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Mass Spectrometry-Based Proteomics of Endoscopically-Collected Pancreatic Fluid in Chronic Pancreatitis Research

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Abstract

Mass spectrometry-based investigation of pancreatic fluid enables the high-throughput identification of proteins present in the pancreatic secretome. Pancreatic fluid is a complex admixture of digestive, inflammatory, and other proteins secreted by the pancreas into the duodenum, and thus is amenable to mass spectrometry-based proteomic analysis. Recent advances in endoscopic techniques, in particular the endoscopic pancreatic function test (ePFT), have improved the collection methodology of pancreatic fluid for proteomic analysis. Here, we provide an overview of mass spectrometry-based proteomic techniques as applied to the study of pancreatic fluid. We address sample collection, protein extraction, mass spectrometry sample preparation and analysis, and bioinformatic approaches and summarize current mass spectrometry-based investigations of pancreatic fluid. We then examine the limitations and the future potential of such technologies in the investigation of pancreatic disease. We conclude that pancreatic fluid represents a rich reservoir of potential biomarkers and that the study of the molecular mechanisms of chronic pancreatitis may benefit substantially from mass spectrometry-based proteomics.

Keywords

chronic pancreatitis; ePFT; mass spectrometry; proteomics; pancreatic juice

Introduction

Proteomics entails the characterization of the complete set of proteins encoded by the genome of a given organism in a given state [1]. Sensitive mass spectrometric analysis of complex mixtures of proteins and peptides allows for high-throughput proteomic analyses. Whereas traditional techniques have focused on only a few proteins per analysis, proteomics attempts to conduct a comprehensive analysis that can identify hundreds or thousands of proteins [2]. Mass spectrometry has become an indispensable analytical tool for studying proteomics. While classical techniques such as co-immunoprecipitation, reciprocal western

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CONFLICTS OF INTERESTS

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blotting, and yeast-two-hybrid assays can typically provide information on binary or in some cases ternary interactions, protein-protein interaction networks can be identified using mass spectrometry without any *a priori* suspicion of interaction. In addition, the proteomic analysis of human body fluids may be a more attractive option than tissue biopsies for the diagnosis and prognosis of disease. Proximal body fluids, which bathe the organ of interest, have the benefit of containing locally secreted proteins, which are likely to include specific markers of disease for that particular organ.

Chronic pancreatitis is a disease for which proteomics may offer a means for enhancing our understanding of its progression and pathogenesis. Chronic pancreatitis is associated with diabetes, pain, malabsorption of fat and protein, and to a lesser degree, the development of pancreatic cancer [3]. The diagnosis of chronic pancreatitis often remains elusive in mild disease due to a scarcity of predictable and reliable radiological and endoscopic abnormalities of the pancreas [4]. 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 obtained endoscopically. The use of proteomic techniques to investigate pancreatic fluid can complement established diagnostic methods to determine pancreatic dysfunction, and may also uncover novel differentially-expressed biomarkers of the various associated clinical manifestations of the disease. In addition, the elucidation of biomolecular pathways of which disease-specific proteins are members will supplement our knowledge of the pathogenesis and progression of chronic pancreatitis.

Below we examine mass spectrometry-based proteomic techniques which are applicable to the study of pancreatic fluid. First, we begin from principles of sample collection and protein extraction, followed by mass spectrometry sample preparation and analysis techniques and bioinformatics. Next, we examine the current progress of mass spectrometry-based proteomics of pancreatic fluid. We include a review of some of our own work emphasizing the need for standardized sample preparation. Finally, we comment on the future potential of mass spectrometry-based proteomic analysis of pancreatic fluid in the evaluation of chronic pancreatitis.

Overview of pancreatic fluid sample preparation and mass spectrometry-based proteomics techniques

In translational research, standardized protocols regarding specimen collection, storage, and processing are often unavailable, but necessary to develop a reproducible proteomics. For instance, in urine proteomics, several recent reviews have stressed the importance of sample handling in reducing experimental variability [5–11]. For pancreatic fluid, such variations are especially pronounced as a result of its inherently high concentration of active proteolytic enzymes and may be further affected by non-standardized sample preparation; however standardized protocols have yet to be established. In the following section, we provide an overview of various methodologies which can be utilized to investigate the proteome of pancreatic fluid. Figure 1 illustrates possible workflows for the proteomics-based pancreatic fluid analysis. Table 1 summarizes the mass spectrometry-based proteomics research of pancreatic fluid which has been published to date, highlighting techniques of sample preparation and mass spectrometric analysis.

