rat exocrine pancreas [42]. Similarly, glyceraldehyde-3-phosphate dehydrogenase partakes in membrane trafficking in the early secretory pathway [43]. It has been shown that differentially expressed alternative splice isoforms of glyceraldehyde-3-phosphate dehydrogenase are detectable in plasma and is a potential candidate biomarker of pancreatic cancer [44].

Likewise, the Arp 2/3 complex subunit 4, plays a role in cellular movement. This protein functions in the binding of the Arp2/3 complex to actin and is involved in regulation of actin polymerization [45]. Future studies may aim to further investigate the roles of these proteins in the development of chronic pancreatitis in *in vitro* and animal model systems.

In addition to these intracellular proteins, certain secreted protease inhibitor inhibitors are also of relatively lower abundance in the pancreatic fluid of chronic pancreatitis subjects. Protease inhibitors are of relevance to chronic pancreatitis, as dysfunction in pancreatic enzymes is a hallmark of the disease. Alpha-1-antichymotrypsin is a serine protease inhibitor, which inactivates neutrophil cathepsin G and mast cell chymase [46]. Similarly, alpha-2-macroglobulin, which is a large protein, can inactivate a variety of proteases, including serine-, cysteine-, aspartic- and metalloproteases [47; 48]. Both alpha-1-antichymotrypsin and alpha-2-macroglobulin may have significant implications in the inactivation of certain proteases that are secreted from the pancreata of chronic pancreatitis subjects. As expected, various pancreatic enzymes are also of lower abundance in the pancreatic fluid of chronic pancreatitis subjects. As enzyme deficiency is a characteristic sign of chronic pancreatitis, the pancreatic enzymes, trypsin-1, carboxypeptidase B, alpha-amylase 1, and triacylglycerol lipase, are generally of lower abundance in the pancreatic fluid from chronic pancreatitis subjects. A correlation may exist between these relatively lower abundance enzymes and inhibitors; however, analyses of these binary or multiplexed interactions must be performed before conclusions regarding these data can be drawn.

As illustrated in Table 2, some proteins, mainly amylase and serum albumin, were determined to be both of relatively higher and lower abundance in chronic pancreatitis compared to chronic abdominal pain controls depending on the specific spot which was analyzed by DiGE-LC-MS/MS. At first, these data appear contradictory; however, plausible explanations exist for these apparent discrepancies. For instance, due to the degree of activity of endogenous proteolytic enzymes in a particular sample of pancreatic fluid, different breakdown products may have been produced. More specifically, the fragmented proteins may be indicative of cohort-specific enzymatic activity of pancreatic fluid. For example, although the theoretical molecular weight of amylase is approximately 58 kDa, it is rarely identified above 50 kDa by our LC-MS/MS analysis. There were several gel spots below 20 kDa which were identified as amylase, and are likely to be cleavage products. Likewise, serum albumin is abundant in pancreatic fluid and was identified to be the highest-scoring protein in several gel spots. Cleavage products of serum albumin, are also prevalent in the lower molecular weight region (<20 kDa) of the gel. Further peptidomic-based investigation [49; 50; 51] may be warranted to compare differences in the endogenous activity of specific pancreas-secreted enzymes in pancreatic disease and non-diseased cohorts, as impairment of these enzymes may be indicative of underlying pancreatic dysfunction..

Unlike one-dimensional SDS-PAGE, 2DGE-based methods (e.g. DiGE) require a substantial amount of time in which the protein solution must equilibrate with the IEF gel strip. Although fractionation occurs under denaturing conditions, it still may be possible for proteolysis to occur during sample processing [52; 53], as is particularly true for the protease-rich pancreatic fluid. To improve the robustness of our DiGE analysis, methods of reducing degradation products using protease inhibitors early in sample preparation for example, may be integrated into our working protocol to decrease the degree of endogenous proteolysis. In regard to pancreatic fluid studies, there is evidence both for and against the addition of protease inhibitors early in the study [12, 27, 32, 33].

