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Proteomic profiling in an animal model of acute pancreatitis

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Abbreviations: AP: Acute pancreatitis, CRP: C-reactive protein, DTE: dithioerythritol, MW: molecular weight, A1I3: alpha 1 inhibitor 3, HSP: heat shock protein.

Keywords: acute pancreatitis, apoptosis, biomarker, inflammation, oxidative stress.

For Peer Review

Abstract

Acute pancreatitis is an inflammatory disease of the pancreas, which evolves in approximately 20% of the patients to a severe illness associated with a high mortality rate. In this study, we performed a comparative proteomic analysis of pancreatic tissue extracts from rats with acute pancreatitis and healthy rodent controls in order to identify changes in protein expression related to the pathobiological processes of this disease. Pancreatic extracts from diseased and control rats were analyzed by 2-DE and MS/MS. A total of 125 proteins were identified from both samples. Comparative analysis allowed the detection of 42 proteins or protein fragments differentially expressed between diseased and control pancreas, some of them being newly described in acute pancreatitis. Interestingly, these changes were representative of the main pathobiological pathways involved in this disease. We observed activation of digestive proteases and increased expression of various inflammatory markers, including several members of the alpha-macroglobulin family. We also detected changes related to oxidative and cell stress responses. Finally, we highlighted modifications of 14-3-3 proteins that could be related to apoptosis regulation. These results showed the interest of proteomic analysis to identify changes characterizing pancreatic tissue damage and, therefore, to highlight new potential biomarkers of acute pancreatitis.

1 Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas, whose severity varies widely from mild forms only affecting the pancreas (80% of the patients) to severe forms associated with multiple organ failure (20% of the patients) [1]. Severe AP is associated with a high mortality rate (15%) and requires the rapid transfer of the patient in an intensive care unit. Laboratory testing is a key element in the management of AP both for diagnosis and assessment of disease severity. Biological diagnosis relies on the detection of enzymes released by damaged pancreatic tissue. The two enzymes currently used in clinical practice are amylase and lipase [2]. However, serum amylase lacks specificity since increased levels are also observed in various diseases associated with intra-abdominal inflammation. For discrimination between mild and severe AP, the best-established laboratory assay is the measurement of blood C-reactive protein (CRP) 48 hours after symptoms onset [3]. However, one important limitation is that organ failures can occur within this delay of 48 hours.

The main processes involved in AP pathobiology are inflammation, edema and necrosis of pancreatic tissue as well as inflammation and injury of extrapancreatic organs [3, 4]. The initial phase of AP is characterized by local inflammation with intracellular activation of digestive enzymes. In mild AP, the disease remains restricted to this stage. However, in severe forms, the pathology evolves towards a second phase with generalized inflammation and systemic inflammatory response syndrome. A third stage corresponds to the development of multiple organ damages. Delayed complications can also result from infected necrosis and sepsis. Pathobiology of AP involves a number of intrapancreatic and extrapancreatic, intracellular and extracellular events that all play an important role in regulating the course of the disease and the extent of the local and systemic damages [4]. The complexity of the interactions between these different pathways makes how those processes are initiated and the relative importance of each one in modulating the course of the disease difficult to understand.

To allow investigating AP pathobiology, numerous animal models have been developed, which differ in the severity of the illness and characteristics of the pathobiological response [4, 5]. However, most studies published until now were focused on a particular aspect of AP pathobiology. Due to its ability to provide an overview of protein expression profiles at cellular or tissue level, proteomic analysis of tissue extracts from such experimental models could provide new insights into the pathobiological processes of AP. Moreover, proteomic analysis could permit the identification of changes in protein patterns characteristic of pancreatic tissue damage and help understanding molecular factors determining the course of the disease. Such findings would help highlighting new potential diagnosis or prognosis biomarkers of AP. In order to demonstrate the interest of proteomics for investigating experimental models of AP, we performed a comparative 2-DE analysis of pancreatic tissue extracts from rats with AP and healthy controls. We used a well-described experimental model in which edematous AP is induced by the administration of cerulein [4]. As expected, our results highlighted numerous changes in protein expression, including several that have not been previously described in AP. The putative relation of the detected changes with key pathobiological pathways of AP will be discussed.