1. Pancreatic fluid for proteomic analysis must be rigorously collected

Systematic sample collection methodology is an essential prerequisite for proteomic analysis of pancreatic fluid. To this end, we have developed a secretin-stimulated endoscopic pancreatic function test (ePFT) fluid collection method that safely and reproducibly collects

large amounts of pancreatic fluid for analysis [12]. The ePFT collection method replaces the gastroduodenal (dual lumen) Dreiling tube with an upper endoscope [13]. Although Dreiling tubes allow for the simultaneous collection of duodenal fluid for analysis and gastric fluid for prevention of acid efflux into the duodenum during secretin-stimulated pancreatic function testing (PFT) [14], 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 [15]. To date, several proteomic investigations of pancreatic fluids have been performed with specimens collected surgically or via ERCP [16–20]. Alternative collection methods are warranted, as ERCP is highly invasive and associated with significant risks (5–10%) to the patients, such as the development of acute pancreatitis [21, 22]. Although restricted to pancreatic cancer research, endoscopic ultrasound-fine needle aspiration (EUS-FNA) is commonly performed for the aspiration of cyst fluids [23], and has been shown to be amenable for proteomic analysis [24].

The ePFT collection method avoids many of the caveats associated with traditional pancreatic fluid collection and is a safe alternative method, with little risk to induce acute pancreatitis. This endoscopic collection method is universally available, and eliminates radiation exposure, while minimizing cost. In addition, the larger amount of fluid attainable via ePFT, when compared to ERCP, is beneficial for comprehensive proteomic analysis [12, 13, 25–28]. Equally important, ePFT fluid collection can be performed readily during routine (esophagogastroduodenoscopy; EGD) or advanced (endoscopic ultrasound; EUS) endoscopy.

ePFT-collected pancreatic fluid is typically stimulated using the hormone secretin [12, 28] (although cholecystikinin (CCK) [13] may also be used) following a 12 hour fasting period. The fluid collected from the duodenum is an admixture of gastric, duodenal, and pancreas secretions. However, duodenal protein secretion is minimal and the efflux of gastric fluid is limited by placing the patient in the left lateral decubitus position. Moreover, both fluids, (collectively known as gastroduodenal fluid) are evacuated prior to ePFT and any remnants are subsequently diluted by the protein-rich secretin-stimulated pancreatic secretions. We have previously investigated the proteome of the gastroduodenal fluid and have determined it to be different from pancreatic fluid, both by SDS-PAGE binding pattern and protein profile, as determined by mass spectrometry [29].

2. Proteins are extracted from pancreatic fluid to eliminate moieties that are incompatible to downstream analyses

Proteins must be isolated from lipids, metabolites, and other non-proteinaceous compounds which interfere with mass spectrometric analysis. In general, salt removal is accomplished via methods such as dialysis (spin, micro) [30], ultrafiltration [31, 32], gel filtration/ electrophoresis, precipitation with trichloroacetic acid (TCA) or organic solvents, and/or solid-phase extraction. Various chemical precipitation methods are available for protein isolation, but the efficiency of protein precipitation varies among different organic solvents. For example, acetone has been determined to precipitate more acidic and hydrophilic proteins, as well as proteins from gastroduodenal fluid [33], whereas ultracentrifugation fractionates more basic, hydrophobic, and membrane proteins [34]. Alternatively, chloroform/ methanol extraction has been used to successfully extract hydrophobic proteins [35]. For pancreatic fluid, the use of acidic precipitation methods, such as TCA, or the pre-acidification of pancreatic fluid may be beneficial to quickly and effectively inactivate pancreatic proteolytic enzymes that are generally active under alkaline conditions [36]. However, due to the potential differences in the types of proteins precipitated, various strategies should be investigated if a particular protein or class of proteins is being targeted, as the chosen strategy is essential for successful and robust proteomic analyses.