In addition, there are several other technical aspects of our DiGE approach that can be further optimized to effectively remove highly abundant proteins, such as albumin. Although

we identify some differentially-expressed proteins in the pancreatic fluid of chronic pancreatitis compared to chronic abdominal pain controls, we do realize that future analyses would benefit from an increased depth of differentially-expressed proteins. Samples were pooled as to control for patient-to-patient variability, as we aimed to investigate the global difference between cohorts, rather than between individuals [54; 55]. However, the high abundance of albumin in our pancreatic fluid samples may be exacerbated by the pooling of samples. To control for this drawback, albumin depletion may be performed using antibodies or dye-based columns, as is often performed with blood samples [56; 57]. A major caveat of albumin removal, is that it functions endogenously as a carrier of less abundant, but more significant proteins and these potential markers of disease may be lost during the depletion process [58]. As such, the denaturation of the protein sample may be a prerequisite to disrupt protein binding to albumin prior to depletion. Nevertheless, by targeted depletion, one adds another step to the sample handling process, one which may increase protein loss and sample variability. However, such optimizations may be necessary if low abundant proteins are to be studied.

There are several advantages to using DiGE for the proteomic analysis of pancreatic fluid, such as i) inherent two-dimensional sample fractionation, ii) better reproducibility, and iii) increased sensitivity and dynamic range. In addition, DiGE resolves some of the drawbacks traditionally associated with two-dimensional gel electrophoresis (2DGE), including gel-to-gel variation, as two or three samples can be conjugated to a specific fluorescent dye (Cy2, Cy3, or Cy5) and analyzed on a single gel [59]. However, along with the aforementioned benefits, the use of DiGE in pancreatic fluid proteomics is also subject to several limitations. Such caveats include: (i) spots containing multiple proteins, (ii) poor spot resolution at high pI values, (iii) very acidic and very basic proteins not being well represented, (iv) very small or very large proteins not being well-resolved, (v) irreproducibility of gels among experiments, and (vi) potential proteolysis resulting from sample handling [60].

Using our methodology, we discovered large changes between the two samples, however for future experiments, higher sensitivity could be attained with improved focusing in the first dimension. Although attempts were made with TCA precipitation to eliminate any non-proteinacious matter, the first dimension focusing showed sub-optimal, diffuse protein pattern. Potential reasons for the diffuse protein patterns include the possibility that non-protein contaminant (bound or unbound to protein) such as nucleic acid may be present, that residual salt present in the sample may have limited the focusing time and not allow steady state to be achieved, or that the samples were not completely solubilized. Improved focusing would enable us to detect more subtle changes in abundance, as opposed to only the major changes that we see in the present study. However, given that we observed dramatic differences between the samples even with the sub-optimal resolution, we chose to identify those proteins which showed these differences, as it would be beneficial to identify potential biomarkers that are abundant and readily detected.

It was our intention to use 2DGE to fractionate, isolate, relatively quantify, and identify proteins that differ in the pancreatic fluid between cohorts of chronic pancreatitis and chronic abdominal pain. However, as pancreatic fluid appears to have a proteome of only several hundred proteins, separation by electrophoretic mobility may be sufficient for fractionation to identify differentially abundant proteins. It follows that the use of one-dimensional gel electrophoresis (1DGE) may provide a technically simpler and more robust method of analyzing pancreatic fluid for downstream mass spectrometry analysis. The proteome of pancreatic fluid is relatively small in comparison to many cellular proteomes, thus the analysis thereof is less likely to benefit from the added dimension of fractionation by isoelectric point, unless certain isoforms or co-migrating proteins require further fractionation. Also, many of the caveats for 2DGE, as discussed above, do not apply to

1DGE. Following our previously–established protocol [21], we analyzed the 6 individual samples via 1DGE, as is depicted in Figure 4. These 1DGE images illustrate sharp, distinct protein banding patterns. We have since pursued the use of 1DGE fractionation of pancreatic fluid proteins in a large scale comparative study investigating chronic pancreatitis (manuscript in preparation). Moreover, the strategy of coupling 1DGE with mass spectrometry is currently the most widely used method of mass spectrometry-based protein identification for pancreatic fluid [20; 61; 62; 63; 64; 65; 66].

In summary, using DiGE-LC-MS/MS, we have demonstrated the feasibility to identify differentially-abundant proteins from ePFT-collected pancreatic fluid of chronic pancreatitis subjects and chronic abdominal pain controls. As a result of the enzyme insufficiency associated with chronic pancreatitis, many of the proteins of relatively lower abundance in the pancreatic fluid from chronic pancreatitis individuals with respect to that of chronic abdominal pain controls are proteolytic enzymes, as is expected. Although evidence supports that 1DGE may be superior to DiGE, we conclude that DiGE does have a role in the proteomic analysis of pancreatic fluid. Further optimization of the methodology that we have described herein enables the development of future comparative analyses of proteins from ePFT-collected fluid, and thereby can further broaden our knowledge of pancreatic disease pathogenesis.