2 Materials and methods

2.1 Experimental induction of acute pancreatitis and sample collection

AP was induced in Sprague-Dawley rats (male, 250 g; n = 3) by two intraperitoneal injections, at 1-hour interval, of a supramaximally stimulating dose (10 µg/kg) of cerulein. Control rats (n = 3) received similar injections of saline solution. The animals were killed 5 hours after the last cerulein or saline injection with pentobarbital sodium injection (50 mg/kg intraperitoneal). Pancreas and serum were collected and stored at –80°C. 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 humane 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). Homogenates were centrifuged and supernatants were stored at –80°C until analysis. Protein concentration was determined according to the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Amylase and lipase activities were measured in serum samples from control and AP rats using Synchron® System reagents and Unicel® Dx C 800 clinical chemistry analyzers (Beckman Coulter, Fullerton, CA).

2.2 Two-dimensional electrophoresis

Pancreatic protein extracts were mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithioerythritol (DTE), 1% ampholytes 4-8) to a final volume of 400 µL and loaded onto commercial 18 cm IPG strips pH 4-7 (GE Healthcare, Chalfont St. Giles, UK) by overnight in-gel rehydration. The amount of proteins applied was 150 µg for analytical gels and 450 µg for preparative gels. For analytical gels, protein extracts from the 3 AP and 3 controls rats were run in duplicate generating a total of 12 gels. IEF was performed under the following running conditions: 300 volts for 1 minute, 300 to 3500 volts for 3 hours, 3500 volts for 3 hours and 5000 volts for 18 hours (total 100 kVh) using a Multiphor system (GE

Healthcare, Chalfont St. Giles, UK). Strips were then equilibrated under gentle agitation for 12 minutes in equilibration buffer (50 mM Tris-HCl pH 8.4, 6 M urea, 30% glycerol and 2% SDS) containing 20 mg/mL of DTE, followed by 5 minutes in equilibration buffer containing 25 mg/mL of iodoacetamide. After equilibration, strips were transferred on the top of in-house manufactured vertical SDS-PAGE gels (12.5% T, 2.6% C) and fixed with 0.5% agarose. The second dimensional separation was performed using a Protean II XL Multi-Cells system (Bio-Rad, Hercules, CA) at 40 mA/gel for 5 hours. Analytical gels were then stained with ammoniacal silver [6]. Preparative gels were fixed with 50% methanol and 7% acetic acid for twice 30 minutes. After fixation, the gels were submerged in Sypro[®] Ruby (Invitrogen Corporation, Carlsbad, CA) for overnight incubation in the dark. Gels were then washed with 10% methanol and 7% acetic acid for 30-60 minutes.

2.3 Image analysis

Silver-stained gels were scanned using a laser densitometer. AP and control group comprised six gels each. Image analysis was carried out with the ImageMaster 2D Platinum software (GE Healthcare, Chalfont St. Giles, UK). Protein spots were detected automatically and improperly detected spots were eliminated by manual editing. For each sample group, a gel was chosen as reference. Gels from the same group were then matched automatically to this reference gel and the correspondence of the spots on each gel was inspected and corrected manually. Comparative analysis of AP and control samples was first performed using the reference gels. Matching spots across these two gels were detected and quantified automatically. The volume and the %volume were automatically computed, including background subtraction in order to correct for differences in gel staining. Differences detected between the two reference gels were then confirmed on the other gels using the same procedure. Detection of differentially expressed polypeptides was done with a minimum mean %volume ratio of 1.8. Only changes consistent between the 6 control and 6 AP gels were taken into account.

2.4 MALDI-TOF-TOF analysis

Spots of interest were excised from preparative gels and destained with twice 100 μ L of 50% acetonitrile, 50 mM ammonium bicarbonate pH 8.0 for 15 minutes. A third washing step was performed with 100 μ L of acetonitrile for 10 minutes and gel pieces were dried for 30 minutes in a vacuum centrifuge. Trypsin digestion was performed as previously described [7].