3. Mass spectrometry sample preparation and analysis may be performed using a wide range of strategies

Various methods of protein fractionation [37–44] are commonly used for proteomic analysis. Prior to the application of mass spectrometry for protein identification, distinct patterns of proteins in pancreatic fluid have been investigated using HPLC [45, 46] and two-dimensional gel electrophoresis [47, 48]. Although such methodologies could determine that specific proteins differed in the fluid of normal and diseased pancreata, the biomolecular mechanisms of disease remain undetermined.

Subsequent studies further investigate pancreatic proteins as diagnostic markers using peptide mass signatures obtained from surface-enhanced laser desorption/ionization (SELDI) mass spectrometry experiments to identify patterns of differentially-expressed proteins [49–51]. More recently, SELDI has been coupled with tandem time-of-flight (TOF/TOF) analyzers to obtain more robust identifications of pancreatic fluid proteins in searching for pancreatic cancer biomarkers [51–53]. Similarly, 2-DE has been used upstream of MALDI-TOF mass spectrometry as a higher-throughput method of protein identification. Using this technique, pancreatic cancer-specific proteins have been identified in both fluid [20, 24] and tissue [54]. MALDI-TOF/TOF analysis has also been used in tandem with DiGE to successfully identify several proteins which were over-expressed in pancreatic fluid from ductal adenocarcinoma patients [19]. However, although these strategies do indicate differences in the pancreatic fluid proteome of diseased and non-diseased individuals, protein identification was limited to only several dozen proteins.

More recently, the coupling of polyacrylamide gel-based protein with mass spectrometry-based techniques has had a valuable, positive impact on the identification of proteins in pancreatic fluid. As the proteome of pancreatic fluid is of relatively moderate complexity, one-dimensional SDS-PAGE has been most often used for protein fractionation, as a result of its simplicity and reproducibility [29, 36]. The strategy of coupling SDS-PAGE with mass spectrometry, termed GeLC-MS/MS, is the most widely used method of mass spectrometry-based protein identification for pancreatic fluid to date [17–20, 24, 29, 55]. Using this technique, proteins are typically digested in-gel and subsequently extracted using standard protocols [56]. Protein digestion may be performed also in-solution, however, as the inactivity of endogenous enzymes is crucial to maintaining sample integrity prior to and during enzymatic digestion. We have observed that for in-solution digests, as performed using standard protocols [57, 58], trypsinization from TCA-precipitated pellets without resuspension in digestion buffer offers an alternative to in-gel digestion (unpublished observation).

The resulting digested peptides may be fractionated in a single dimension (e.g., reversed-phase-HPLC [59] or ultra-high performance liquid chromatography, UHPLC [60–62]) or in multiple dimensions [63–65]. Several recent studies have compared various fractionation methods [66–70]; however no consensus has been established. Due to the relatively modest sample complexity of pancreatic fluid, a single dimension of peptide fractionation (e.g., reversed-phase-(U)HPLC) is sufficient, although using multi-dimensional separation may be advantageous in enhancing analytical depth, particularly for identifying low-abundance proteins. As a result of proteolytic digestion and peptide fractionation, substances which interfere with downstream mass spectrometric acquisition may require removal by using C18 or strong cation exchange resin in cartridge, on-line columns, spin column, or pipette tip format [71, 72]. In general when using GeLC-MS/MS, further sample clean-up is not required, however, in-solution digests do benefit from these methods.

The proteomic analysis of pancreatic fluid is not limited to a single mass spectrometric modality, as several different combinations of ion sources, mass analyzers, and ion detectors

are available and have been used for the analysis of pancreatic fluid [17–20, 33, 55]. We show a representative peptide identification obtained by our GeLC-MS/MS analysis strategy, in which the peptide NLSQIVDIDGIWEGTR originating from pancreatic triacylglycerol lipase is sequenced (Figure 2). We refer the reader to several recent comprehensive mass spectrometry reviews for further technical details [73–75].

4. Bioinformatic techniques assign peptide sequences to acquired mass spectra and can be used to characterize protein lists

The complexity of tandem mass spectra for large-scale experiments limits the feasibility of manual interpretation and false discovery rate analysis of all spectra [76, 77]. As such, several computational algorithms and software packages [78–83] have been developed for automated sequence identification and database searching which assign these peptides to the corresponding proteins with set significance thresholds. The authors direct the reader to the references listed above for technical details concerning the aforementioned software. These search algorithms can generate lists of several hundreds or thousands of proteins; the task is then to understand the functions of these proteins in biological pathways. Several software packages are available to extract this information including: Ingenuity Pathways Analysis (IPA; IngenuitySystems), GeneGO [84], DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Database [85, 86], KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis [87–89], and GoFact [90]. Such software greatly improves the ease by which biologically relevant knowledge can be extracted from the data and is applicable to comparative proteomic analyses, which is an integral aspect of biomarker discovery.