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LIST OF ABBREVIATIONS

2DGE	two-dimensional gel electrophoresis
CP	chronic pancreatitis
CAP	chronic abdominal pain
DiGE	difference imaging gel electrophoresis
ePFT	endoscopic pancreatic function test
GO	gene ontology
IEF	isoelectric focusing
IPG	immobilized pH gradient
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
MW	molecular weight
pI	isoelectric point
TCA	trichloroacetic acid

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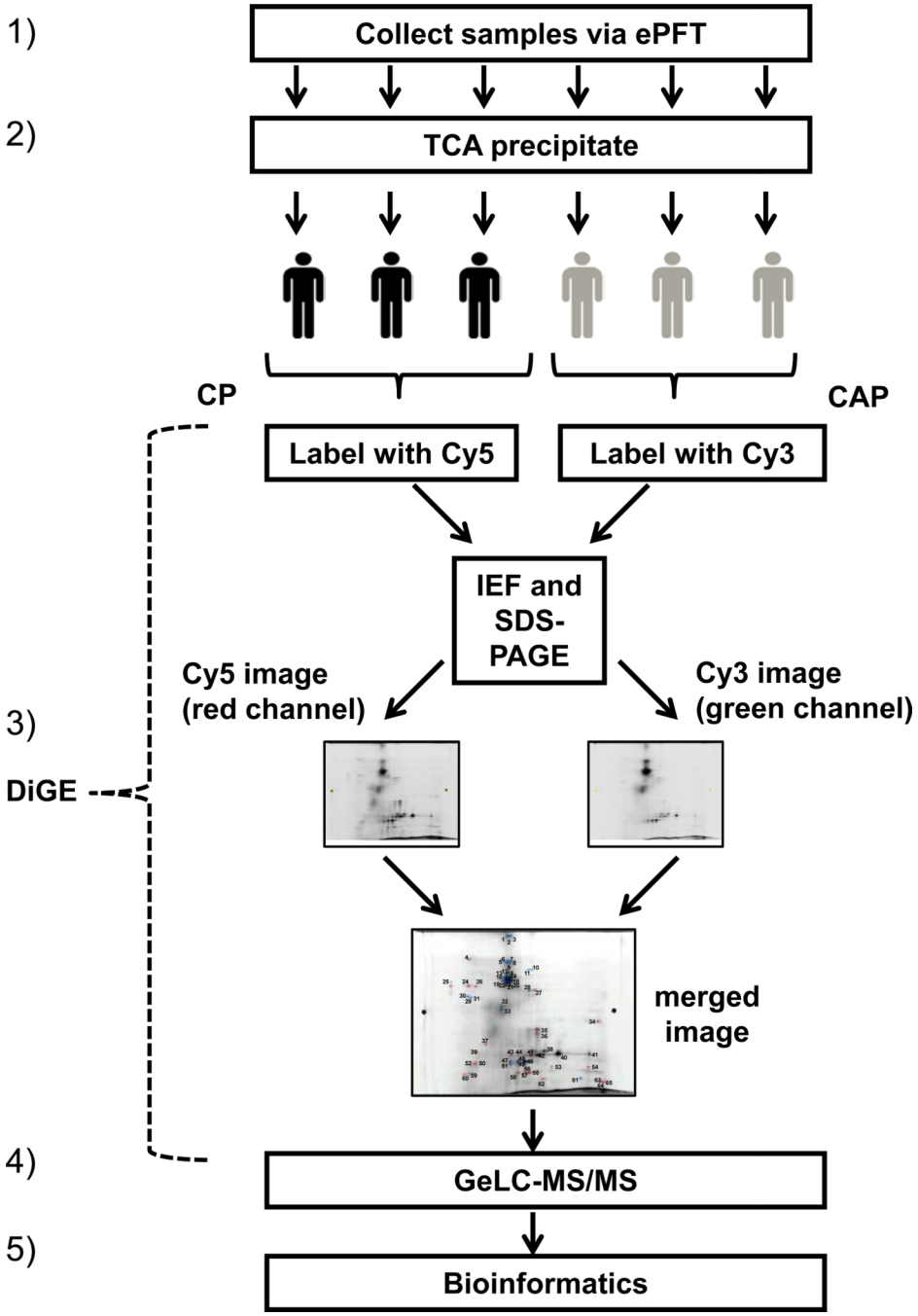

Figure 1. Experimental workflow

The procedure was as follows: (1) pancreatic fluid was collected from chronic pancreatitis subjects (n=3) and chronic abdominal pain controls (n=3) with the ePFT method, (2) proteins were extracted with TCA precipitation, (3) DiGE was performed with differentially-labeled samples from the two cohorts, (4) gel spots indicating differentially abundant proteins were excised and processed using GeLC-MS/MS techniques, and (5) bioinformatics analysis of the data was performed.