Peptides were analyzed on a 4800 MALDI-TOF/TOF™ analyzer (Applied Biosystems, Foster City, CA). Each sample (0.5 μ L) was deposited twice on an Opti-TOF™ plate (Applied Biosystems, Foster City, CA) and dried under vacuum. Equal volumes of matrix (5 mg/mL α -cyano-4-hydroxycinnamic acid in 50% CH₃CN, 0.1% TFA, 10 mM NH₄H₂PO₄) were added on top of sample spots and dried under vacuum. Argon was used as the collision gas. A MS scan was conducted from 800 to 4000 m/z, and the 20 most abundant peaks with signal to noise ratios >10 were selected for MS/MS.

2.5 Database search

Peak lists were generated using the 4000 Series Explorer™ software (Applied Biosystems, Foster City, CA). Resulting peak lists were searched against the UniProtKB combined Swiss-Prot and TrEMBL database (release 12.0) restricted to *rattus norvegicus* using Mascot (version 2.2.03, Matrix Science Ltd, London, UK) and Phenyx (version 2.5, GeneBio, Geneva, Switzerland) softwares. Mascot was used with average mass selected, a precursor mass error of 1.0 Da and a peptide mass error of 0.6 Da. Trypsin was selected with a single potential missed cleavage. MALDI-TOF/TOF was selected as the instrument.

Carbamidomethyl was selected as fixed modification and oxidized methionine and deamidation as variable modifications. For Phenyx, MALDI-TOF/TOF was selected as the instrument. Two search rounds were used, both with trypsin selected, carbamidomethyl as fixed modification and oxidized methionine as variable modification. In the first round, one missed cleavage was allowed and the normal cleavage mode was used. This round was selected in “turbo” search mode. In the second round, three missed cleavages were allowed, the cleavage mode was set to half-cleaved and deamidation was added as variable

modification. The minimum peptide length allowed was 6 aminoacids and the parent ion tolerance was 1.0 Da in the first round and 0.8 Da in the second round. The acceptance criteria were slightly lowered in the second round search (round 1: AC score 8.0, peptide Z-score 7.0, peptide p-value 1.0 E-7; round 2: AC score 8.0, peptide Z-score 6.0, peptide p-value 1.0 E-6).

2.6 Immunoblotting assay

Ten μg of pancreatic protein extracts were separated by SDS-PAGE on Tris-Glycine gels (12.5% T, 2.6% C). Proteins were then electroblotted onto a nitrocellulose membrane essentially as described by Towbin *et al.* [8]. 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 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 dilution in blocking buffer: 1:1000 for anti-alpha 1 inhibitor 3 (ab61338, Abcam, Cambridge, UK) and peroxiredoxin 6 (ab16947, Abcam, Cambridge, UK), 1:3000 for anti-14-3-3 ϵ antibodies (ab43057 and ab40117, Abcam, Cambridge, UK), 1:5000 for anti-peroxiredoxin 3 and 4 (LF-PA0030 and LF-PA0009, Lab Frontier, Seoul, Korea). Membranes were washed with blocking buffer and incubated with secondary antibody (Dako, Glostrup, Denmark) at 1:2000 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.

To confirm the induction of AP, serum amylase and lipase activities were measured 5 hours after the second cerulein injection. Results showed that both enzymes were strongly increased in AP rats compared to healthy controls (Table 1).