Optimization of pancreatic fluid sample preparation

In efforts to standardize sample processing strategies, we initiated a study using SDS-PAGE protein profiling to establish a protocol for pancreatic fluid analysis for future studies. These experiments were designed to maximize the integrity of our sample, as protein degradation is expected in this endogenously protease-rich body fluid [36]. We have shown that if such a sample is incubated at 37°C, it shows progressive degradation, most noticeably for time points beyond 1 hour. We investigated (1) ePFT as a viable collection method for pancreatic fluid, (2) protein extraction techniques (vacuum centrifugation, PD-10 column, 5kDa molecular weight cut-off Centricon filtration, C4 trapping column, trifluoroacetic acid/ acetonitrile precipitation, trichloroacetic acid precipitation, ethanol precipitation) to maximize protein extraction, (3) auto-digestion of pancreatic fluid following prolonged exposure to a range of temperatures, (4) effects of multiple freeze-thaw cycles on protein stability, and (5) the utility of protease inhibitors. Analogous experiments may be useful beyond pancreatic fluid, as other body fluids are also susceptible to varying degrees of degradation.

We have shown that ePFT is indeed an excellent option to collect pancreatic fluid for proteomic analysis [29]. TCA precipitation maximizes protein extraction and instantaneously inactivates proteolytic enzymes, when compared to the other methods investigated, thus eliminating the need for protease inhibitors. Accordingly, auto-digestion is minimal if samples are efficiently handled or stored on ice for up to 8 hours. In addition, the protein profiles are not affected by up to 5 freeze-thaw cycles. In Figure 3, we outline our optimized sample preparation strategy for SDS-PAGE (Figure 3A) and GeLC-MS/MS (Figure 3B). We have applied this methodology to pancreatic fluid from various individuals and produced robust and reproducible SDS-PAGE protein banding patterns, ideal for GeLC-MS/MS analysis (Figure 4). Figure 4 illustrates pancreatic fluid that has been analyzed from different patients. Although there is some expected intrinsic variability among the lanes in Figure 4, the overall banding pattern is similar. Similar person-to-person variability has been

seen in urine proteomics [91–93]. Consideration must be taken that the samples remain stable during collection, transportation from site of collection to site of analysis, storage, and preanalytical preparation to eliminate any post-collection sample variability. To this end, we suggest the meticulous recording of the conditions for sample preparation and handling thereby allowing the tracking of all preanalytical variation. This optimized sample preparation strategy can be utilized in future studies investigating the proteome of pancreatic fluid, and to provide/ a basis for the further optimization of pancreatic fluid proteome analysis for a range of pancreatic diseases.

Limitations of mass spectrometry-based pancreatic fluid proteomics

As a result of endogenous protease activity, careful and consistent sample handling is essential. The proteases, if they remain active, can degrade a considerable amount of pancreatic fluid protein, adversely affecting the protein migration patterns during SDS-PAGE and subsequent proteomic analyses. Whether or not to use protease inhibitors and/or acidification must be determined for a particular experiment or assay, as their addition may result in irreversible modifications and may be detrimental toward downstream analyses. For example, several small molecule inhibitors, such as PMSF (phenylmethanesulfonylfluoride) and AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride), have been shown to form aberrant covalent bonds with proteins [94], thereby changing protein pI and electrophoretic mobility [95]. In addition, many protease inhibitor cocktails contain small molecule or peptide inhibitors, which can interfere with subsequent peptide ionization [96]. However, protease inhibitors may be useful in applications requiring extended periods of incubations, such as immunoprecipitations and ELISAs (enzyme linked immunosorbent assays). Moreover, in general, standardization in both sample collection and mass spectrometry analysis is needed to improve the reproducibility of the data. Confounders, such as gender, age, etiology, smoking, alcohol consumption, and race must also be taken into consideration; as such demographic differences may result in additional variability. Cohorts should be chosen carefully and matched as closely as possible.