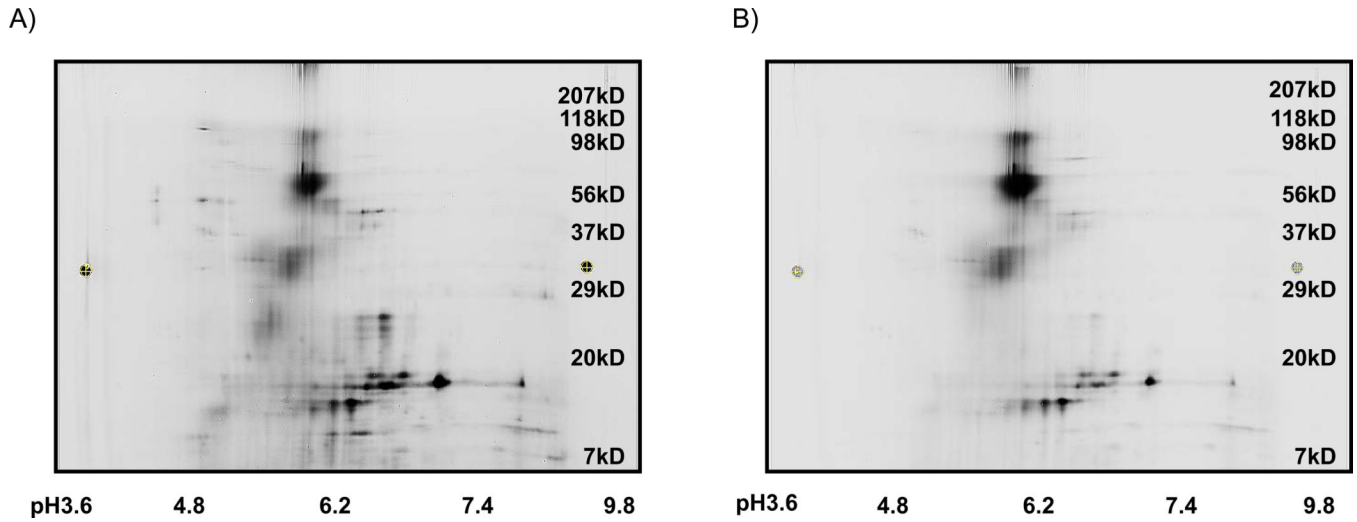

Figure 2. DiGE images for chronic abdominal pain and chronic pancreatitis pancreatic fluid samples

A) Image of Cy-3-labeled chronic abdominal pain pancreatic fluid sample. B) Image of Cy5-labeled chronic pancreatitis pancreatic fluid sample. The molecular weight (kDa) of the proteins may be approximated using the scale on the right of the gel and the values corresponding to the pI on the IPG strips are listed below the gel image.

