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Analysis of the pancreatic low molecular weight proteome in an animal model of acute pancreatitis

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Abbreviations: LMW: low molecular weight; IEF: isoelectric focusing; ACN: Acetonitrile; FA: formic acid; PBS: phosphate buffer saline; OGE: OFFGEL electrophoresis; CV: coefficient of variation; HSP: heat shock proteins.
Abstract

We used a peptidomic approach for the analysis of the low molecular weight proteome in rat pancreatic tissue extracts. The goal was to develop a method that allows identifying endogenous peptides produced in the pancreas in the course of acute pancreatitis. The workflow combines peptides enrichment by centrifugal ultrafiltration, fractionation by isoelectric focusing, and LC-MS/MS analysis without prior enzymatic digestion. The method was assessed on pancreatic extracts from 3 rats with caerulein-induced pancreatitis and 3 healthy controls. A qualitative analysis of the peptide patterns obtained from the different samples was performed to determine the main biological processes associated to the identified peptides. Comparison of peptidomic and immunoblot data for alpha-tubulin, beta-tubulin and coatomer gamma showed that the correlation between the number of identified peptides and the protein abundance was variable. Nevertheless, peptidomic analysis highlighted inflammatory and stress proteins, which peptides pattern was related to acute pancreatitis pathobiology. For these proteins, the higher number of peptides in pancreatitis samples reflected an increase in protein abundance. Moreover, for murinogluolin-1 or carboxypeptidase B, peptide pattern could be related to protein function. These data suggest that peptidomic analysis is a complementary approach to proteomics for investigating pathobiological processes involved in acute pancreatitis.
1. Introduction

Acute pancreatitis is an inflammatory disease of the pancreas characterized by a great variability in its clinical presentation and severity. In most patients, the pathology evolves favorably without any complication but in up to 20% of the cases, the disease is severe with a potentially fatal outcome [1]. The molecular mechanisms involved in the initial steps of the disease are not completely understood. However, it has been shown that uncontrolled activation of trypsin within pancreatic acinar cells is an early and crucial event [1, 2]. Activation of trypsin and other digestives proteases leads to pancreatic tissue injury and local inflammation. Severe forms of the disease are related to the occurrence of an extensive necrosis of the pancreas and/or a systemic inflammatory response syndrome associated with remote organ injuries, in particular lung and kidney failures [1, 3]. Different animals models have been developed to study the pathobiology of acute pancreatitis and the main pathobiological processes involved in the disease have been identified [2]. However, the complexity of the interactions between the different pathobiological pathways makes difficult to understand which are the key molecular factors modulating disease course and severity.

Digestive proteases activation was shown to play a central role in initiation and progression of acute pancreatitis [4-6]. In the caerulein-induced acute pancreatitis model, trypsinogen activation occurs within 15 minutes following supramaximal caerulein stimulation and precedes other pathological features, such as hyperamylasemia, pancreatic edema or acinar cells vacuolization [5, 6].
Several studies demonstrated that, once activated, digestive proteases are important effectors of acinar cells injury [7, 8]. The role of digestive proteases on disease severity was further supported by the observation that prophylactic administration of protease inhibitors had a partial protective effect in experimental models of acute pancreatitis [7-9]. In addition to digestive proteases, several other proteolytic enzymes are also involved in the pathobiology of acute pancreatitis. Firstly, the lysosomal cysteine proteinase cathepsin B is believed to play an essential role in trypsinogen activation and disease initiation [10, 11]. According this "co-localization hypothesis", digestive enzyme zymogens and lysosomal hydrolases become co-localized within the same intracellular compartment leading to digestive proteases activation [12]. Secondly, the cytosolic cysteine protease calpain was shown to be a factor of acinar cell damage during caerulein-induced acute pancreatitis. Calpain is activated immediately after caerulein stimulation with a concomitant decrease of its endogenous inhibitor calpastatin [13]. Calpain activation results in the degradation of several actin-associated proteins and the alteration of the actin skeleton organization [14]. Thirdly, recruitment and activation of inflammatory cells contribute to the increased proteolytic activity in pancreatic tissue. In the initial phase of experimental pancreatitis, polymorphonuclear leukocyte elastase dissociates cell-cell contacts at adherens junctions and, thereby, allows the transmigration of leukocytes into the epithelial tissue [15]. Several studies showed neutrophil proteinases are important mediators of tissue injury and that administration of inhibitors of these enzymes decreased the severity of
experimental acute pancreatitis [16,17]. Furthermore, serum level of
polymorphonuclear elastase positively correlates with disease severity [18, 19].
The deleterious effects of polymorphonuclear elastase and digestive proteases
result from their proteolytic activity but also from effects on other pathobiological
processes, such as pancreatic microcirculation disturbance [16, 20] or lung
inflammation [21].
Unexpectedly, proteomic analysis of pancreatic tissue extracts from a rat model
of experimental pancreatitis showed only moderate proteome degradation [22].
Proteolysis of a limited number of proteins was observed suggesting that, at least
in the early steps of the disease, proteolytic processes could target specific
proteins. In this context, identification of proteolytic fragments generated during
the course of acute pancreatitis appears of great interest. It could allow a better
understanding of the role of proteases in acute pancreatitis pathobiology. We
therefore developed a method for the analysis of the low molecular weight (LMW)
proteome in pancreatic tissue extracts. LMW proteins and peptides were
enriched by centrifugal ultrafiltration [23, 24] and fractionated by isoelectric
focusing (IEF). Each IEF fraction was analyzed by LC-MS/MS without prior
enzymatic digestion since we wanted to identify endogenous peptides produced
in the course of acute pancreatitis. The validity of the peptidomic approach for
investigation of acute pancreatic pathobiology was evaluated by analyzing
peptides patterns identified in pancreatic tissue extracts from 3 rats with
caeerulein-induced pancreatitis and 3 healthy controls.
2. Experimental section