Similarly, another aspect of sample preparation which requires further investigation is the depletion of abundant proteins. Many human body fluids, including pancreatic fluid, have nearly mg per mL levels of serum albumin and α 2-macroglobulin, which may necessitate depletion [97–99]. Conversely, many important differentially expressed proteins are present at very low levels in pancreatic fluid. For example, cytokines are present generally on the order of pg per mL in pancreatic fluid [100–103]. More targeted assays, such as western blotting, protein microarrays, and ELISA, may be required to detect, in a targeted manner, such proteins and identify differences in pancreatic fluid protein content between normal and diseased pancreata. In addition, differential protein analysis may require quantitative methods, as there may be basal levels of expression of a targeted protein regardless of the diseased state of the pancreas.

Outlook for the Proteomic Analysis of Pancreatic Fluid

The combination of high-resolution separation techniques and powerful mass spectrometric analysis allows previously unattainable information about the pancreatic fluid proteome to be acquired and interpreted. Recent developments in high-throughput mass spectrometry can facilitate the elucidation of proteins which regulate the pathogenesis of disease and facilitate the discovery of clinically-relevant biomarkers of pancreatic disease [104–107]. However, the quality of proteomic results depends heavily on the methodology by which samples are prepared and analyzed.

Some mass spectrometry-based studies of pancreatic fluids investigating either pancreatic cancer [16, 24, 108–110] or chronic pancreatitis have utilized GeLC-MS/MS [18], SELDI

[111], and isotope-coded affinity tags (ICAT) [55]. Based on the methodology utilized, the number of proteins identified ranged from 22 to over 170. Although there are some overlapping proteins among the lists, there is little consensus, thus conclusions are difficult to derive. As the collection of pancreatic fluid is a relatively invasive procedure when compared to the drawing of blood or the collection of urine, it is common practice to use “surrogate” controls, such as dyspepsia/chronic abdominal pain patients undergoing upper endoscopy, as substitutes for healthy patients, who have no need to undergo an upper endoscopy. Such a strategy may introduce confounding factors, which can potentially hinder biomarker discovery, as pain may be an early indicator of pancreatic disease. The data collected to date are promising, but improvements in the standardization of methodologies and the establishment of a baseline healthy pancreatic fluid proteome must be made to improve the specificity and reproducibility of the findings.

Quantitative proteome profiling is valuable when comparatively analyzing pancreatic fluid from normal subjects and pancreatic disease patients, as similar proteins may be present in both states, but at significantly different concentrations. Without quantitative information, the significance of certain proteins as biomarkers may be overlooked. In comparative proteomics, sample preparation is of utmost importance as minor differences in experimental and control samples may be the key to understanding the mechanisms that underlie a particular disease. For example, in pancreatic fluid, we and others have identified pancreatic enzymes in both chronic pancreatitis and non-chronic pancreatitis samples [29]. Quantitatively, however, these levels may differ significantly between these two states, as evidenced by known enzyme insufficiency in chronic pancreatitis patients. Techniques, such as ^{18}O labeling [112], iTRAQ (isobaric tag for relative and absolute quantitation) [113], TMT (tandem mass tag) [114], AQUA (absolute quantitation) [115–117], and ICAT [118, 119], may be useful in the study of quantitative differences between diseased and non-diseased cohorts. Whereas the genome is relatively stable and identical in all cells, the proteome varies over time, by organ, cell, subcellular location, and as a result of stimuli (e.g., changes in health, diet, and environment). In depth quantitative proteomic interrogation of pancreatic fluid can assist in determining if such variations are indeed a result of a particular disease state of interest (i.e., chronic pancreatitis).

Pancreatic fluid collected using ePFT is attractive for biomarker discovery due to the relative ease of collection, safety profile, and the potential for serial measurements over time, all of which can lead to the development prognostic and/or diagnostic tests [120]. Pancreatic fluid is a rich reservoir of proteins, which represent the end product of genomic expression. As one gene may produce more than one protein, a genome of 30,000 genes can produce over 100,000 proteins, when alternative splicing is considered [121]. In addition, differential posttranslational modifications of proteins, such as phosphorylation and glycosylation, further expand the number of possible protein isoforms to be identified [122, 123]. Once biomarkers and molecular pathways of disease have been elucidated, efforts must be focused on validation of these mass spectrometry-based data. Disease-specific biomarkers, for example, may be targeted in large numbers of patients and controls, while longitudinal studies can be developed that examine the appearance, disappearance, or modulation of expression over the course of the disease. In addition, biomolecular pathways that may be altered during the course of disease progression may be investigated further using the more controlled environment of cell culture and/or animal models. With further methodological and technological advances, mass spectrometry-based proteome analysis of pancreatic fluid offers phenomenal potential for investigating the pathogenesis and progression of pancreatitis for the purpose of early diagnosis, retarding disease progression, and prevention of long-term disease complications.