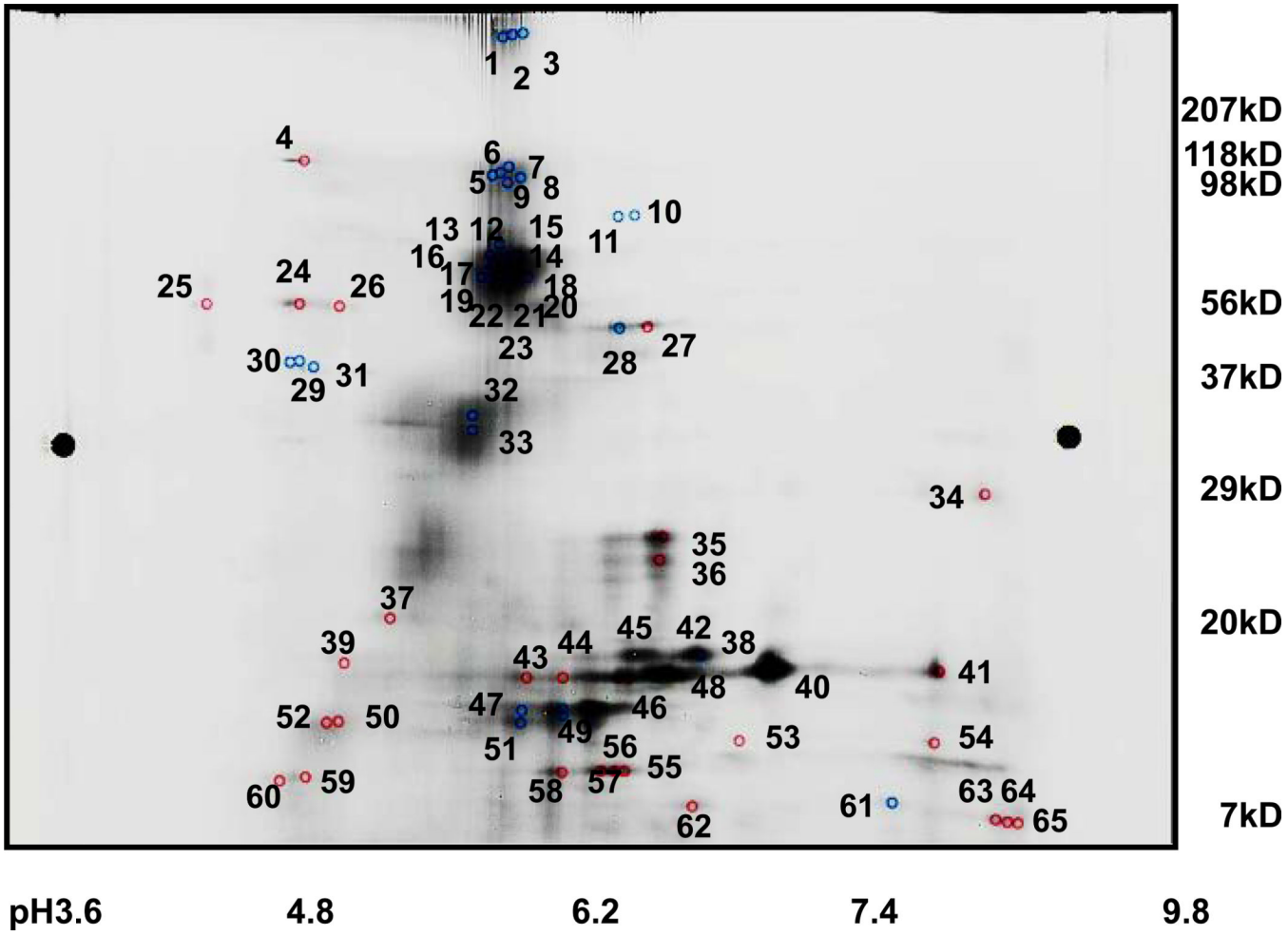

Figure 3. Overlay of DiGE images

The numerical values indicate gel spots that were excised and processed via GeLC-MS/MS. These numbers correspond to those in the leftmost column of Table 2.