2.1. Experimental induction of acute pancreatitis and sample collection

Acute pancreatitis was induced in Sprague-Dawley rats (male, 250 g) by two intraperitoneal injections, at 1-h interval, of a supramaximally stimulating dose (10 µg/kg) of caerulein. Control rats received similar injections of saline solution. The animals were killed 5 h after the last caerulein or saline solution injection with pentobarbital sodium injection (50 mg/kg intraperitoneal). Pancreas tissue were immediately collected and snap-frozen in liquid nitrogen. Pancreas and serum samples were stored at -80 °C until analysis. The animal welfare committee of the University of Geneva and the veterinary office approved the protocol, and the study conformed to the American Veterinary Medical Association guidelines on human treatment of laboratory animals. Pancreatic tissue extracts were prepared by homogenization with a T18 Basic Ultra-Turrax disperser (IKA®-Werke, Staufen, Germany) in 1 mL of PBS containing a protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Basel, Switzerland). Samples and solutions were kept on ice during all the process. Homogenates were centrifuged at +4 °C and supernatants were stored at -80 °C until analysis. Pancreatitis 1, pancreatitis 2 and pancreatitis 3 pancreatic extracts were prepared from 750 mg, 510 mg and 640 mg of tissue, respectively. Control 1, control 2, and control 3 pancreatic extracts were prepared from 540 mg, 755 mg and 895 mg of tissue, respectively. Amylase and lipase activities were measured in serum samples from control and pancreatitis rats using Synchro® System reagents and Unicel® DxC 800 clinical chemistry analyzers (Beckman Coulter, Fullerton, CA).
2.2. Peptides enrichment and fractionation

Pancreatic tissue extract (500µl) was loaded onto a Microcon Ultracon YM-10 ultrafiltration device having a 10kDa molecular weight cut-off (Millipore, Billerica, MA, USA) and centrifuged 1h30 at 10,000g. The flow-through was dried under vacuum and the resulting pellet was dissolved in 200µl of 5% acetonitrile (ACN), 0.1% formic acid (FA). The sample was then desalted using C18 Macro SpinColumns (Harvard apparatus, Holliston, MA, USA). After sample loading, the column was washed with 5% ACN, 0.1% FA and elution was performed with two times 150µl of 50% ACN, 0.1% FA. The flow-through was dried under vacuum and dissolved in 720 µl of deionized water. A solution containing 6% glycerol and 0.15% IPG Buffer pH 3-10 (Agilent, Santa Clara, CA, USA) was added to a final volume of 3.6 ml. Peptides were fractionated according to their pI on an Agilent 3100 OFFGEL Fractionator (Agilent) using commercial 24 cm IPG pH 3-10 linear strips (GE Healthcare, Chalfont St. Giles, UK). The strip was rehydrated with 20 µl of rehydration solution (4.8% glycerol, 0.12% IPG Buffer pH 3-10) per well and incubated for 15 minutes. A volume of 150 µl of the sample solution was loaded per well. The isoelectric focalization was carried out at 20°C until a total voltage of 50 kV/h with a maximum current of 50 µA and a maximum power of 200 mW.