Abbreviations

CP	chronic pancreatitis
CAP	chronic abdominal pain
DiGE	difference imaging gel electrophoresis
ePFT	endoscopic pancreatic function test
ERCP	endoscopic retrograde cholangiopancreatography
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
MW	molecular weight
PFT	pancreatic function test
SCX	strong cation exchange
TCA	trichloroacetic acid
Th	Thomson

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Figure 1. General workflow for mass spectrometry sample processing

Pancreatic fluid may be processed for mass spectrometry-based proteomic analysis using the general workflow as presented in this diagram. 1) The pancreatic fluid is collected, 2) protein is extracted from lipids and small molecule metabolites, 3) mass spectrometry sample preparation and data analysis is performed, and 4) bioinformatics techniques are used for protein identification and characterization.

Figure 2. Representative GeLC-MS/MS analysis of the peptide NLSQIVDIDGIWEGTR from pancreatic triacylglycerol lipase

A) Pancreatic proteins are separated by SDS-PAGE and gel bands are excised. B) Trypsin-digested peptides extracted from each gel band are separated via HPLC producing such a chromatogram. C) MS spectra are recorded as peptides are eluted/ionized from the LC system (retention time=50.09 min, as depicted). D) A predetermined number ion peaks are selected (peak at 1079.571 Th is selected for illustration), fragmentation by collision-induced dissociation is performed resulting in an MS/MS spectrum. Computational methods are used to determine the sequence of the fragmented peptide.

Figure 3. Optimized workflow for proteomic analysis of pancreatic fluid

A) 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. B) 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 C18 reversed-phased 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.

Figure 4. SDS-PAGE image of pancreatic fluid processed using our optimized sample preparation protocol

Pancreatic fluid from 12 patients processed as illustrated in Figure 3A, showing the distinct and well-resolved protein banding patterns.

Summary of the strategies used for peptide mass spectrometry-based proteomic analysis of pancreatic fluid.

Table 1

Study	Fluid collection	Protein extraction	Protein fractionation	Digestion	Peptide fractionation	Mass spectrometry modality	Bioinformatics	Ref.
Chen, et al 2006	ERCP	N/A	N/A	Trypsin	SCX-RP	LC-nESI-MS/MS	SEQUEST	[17]
Chen, et al 2007	ERCP	N/A	N/A	Trypsin	SCX-RP	LC-nESI-MS/MS	SEQUEST	[55]
Gronborg, et al 2004	surgery	N/A	SDS-PAGE	Trypsin	RP	LC-nESI-MS/MS	Mascot	[18]
Ke, et al 2009	EUS-FNA	acetone	ultrafiltration	Trypsin	N/A	MALDI-TOF-MS	Mascot	[24]
Paulo, et al 2010	ePFT	acetone	2-DE & GeLC-MS/MS	Trypsin	RP	LC-nESI-MS/MS	Mascot	[24]
Tian, et al 2008	ERCP	TCA	SDS-PAGE	Trypsin	RP	LC-nESI-MS/MS	Mascot	[29]
Zhou, et al 2007	ERCP	acetone	DiGE	Trypsin	N/A	MALDI-TOF-MS/MS	Mascot	[19]
		acetone	2-DE	Trypsin	N/A	MALDI-TOF-MS	Mascot	[20]

Abbreviations: ERCP, endoscopic retrograde cholangiopancreatography; ePFT, endoscopic pancreatic function test; DiGE, difference gel electrophoresis; SCX, strong cation exchange; RP, reversed-phase; EUS-FNA, endoscopic ultrasound-fine needle aspiration N/A, not available; nESI, nanoelectrospray ionization; MS/MS, tandem mass spectrometry; TOF, time of flight; MALDI, matrix-assisted laser desorption/ionization; not available.