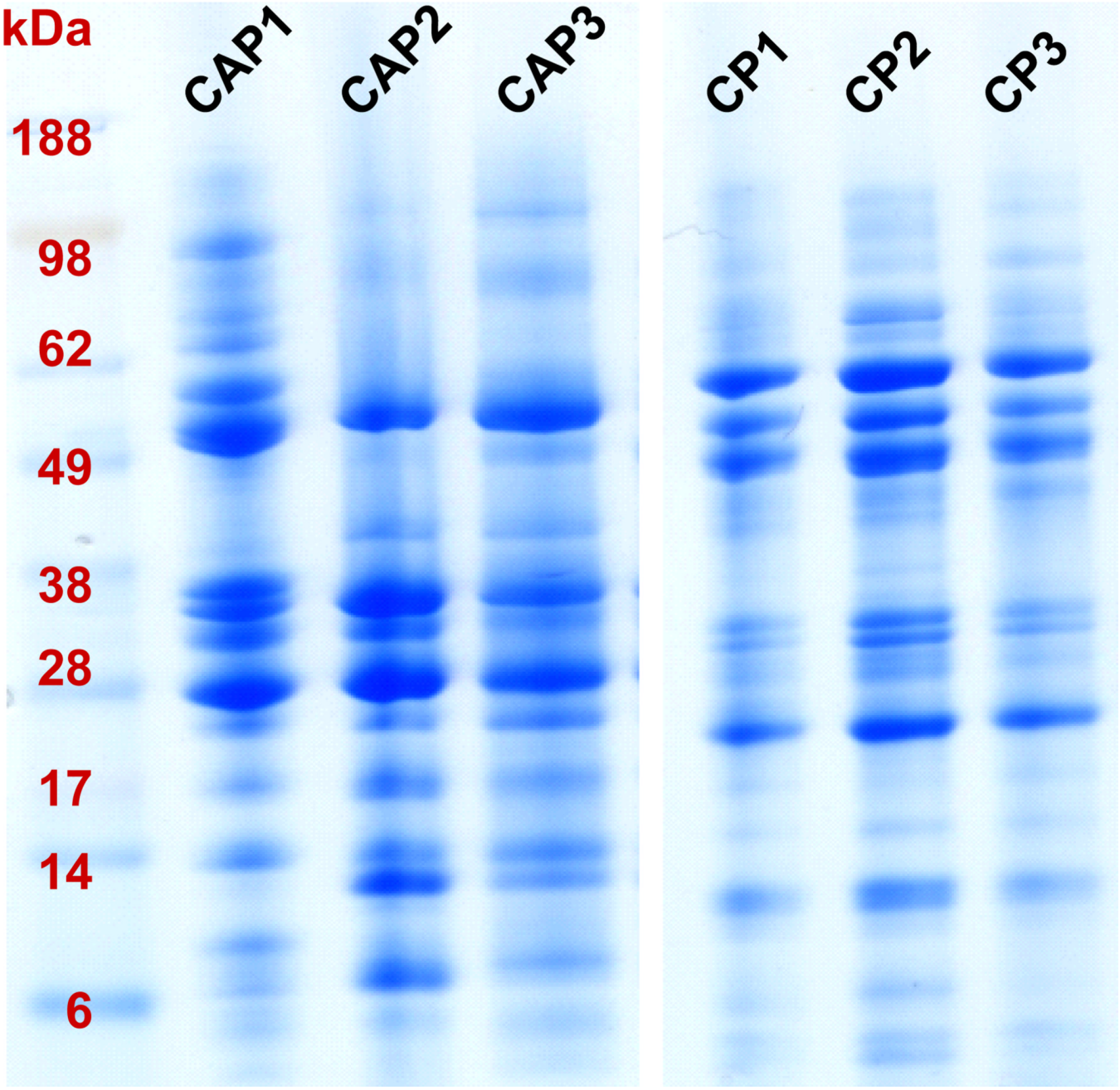

Figure 4. 1DGE protein fractionation

Each gel lane represents ePFT-collected pancreatic fluid that has been TCA precipitated from a particular patient (Table 1). CAP, chronic abdominal pain; CP, chronic pancreatitis.

Electrophoresis. Author manuscript; available in PMC 2012 June 13.

Table 1

Demographic and clinical data for study cohort.

ID	Reason for referral	Age (yr)	Gender	CT Scan	MRI	Pancreatic elastase – 1 (units/mg)	EUS Score (0–9)	ePFT Peak Bicarbonate (meq/L)	Pancreatic fluid protein conc. (mg/mL)	Endoscopic Biopsy (gastric, duodenum)
CAP1	abdominal pain	28	male	normal	normal	n/a	3	84	1.2	normal
CAP2	abdominal pain	53	female	normal	normal	148 (diarrhea)	1	92	0.8	gastropathy
CAP3	abdominal pain	54	male	normal	normal	202	n/a	114	0.6	normal
CP1	chronic pancreatitis	64	female	atrophy, calcifications, strictured and dilated duct	atrophy, dilated and strictured duct, filling effects, low T1 signal, dilated side branches	221	n/a	37	1.1	duodenitis, gastropathy
CP2	chronic pancreatitis	60	female	atrophy, calcifications, strictured and dilated duct	n/a	n/a	8	54	0.6	normal
CP3	chronic pancreatitis	52	female	n/a	atrophy, dilated and strictured duct, low T1 signal, dilated side branches	<15	5	38	0.7	normal

CT, indicates computed tomography; MRI, magnetic resonance imaging; EUS, endoscopic ultrasound; ePFT, endoscopic pancreas function test; CP, chronic pancreatitis; conc., concentration; n/a, not available.

Table 2

Proteins identified with the highest Mascot scores following LC-MS/MS analysis from each excised DiGE gel spot. The “dye ratio” represents Cy5/Cy3 and −Cy3/C5 intensity ratios for proteins of higher and lower abundance, respectively, in chronic pancreatitis (Cy5) with respect to chronic abdominal pain controls (Cy3).