After the focalization, peptidic fractions were recovered in separate tubes and pH values were measured to check the accuracy of the pH gradient. Each fraction was dried under vacuum, dissolved in 5% CAN, 0.1% FA and loaded onto a C18 Micro SpinColumn (Harvard Apparatus). Elution was performed as previously
described. The 24 peptide fractions were evaporated, dissolved in 25 µl of 5% ACN, 0.1% FA and then stored at -80°C until MS analysis.

2.3 LC-MS/MS analysis

The 24 peptide fractions were analyzed using a LTQ Orbitrap XL (Thermo Scientific, San Jose, CA, USA) equipped with a NanoAcquity UPLC system (Waters). Peptides were trapped on a home-made 0.1 × 2 mm pre-column packed with Magic C18 AQ 5µm, 200Å stationary phase (Michrom Bioresources, Auburn, CA, USA) and then separated on a home-made 0.75 × 15 mm column packed with Magic C18 AQ 5µm 100Å stationary phase (Michrom Bioresources). The analytical separation was run for 65 min using a gradient of H₂O/FA 99.9%/0.1% (solvent A) and ACN/FA 99.9%/0.1% (solvent B). The gradient was run at a flow rate of 220 nL·min⁻¹ as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min. For MS survey scans, the Orbitrap resolution was set to 60’000 and the ion population was set to 5 × 10⁵ with an m/z window from 400 to 2’000. Maximum of 3 precursors were selected for both collision-induced dissociation in the LTQ and high-energy C-trap dissociation with analysis in the Orbitrap. For MS/MS in the LTQ, the ion population was set to 1 × 10⁴ (isolation width of 2 m/z) while for MS/MS detection in the Orbitrap, it was set to 2 × 10⁵ (isolation width of 4 m/z), with a resolution of 7’500, first mass at m/z = 100, and a maximum injection time of 750 ms. The normalized collision energies were set to 35% for CID and 50% for HCD.
2-4 Database searching

Peak lists were generated from raw data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [25]. The corrected mgf files, combined from the 24 analyzed OFFGEL fractions, were searched against UniProt_Swiss-Prot database (57.10 of 03-Nov-2009) using Phenyx software (GeneBio, Geneva, Switzerland). *Rattus norvegicus* taxonomy was specified for database searching. The parent ion tolerance was set to 25 ppm. Variable amino acid modification was oxidized methionine. Unspecific was selected as the enzyme, with 35 potential missed cleavages, and the normal cleavage mode was used. Only one search round was used with selection of “turbo” scoring. The peptide p value was 1 E-4 for LTQ-Orbitrap data. False-positive ratios were estimated using a reverse decoy database [26]. All datasets where searched once in the forward and once in the reverse database. Separate searches were used to keep the database size constant. Protein and peptide scores were then set up to maintain the false positive peptide ratio below 1%. This resulted in a slight overestimation of the false-positive ratio [26].

2-5 Immunoblotting

Ten µg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T, 2.6% C). Proteins were then electroblotted onto a nitrocellulose membrane essentially as described by Towbin et al. [27]. Membranes were stained in Ponceau red, destained in water and
scanned to control homogeneity of sample loading. Immunodetection was performed as follows. Membranes were blocked in phosphate buffer saline (PBS), 0.05% Tween 20, 5% nonfat milk for 1 hour at room temperature and incubated with primary antibody overnight at 4°C. Primary antibodies were used at the following dilutions in blocking buffer: 1:1000 for rabbit polyclonal anti-alpha 1 inhibitor 3 (ab61338, Abcam, Cambridge, UK), 1:10’000 for rabbit monoclonal anti-alpha-tubulin (ab52866, Abcam), 1:500 for mouse monoclonal anti-beta-tubulin (sc-53140, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 for goat polyclonal anti-coatomer gamma (sc-14167, Santa-Cruz Biotechnology). Membranes were washed with blocking buffer and incubated with secondary antibody (Dako, Glostrup, Denmark) at 1:2’000 in blocking buffer for 1 hour at room temperature. Membranes were washed in PBS, 0.05% Tween 20, developed with BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland), and visualized on X-ray films.
3. Results

3.1. Induction of acute pancreatitis

Induction of experimental acute pancreatitis was assessed by measuring serum amylase and lipase activities 5 hours after the second caerulein injection. As shown in Figure 1, these two markers of pancreatic damage were strongly increased in pancreatitis rats compared to healthy controls.

3.2. Peptides identification

Combination of centrifugal ultrafiltration, OFFGEL electrophoresis (OGE) fractionation and LC-MS/MS analysis allowed to identify 1216 unique peptides corresponding to 282 proteins in control sample 1 (Supplementary Table 1), 4189 unique peptides corresponding to 635 proteins in control sample 2 (Supplementary Table 2), 2945 unique peptides corresponding to 512 proteins in control sample 3 (Supplementary Table 3), 2978 unique peptides corresponding to 508 proteins in pancreatitis sample 1 (Supplementary Table 4), 1530 unique peptides corresponding to 323 proteins in pancreatitis sample 2 (Supplementary Table 5), and 2155 unique peptides corresponding to 471 proteins in pancreatitis sample 3 (Supplementary Table 6). The efficiency of OGE peptide fractionation was evaluated by calculating the number of peptides identified in each fraction. The pH of each fraction was also measured to check that the pH gradient was correctly established during IEF. Results obtained for pancreatitis sample 1 are presented in Figure 2. The number of peptides identified in each fraction was not even but the overall pattern was similar to results observed for other proteomes.
[28, 29]. These data also confirmed that the pH gradient was correctly established all along the IPG strip during IEF. As previously indicated, MS data were searched against UniProt_Swiss-Prot database without specifying any proteolytic enzyme in order to maximize the number of identified peptides, irrespective of the mechanism by which they were obtained. MS data were also search against UniProt_Swiss-Prot database with trypsin specified as the proteolytic enzyme (data not shown). Only a few peptides were identified, indicating that almost all peptides detected in pancreatic extracts were not tryptic peptides. Examples of spectra obtained for peptides identified from carboxypeptidase B and murinoglobulin 1 are presented in Figure 3.

3.3. Biological processes

For further analysis of peptide identification results, we made a selection from the total list of identifications. We excluded peptides from proteins identified in a only one pancreatic extract, peptides from proteins identified in a several samples but with only a single peptide, and peptides from proteins identified in two samples with only two and one unique peptides, respectively. The goal was to focus on peptides from proteins identified with a high level of confidence. The 487 proteins matching these criteria are listed in Supplementary Table 7. Each of these proteins was attributed a biological function using data from Swiss-Prot and Gene Ontology databases. The number of peptides associated to each functional category was calculated for the pancreatitis and the control samples. The functional classification of these peptides is presented in Figure 4. The most
represented functional categories in control samples were cytoskeleton, translation, metabolic processes and unknown. These observations were consistent between samples with coefficients of variation (CV) of 21% for cytoskeleton, 21% for metabolic processes, 23% for translation, and 5.9% for unknown. Cytoskeleton, metabolic processes, and translation represent nearly 50% of the identified peptides in control samples, which is not surprising when considering cellular abundance of the corresponding proteins. In pancreatitis samples, cytoskeleton and translation were the only two functional categories above 10%, with cytoskeleton representing more than one fourth of the identifications. The proportion of peptides from metabolic process and unknown were decreased to 9.1% and 7.8%, respectively. As observed for controls, inter-samples reproducibility was quite good with CVs of 4.7% for cytoskeleton, 48% for metabolic processes, 11% for translation, and 28% for unknown. The two main differences between the two groups of samples were peptides from cytoskeletal proteins, which changed from 19.3% in controls to 27.6% in pancreatitis samples and peptides from proteins involved in inflammatory response, which changed from 0.7% in controls to 7.8% in pancreatitis samples. Inter-samples variability was relatively high for inflammatory peptides with CVs of 36% for controls and 58% for pancreatitis samples but results were nevertheless reliable with values ranging from 0.48% to 0.99% for controls and from 4.6 to 13% in pancreatitis samples.