Spot#	Mascot result				Calculated		Estimate from gel		dye ratio
	protein name	IPI number	score	peptides	MW (kDa)	pI	MW (kDa)	pI	
1	Desmoplakin	IPI00013933	426	57	332	6.4	250	5.7	1.4277
2	Serum albumin	IPI00745872	541	28	69	5.9	250	5.7	2.7033
3	Serum albumin	IPI00745872	176	16	69	5.9	250	5.7	2.3701
4	Endoplasmin	IPI00027230	262	28	92	4.8	98	4.8	−2.7721
5	Serum albumin	IPI00745872	441	25	69	5.9	98	5.7	3.0376
6	Serum albumin	IPI00745872	328	23	69	5.9	98	5.7	2.9618
7	Serum albumin	IPI00745872	443	28	69	5.9	98	5.7	3.5638
8	Serum albumin	IPI00745872	446	30	69	5.9	98	5.7	2.6246
9	Serum albumin	IPI00745872	441	26	69	5.9	98	5.7	3.6763
10	TF Serotransferrin	IPI00022463	174	15	77	6.8	85	6.2	4.7389
11	TF Serotransferrin	IPI00022463	71	4	77	6.8	85	6.2	6.2057
12	Serum albumin	IPI00745872	309	19	69	5.9	56	5.7	2.7793
13	Serum albumin	IPI00745872	477	34	69	5.9	56	5.7	3.4765
14	Serum albumin	IPI00745872	567	34	69	5.9	56	5.7	2.858
15	Serum albumin	IPI00745872	550	40	69	5.9	56	5.7	3.2367
16	SNC73 protein	IPI00386879	63	5	53	6.5	56	5.7	2.7797
17	SNC73 protein	IPI00386879	82	5	53	6.5	56	5.7	2.1518
18	TF Serotransferrin	IPI00022463	620	42	77	6.8	56	5.7	2.476
19	Serum albumin	IPI00745872	433	34	69	5.9	56	5.7	3.7413
20	Serum albumin	IPI00745872	613	39	69	5.9	56	5.7	2.7902
21	Actin	IPI00021439	108	6	42	5.3	56	5.7	1.8866
22	Serum albumin	IPI00745872	466	31	69	5.9	56	5.7	4.0114
23	Serum albumin	IPI00745872	346	30	69	5.9	56	5.7	2.8934
24	Serum albumin	IPI00745872	346	30	69	5.9	56	4.8	−4.208
25	Protein disulfide-isomerase	IPI00010796	184	16	57	4.8	56	4.5	−4.401

Electrophoresis. Author manuscript; available in PMC 2012 June 13.

Spot#	Mascot result				Calculated		Estimate from gel		dye ratio
	protein name	IPI number	score	peptides	MW (kDa)	pI	MW (kDa)	pI	
26	Alpha-1-antichymotrypsin	IPI00550991	72	3	51	5.3	56	4.8	−3.9172
27	Pancreatic alpha-amylase	IPI00025476	342	22	58	6.6	50	6.2	−1.4011
28	Pancreatic alpha-amylase	IPI00025476	436	20	58	6.6	50	6.4	1.0452
29	Alpha-amylase 2B	IPI00021447	111	4	58	6.6	37	4.8	1.5795
30	Alpha-1-antitrypsin	IPI00553177	229	10	47	5.4	37	4.8	1.7298
31	Alpha-1-antitrypsin	IPI00553177	115	10	47	5.4	37	4.8	1.4306
32	Serum albumin	IPI00745872	99	12	69	5.9	34	5.5	1.7838
33	Serum albumin	IPI00745872	255	19	69	5.9	34	5.5	1.792
34	Glyceraldehyde-3-phosphate dehydrogenase	IPI00219018	81	12	36	8.6	29	8.0	−2.1575
35	Pancreatic alpha-amylase	IPI00025476	132	9	58	6.6	25	6.4	−2.4613
36	Alpha-amylase 2B	IPI00021447	146	6	58	6.6	25	6.4	−3.0721
37	Serum albumin	IPI00745872	251	20	69	5.9	18	5.0	−2.5469
38	Pancreatic alpha-amylase	IPI00025476	158	7	58	6.6	20	6.4	1.163
39	Pancreatic alpha-amylase	IPI00025476	127	5	58	6.6	17	5.8	−1.1942
40	Trypsin-1	IPI00011694	287	28	27	6.1	16	7.2	−1.5565
41	Serum albumin	IPI00745872	130	7	69	5.9	16	7.7	−1.1998
42	Trypsin-1	IPI00011694	283	29	27	6.1	16	6.5	−1.5206
43	Serum albumin	IPI00745872	180	12	69	5.9	16	6.2	−1.2733
44	Pancreatic alpha-amylase	IPI00025476	132	6	58	6.6	16	6.4	−1.3937
45	Trypsin-1	IPI00011694	67	6	27	6.1	16	6.6	−1.1982
46	Pancreatic alpha-amylase	IPI00025476	173	7	58	6.6	12	6.2	1.4043
47	Serum albumin	IPI00745872	175	15	69	5.9	12	5.8	2.5705
48	Alpha-amylase 2B	IPI00021447	108	4	58	6.6	12	5.8	1.9996
49	Alpha-amylase 2B	IPI00021447	148	5	58	6.6	12	5.8	1.9578
50	Serum albumin	IPI00745872	181	16	69	5.9	10	4.8	−1.7044
51	Serum albumin	IPI00745872	211	20	69	5.9	12	5.8	1.876
52	Serum albumin	IPI00745872	191	16	69	5.9	10	4.8	−1.7595
53	Pancreatic triacylglycerol lipase	IPI00027720	58	4	51	6.3	10	7.0	−2.1418
54	Carboxypeptidase B	IPI00009826	106	4	47	6.2	10	7.8	−1.8589
55	Pancreatic alpha-amylase	IPI00025476	82	3	58	6.6	15	6.0	−2.7219