3.4 Comparison of immunoblot and peptidomic data for selected proteins
At the level of individual proteins, an important variability in the number of identified peptides was often observed between samples from the same group. Furthermore, an overlap was often found between control and acute pancreatitis groups (Supplementary Table 7). Normalizing these data to the total number of peptides identified in the pancreatic extract decreased inter-samples variability for some proteins but, in numerous cases, did not change the situation. In this context, the question was to know whether peptidomic results could be indicative of the concentration of a native protein or of its degradation status in pancreatic extracts. We therefore compared, for selected proteins, immunoblot data with peptidomic results, using both the absolute and relative numbers of identified peptides. We first considered alpha and beta tubulin chains, which have been detected with a large number of unique peptides in both control and pancreatitis samples. For tubulin alpha chains, the mean number of identified peptides and the mean ratio, obtained by normalization to the total number of identified peptides, were increased by approximately 2-fold in pancreatitis samples compared to controls (Table 1). There was an overlap between the control and pancreatitis groups for the absolute numbers of identified peptides but not for the ratios. Immunoblot performed for alpha tubulin showed a strong decrease in the native protein concentration in pancreatitis extracts with faint bands of lower molecular weight, probably corresponding to proteolytic products (Figure 5). Degradation products were also detected in control samples. For tubulin beta chains, the mean number of identified peptides and the mean normalized ratio were slightly higher in pancreatitis samples compared to controls but there was
an overlap between the two groups for both parameters (Table 1). Immunoblot results indicated that the beta tubulin concentration was reduced in pancreatitis samples compared to controls (Figure 5). Band intensities suggested that it was, however, to a lower extent than for alpha tubulin. Taken together, these data suggest that the decrease in native protein concentration observed for alpha and beta-tubulin chains in pancreatitis samples were associated with modifications of their peptide pattern detected by peptidomic analysis. This correlation appeared to be better when normalizing to the total number of identified peptides rather than with absolute numbers of identified peptides. A comparison of peptidomic and immunoblot data was also performed for coatamer gamma. Peptides from this protein were identified in two controls and in two pancreatitis samples. The inter-samples variability was high either considering the total number of identified peptides or normalized ratios (Table 1). In contrast, homogenous results were obtained by immunoblot for both control and pancreatitis groups. In controls, the native protein was clearly visible along with faint bands of lower molecular weight suggesting the presence of proteolytic products. In pancreatitis samples, the native proteins was not or faintly visible with a strong pattern of degradation. In the case of coatamer gamma, peptidomic data were therefore not reflecting changes in the concentration and the degradation status of the native protein.

3.5 Pancreatitis specific peptide patterns

Peptidomic analysis identified several proteins, which proteolytic pattern was increased in pancreatitis samples. Peptides from these proteins were detected in
all pancreatitis samples and the number of identified peptides was at least 2
times higher than in controls (Table 2). For half of them, no peptides were
detected in controls extracts. Most of these proteins were related to inflammatory
response. The list also includes inducible heat shock proteins (HSP) and
digestive proteases. Sequences analysis showed that for some of these proteins,
the peptides identified using peptidomics were located only or predominantly
within a specific region of the protein sequence. This suggests that proteolysis
could be related to a particular function of the protein. Murinoglobulin 1 is one
example of such proteins. Of the 19 unique peptides identified in pancreatitis
samples for this protein, 18 were located within a small sequence of 21
aminoacids (Figure 6). This sequence is part of a region that is directly involved
in the anti-protease activity of murinoglobulin 1. Similar findings were obtained for
alpha-1-inhibitor 3, a protein homolog to murinoglobulin 1. Carboxypeptidase B, a
digestive protease specifically synthesized in pancreatic acinar cells, gave
another example of a peptide pattern related to a particular protein function. Of
51 peptides identified for this protein in pancreatitis samples, 46 were localized
within the activation peptide that is cleaved upon activation of the zymogen
(Supplementary Figure 1).
4. Discussion

We developed a peptidomic method for the analysis of the LMW proteome in rat pancreatic tissue extracts. The objective was to set up an analytical tool that allows monitoring proteolytic processes during the course of acute pancreatitis. In order to evaluate the reliability of this peptidomic workflow, we performed a qualitative analysis of peptide patterns in pancreatic extracts from rats with experimental acute pancreatitis and healthy controls. As exemplified by peptide sequences presented for murinoglobulin or carboxypeptidase B, analysis of MS data showed that almost all identified peptides were not tryptic. This was confirmed by the fact that database searching with trypsin selected as the proteolytic enzyme yielded only a few identifications (data not shown).

Considering the multiple endo- and exoproteases present in the pancreas, these data suggest that the peptides identified in this study resulted from various proteolytic cleavages.

The results we obtained underlined the complexity of peptidomic analysis and potential problems for results interpretation. Inter-samples variability was high in both control and pancreatitis groups regarding the total number of peptides identified. The fact that extracts were prepared from different amounts of tissue could not fully explain this variability, in particular for control samples. In addition, contrary to what one should expect, the number of unique peptides identified was not higher in pancreatitis samples. These observations were also true, in many cases, when peptides patterns detected for individual proteins were considered. Different factors, probably acting in combination, can explain these results.
Firstly, biological variability between animals and differences in the response to the experimental model can cause inter-samples variability. However, no direct correlation between peptidomic results and serum amylase or lipase level was found. Secondly, technical variability, for example during tissue extracts preparation or OGE fractionation, can introduce some bias. Thirdly, a part of the identified peptides may correspond to the so-called “degradome”. Such peptides are not related to biological or pathobiological processes but are produced post-mortem during tissue collection and sample processing. Pancreas was shown to be a tissue that may produce a relatively high amount of such degradation products when using a snap-freezing tissue sampling procedure [30]. A number of peptides detected in controls and pancreatitis extracts may therefore correspond to degradome products. Finally, the peptidomic method was not quantitative and the number of peptide detected in pancreatitis extracts was not reflecting absolute amounts present in the sample. Due the very high sensitivity of the Orbitrap mass spectrometer, similar numbers of peptides could have been detected in controls samples using data-dependent acquisition despite the fact that peptide concentrations were much lower than in pancreatitis samples. This could explain that results obtained for the different functional categories, as well as for individual proteins such as tubulin alpha chains, appeared more consistent between samples from the same group when normalizing to the total number of identified peptides. Taken together, these data stress the need for developing reliable normalization strategies for further comparative peptidomic studies using quantitative approaches, such as label-free quantification.
Despite these limitations, a number of results obtained in this study were fully relevant in the context of acute pancreatitis. Analysis of the relation between identified peptides and biological processes showed that the proportion of peptides from proteins associated to inflammation, a key pathobiological process of acute pancreatitis, was strongly increased in pancreatitis samples. This phenomenon can be explained by the fact that the concentration of inflammatory proteins is increased in the pancreas following disease onset and, as a consequence, the number of proteolytic peptides detected by MS for these proteins was increased. Most of the inflammatory-related peptides detected in acute pancreatitis samples were from plasma proteins, such as apolipoproteins AI and AIV, alpha-1-inhibitor 3, murinoglobulin1, alpha-2-HS-glycoprotein, complement C3, fibrinopeptides A and B, or serine protease inhibitor A3K, which arose in pancreatic tissue due to plasma extravasation and edema [2]. Increased pancreatic levels of apolipoprotein AI, alpha-1-inhibitor 3, murinoglobulin1 and complement C3 have been previously described in caerulein-induced pancreatitis using 2-DE and immunoblotting [22]. We also detected in pancreatitis samples, but not in controls, peptides from proteins whose expression is induced in the pancreas in response to pancreatic tissue injury and acute pancreatitis: clusterin [31-33], Heat shock 70 kDa protein 1A/1B and HSP beta-1 (HSP27) [34, 35]. HSP70 and HSP27 were shown to exert an endogenous protective effect against pancreatic injury [36-38]. An increased proportion of peptides from cytoskeletal proteins was also found in pancreatitis samples. This finding is in accordance with the fact that microtubules
and microfilaments are degraded in the early steps of acute pancreatitis, which
results in the disassembly of acinar cell cytoskeleton [39, 40]. This process is
believed to be responsible for the inhibition of digestive enzyme secretion by
interfering with intracellular vesicular transport [39-41]. Using immunoblot
analysis, we confirmed that the change in peptidomic pattern observed in
pancreatitis samples for alpha and beta-tubulin chains was correlated with a
decreased expression of the native proteins. However, probably because
cytoskeletal proteins are constitutively expressed in the pancreas, numerous
peptides were also found in control samples, making interpretation of peptidomic
data more difficult than for inflammatory or stress inducible proteins. In addition,
immunoblot analysis performed for the Golgi-associated protein coatamer
gamma showed that, for some proteins, peptidomic results were not indicative of
the protein condition in pancreatic extracts. Indeed, peptidomic data obtained for
this protein were not informative while immunoblotting clearly showed that it was
degraded in pancreatitis samples, potentially as a consequence of the Golgi
apparatus disorganization [42].

Finally, peptidomic analysis identified a few proteins with an increased peptide
pattern in pancreatitis samples, which, to our knowledge, have never been
described in acute pancreatitis. One of them is a stress protein called serpin H1
or HSP47. Peptides from this protein were identified in pancreatitis samples
only. Interestingly, HSP47 is a collagen-binding stress protein, which was shown
to be over-expressed in various models of tissue injury [43-45]. Another protein
detected in pancreatitis only was peflin, a calcium-binding protein of unknown
function [46]. Finally, histone H1.2 was identified in pancreatitis extracts with a number of peptides at least two times higher than in controls. Histone H1.2 is directly involved in the regulation of apoptosis and may triggers caspases activation [47-49]. Interestingly, analysis of peptidomic data obtained for some proteins provided information on specific molecular mechanisms related to acute pancreatitis pathobiology. The first examples were murinoglobulin 1 and alpha-1-inhibitor 3. These proteins, highly homologous to human alpha-2-macroglobulin, are protease inhibitors that function through a trapping mechanism. The target protease cleaves the alpha-macroglobulin within a specific sequence called the "bait region", thereby triggering a conformational change of the alpha-macroglobulin and entrapment of the endopeptidase [50]. In a previous study, we showed using 2-DE, MS analysis and immunoblotting that high levels of amino- and carboxy-terminal fragments of alpha-1-inhibitor 3 and murinoglobulin 1, probably resulting from protein cleavage within the bait region, were present in pancreatic extracts from rats with acute pancreatitis [22]. Peptidomic analysis confirmed these findings since 18 of the 19 peptides identified for murinoglobulin 1 were located within the bait region. These data suggest that alpha-1-inhibitor 3 and murinoglobulin 1 may play an important role in the control of proteolytic activity during acute pancreatitis. Another example of peptidomic data correlated with a pathobiological mechanism was carboxypeptidase B. The peptide pattern identified for this digestive protease reflects its activation following induction of acute pancreatitis. Indeed, 46 of the 51 peptides identified for this protein were
localized within the activation sequence, which is cleaved upon activation of the 
zymogen. Again, peptidomic results were complementary to 2-DE analysis, which 
allowed visualizing the raise of active carboxypeptidase B in pancreatic extracts 
following caerulein treatment [22].