Spot#	Mascot result				Calculated		Estimate from gel		dye ratio
	protein name	IPI number	score	peptides	MW (kDa)	pI	MW (kDa)	pI	
56	Alpha-amylase 1 precursor	IPI00025476	70	4	58	6.6	15	6.0	−2.2757
57	Serum albumin	IPI00745872	155	12	69	5.9	15	6.0	−3.0823
58	Pancreatic alpha-amylase	IPI00025476	141	7	58	6.6	15	6.0	−1.992
59	Alpha-2-macroglobulin	IPI00478003	71	6	163	6.0	7	4.4	−4.1582
60	Serum albumin	IPI00745872	137	13	69	5.9	7	4.4	−3.8268
61	Trypsin-1	IPI00011694	84	6	27	6.1	8	7.6	1.1487
62	Trypsin-1	IPI00011694	88	6	27	6.1	8	6.6	−1.45
63	Serum albumin	IPI00745872	85	10	69	5.9	7	8.7	−2.6158
64	Arp2/3 complex 20 kDa subunit	IPI00554811	92	6	20	5.3	7	8.7	−3.2895
65	Serum albumin	IPI00745872	211	15	69	5.9	7	8.7	−3.2561

Abbreviations: Spot#, gel spot as numbered in Figure 3; score, Mascot score; peptides, number of peptides identified per protein; MW, molecular weight; pI, isoelectric point.

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Table 3

Proteins consistently of either higher or lower abundance in pancreatic fluid from chronic pancreatitis individuals with respect to chronic abdominal pain controls.

dye ratio (mean)	abundance in CP	protein name	IPI number	subcellular localization	biological function	spot #(s)
1.8866	higher	Actin	IPI00021439	cytoskeleton	structural	21
1.5802	higher	Alpha-1-antitrypsin	IPI00553177	extracellular	protease inhibitor	30, 31
1.4277	higher	Desmoplakin	IPI00013933	cytoskeleton	structural	1
2.4658	higher	SNC73 protein	IPI00386879	extracellular	n/a	16, 17
4.4735	higher	TF Serotransferrin	IPI00022463	extracellular	cell proliferation	10, 12, 18
−3.2895	lower	Arp2/3 complex 20 kDa subunit	IPI00554811	cytoskeleton	structural	64
−3.9172	lower	Alpha-1-antichymotrypsin	IPI00550991	extracellular	protease inhibitor	26
−4.1582	lower	Alpha-2-macroglobulin	IPI00478003	extracellular	protease inhibitor	59
−2.2757	lower	Alpha-amylase 1 precursor	IPI00025476	extracellular	enzyme	56
−1.8589	lower	Carboxypeptidase B	IPI00009826	extracellular	enzyme	54
−2.7721	lower	Endoplasmin	IPI00027230	endoplasmic reticulum	chaperone	4
−2.1575	lower	Glyceraldehyde-3-phosphate dehydrogenase	IPI00219018	cytoplasm	metabolic enzyme	34
−2.1418	lower	Pancreatic triacylglycerol lipase	IPI00027720	extracellular	enzyme	53
−4.4010	lower	Protein disulfide-isomerase	IPI00010796	endoplasmic reticulum	enzyme	25