This study demonstrated that peptidomic analysis of pancreatic tissue extracts is 
an interesting approach for investigating pathobiological processes involved in 
acute pancreatitis. Analysis of peptide patterns from pancreatitis and control 
samples allowed highlighting a number of differences that were relevant to acute 
pancreatitis pathobiology. In addition, proteolytic fragments from a few proteins 
that have never been described in acute pancreatitis were detected in 
pancreatitis samples but not in the healthy control, which suggests that analysis 
of the LMW proteome could bring new insights in disease mechanisms.

Nevertheless, this study also underlined the complexity of peptidomic analysis 
and identified a number of pitfalls that can lead to experimental bias. Therefore, 
peptidomic analysis should be used in combination with proteomic techniques 
and careful bibliographical and database searches should be done to help for 
data interpretation. Combination of this peptidomic workflow with label-free 
quantitative LC-MS/MS would probably be of great interest for the study of 
experimental models of acute pancreatitis but performing reliable comparative 
studies will require establishing suitable normalization procedures. Including rats 
treated with protease-inhibitors in future peptidomic studies could also be of 
interest to better understand the role of proteolytic processes in acute 
pancreatitis pathobiology.
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Tables:

Table 1: Absolute and relative numbers of peptides identified in acute pancreatitis and control samples for alpha tubulins, beta tubulins and coatamer gamma. Numbers given for tubulin alpha chains correspond to the sum of peptides identified for tubulin alpha-1A, alpha-1C, alpha-4A, and alpha-8 chains. Numbers given for tubulin beta chains correspond to the sum of peptides identified for tubulin beta-2A, beta-2C, and beta-5 chains. Ratios were obtained by normalizing peptide numbers for alpha tubulins, beta tubulins and coatamer gamma to the total number of peptides identified in the pancreatic extract. CTL: control, AP: acute pancreatitis sample, CV: coefficient of variation.

<table>
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<th>Peptides</th>
<th>CTL 1</th>
<th>CTL 2</th>
<th>CTL 3</th>
<th>Mean</th>
<th>CV</th>
<th>AP 1</th>
<th>AP 2</th>
<th>AP 3</th>
<th>Mean</th>
<th>CV</th>
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<td></td>
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<td></td>
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<tr>
<td>Absolute number</td>
<td>28</td>
<td>86</td>
<td>26</td>
<td>46</td>
<td>73%</td>
<td>111</td>
<td>58</td>
<td>101</td>
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<tr>
<td>Ratio %</td>
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<td>1.74</td>
<td>43%</td>
<td>3.73</td>
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<td><strong>Tubulin beta chains</strong></td>
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<tr>
<td>Absolute number</td>
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<td>87</td>
<td>108</td>
<td>44%</td>
<td>159</td>
<td>102</td>
<td>127</td>
<td>129</td>
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<td>2.95</td>
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<td><strong>Coatomer gamma</strong></td>
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<tr>
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<td>2.5</td>
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<td>Ratio %</td>
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<td>0.00</td>
<td>0.05</td>
<td>0.11</td>
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Table 2: Proteins with different proteolytic patterns in acute pancreatitis and control samples. Proteins were selected based on the following criteria: (i) peptides were identified all pancreatitis samples, (ii) the number of peptides was at least 2-fold higher in pancreatitis samples than in controls. The number of peptides identified in each sample and the biological function of the proteins, determined from Swiss-Prot and Gene Ontology databases, are indicated. AP: acute pancreatitis sample. CTL: control.

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<tr>
<th>Swiss-Prot Entry</th>
<th>AC</th>
<th>Protein Name</th>
<th>AP 1</th>
<th>AP 2</th>
<th>AP 3</th>
<th>CTL1</th>
<th>CTL2</th>
<th>CTL3</th>
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<td>CBPA1_RAT</td>
<td>P00731</td>
<td>Carboxypeptidase A1</td>
<td>11</td>
<td>9</td>
<td>6</td>
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<td><strong>DNA binding protein/transcription</strong></td>
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<td>11</td>
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<td>5</td>
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Figures captions

Figure 1. Serum amylase and lipase activities in control and pancreatitis rats. Acute pancreatitis was induced in rats by two intraperitoneal injections of caerulein, at 1-hour interval. Control rats received similar injections of saline solution. The animals were killed 5 hours after the last injection. Serum were collected and stored at –80°C until analysis. Enzymatic assays were performed on a clinical chemistry analyzer Unicel® DxC 800 using Synchron® System amylase and lipase reagents (Beckman Coulter). A single measurement was done. Means and standard deviations are indicated on the graph. CTL: control, AP: acute pancreatitis.

Figure 2. Peptide fractionation using OFFGEL electrophoresis. Pancreatic peptides were enriched by centrifugal ultrafiltration using a 10kDa molecular weight cut-off. Peptides were fractionated according to their pI on an Agilent 3100 OFFGEL Fractionator using commercial 24 cm IPG pH 3-10 linear strips (GE Healthcare). Peptidic fractions were recovered in separate tubes and pH values were measured to check accuracy of the pH gradient. Peptide fractions were analyzed using a nanoRP-UPLC system (Waters) connected to an LTQ-Orbitrap mass spectrometer (Thermo Scientific). Peptides identification was carried out using Phenyx software (Genebio). The number of peptides identified and the pH measured after IEF are indicated for each OFFGEL fraction from pancreatic extract of pancreatitis sample 1.
Figure 3. MS spectra of peptides identified from carboxypeptidase B and murinoglobulin 1. MS analysis was performed on a LTQ Orbitrap XL (Thermo Scientific) as described in Material and Methods section. Peak lists were searched against UniProt_Swiss-Prot database using Phenyx software (GeneBio). *Rattus norvegicus* taxonomy was specified for database searching. False positive peptide ratio for protein and peptide identifications was below 1%.

Exp. data: experimental data; b, y: b and y ions.

Figure 4. Biological functions associated to the peptides identified in control and pancreatitis samples. 487 proteins were selected from the total list of identifications by excluding peptides from proteins identified in a only one pancreatic extract, peptides from proteins identified in a several samples but with only a single peptide, and peptides from proteins identified in two samples with only two and one unique peptides, respectively (Supplementary Table 7). A biological function was attributed to each of these proteins using data from Swiss-Prot and Gene Ontology databases. The number of peptides associated to each functional category was calculated for the pancreatitis and the control groups. Percentages corresponding to each functional category are presented as pie charts.

Figure 5. Immunoblot of alpha-tubulin, beta-tubulin and coatomer gamma. Ten µg of pancreatic protein extracts were separated by SDS-PAGE on
homemade Tris-Glycine gels (12.5% T). Proteins were then electroblotted onto a
nitrocellulose membrane as described by Towbin et al. [27]. Immunodetection
was performed as described in Experimental section using antibodies against
alpha-tubulin, beta-tubulin and coatomer gamma. BM Chemiluminescence
Blotting Substrate (Roche, Basel, Switzerland) was used for detection. CTL:
control, AP: acute pancreatitis.

Figure 6. Peptides identified for murinoglobulin 1 located within the bait
region. On the left, the complete sequence of the protein is given with the bait
region indicated in bold italic letters. On the right, an enlargement is shown with
the sequence covered by LC-MS/MS analysis in the bait region indicated in bold
italic letters. Sequences of the 18 unique peptides identified in the bait region are
given below.
References


41. Ueda, T.; Takeyama, Y.; Kaneda, K.; Adachi, M.; Ohyanagi, H.; Saitoh, Y.


Synopsis:

We analyzed the low molecular weight proteome in pancreatic extracts from rats with experimental pancreatitis and controls. A qualitative analysis was performed to evaluate the reliability of the approach. Peptidomic analysis highlighted proteins, which peptides pattern was related to acute pancreatitis pathobiology. Moreover, for some proteins, peptide pattern could be related to protein function. These data suggest that peptidomic analysis is an interesting approach for investigating acute pancreatitis pathobiology.

Q03626, Murinoglobulin-1, Rattus norvegicus (Rat)
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78x182mm (400 x 400 DPI)
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177x125mm (400 x 400 DPI)
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145x134mm (400 x 400 DPI)