3.2 Pancreatic tissue 2-DE maps

Whole pancreas protein extracts prepared from AP and control rats were analyzed on analytical 2-D gels. Computer analysis of gel images showed a good matching between analytical replicates and between samples from control or AP group (data not shown). Two-DE protein maps of control and AP samples are shown in Figures 1 and 2, respectively. Tandem-MS analysis allowed identifying a total of 125 proteins from AP and control samples (Supporting Information 1 and 2). Some of the spots labeled on figures 1 and 2 were not identified by MS from the corresponding sample but were named according to matching of AP and control 2-D maps. The list of spots actually identified by MS is given in Supporting Information 2 and their localization on AP and control maps is shown in Supporting Information 3. It is noteworthy that one of the proteins identified in AP extracts, carboxypeptidase B activation peptide, was not labeled on AP 2-DE map. Indeed, this protein was detected on preparative gels stained with Sypro[®] Ruby but was not visible on silver stained gels. The putative tissue and cellular localizations, molecular function and biological processes of the identified proteins are listed in Supporting Information 4. Most of them were ubiquitous or widely expressed intracellular proteins, including metabolic enzymes, structural proteins, proteins involved in redox homeostasis, signal transduction or protein synthesis. A number of pancreatic digestive enzymes were also identified: trypsin 1, 2, 4 and V-A, carboxypeptidase A1, A2 and B, chymotrypsin B, triglyceride lipase, pancreatic lipase-related protein 1, and cholesterol esterase. Pancreatic amylase was not detected on our gels due to the basic pH of the mature chain. However, the corresponding spot was observed on gels

prepared using pH 3-10 IPG strips (data not shown). In addition, a few plasmatic proteins, in particular serum albumin, were detected.

3.3 Comparative 2-DE analysis

Comparative 2-DE analysis allowed identifying a total of 42 differentially expressed proteins or protein fragments between AP and normal pancreatic tissue (Table 2 and Supporting Information 5). Of them, 14 were decreased in AP samples and 22 were increased. Three other changes were labeled as probable post-translational modifications. Finally, *pI* and *Mr* shifts observed for 14-3-3 proteins were further shown to be the consequence of a limited proteolysis. Enlargements of 2-DE maps showing examples of changes in protein expression between AP and control pancreatic tissue are presented in Figure 3. In addition, Supporting Informations 6 to 11 provide enlargements of the six AP and six control gels showing all the differentially expressed spots. One interesting finding was the identification in AP samples of carboxypeptidases A1, A2, and B spots that were absent in controls (Figure 3). In those spots, peptides located within the activation sequence of the proteases were lacking while there have been found in zymogens spots present on both AP and control gels (Supporting Information 12). These data strongly suggest that these additional spots correspond to the active forms of the digestive proteases. This assumption was further supported by the fact that *Mr* and *pI* of these spots matched to the theoretical molecular weight (MW) and *pI* values for active carboxypeptidases A1, A2, and B. A faint spot of active carboxypeptidase B was also observed in healthy controls, which could be explained by a low rate of activation of this enzyme during tissue sampling. Another digestive protease, trypsin 4, was identified in a spot strongly increased in AP samples. However, in that case, it was not possible to determine whether this spot was corresponding to the active form of the enzyme. Firstly, differences of MW and *pI* between zymogen and active form of trypsin 4 are too weak. Secondly, proteasome subunit alpha type 6 was identified in the same spot than trypsin 4. Other proteins, such as C reactive protein, lithostathine or pancreatitis-associated protein 2, involved in inflammatory or cellular stress responses in AP were also identified. In addition,

we detected a number of changes in protein expression that, to our knowledge, have never been described in AP. For some changes, such as those observed for 14-3-3 proteins, leukocyte elastase inhibitor A, RSSA, or peroxiredoxins, a connection with a pathobiological process involved in the disease can be proposed. In other cases, such as Phosphatidylethanolamine-binding protein 1, Lymphocyte cytosolic protein 1, Protein disulfide-isomerase A6, or Heterogeneous nuclear ribonucleoproteins F and K, the potential link with AP could not be established.

3.4 Immunoblots

Gel analysis allowed detecting additional spots for 14-3-3 proteins ϵ , θ and ζ in AP samples compare to healthy controls. In all cases, new spots found in AP samples had a higher pI and a slightly lower Mr (Figure 3 and Supporting Information 9). Interestingly, the observed shift was compatible with the cleavage of a short carboxyterminal peptide by caspases [9,10]. In order to confirm this hypothesis, immunoblots were performed using two anti-14-3-3 ϵ antibodies raised against aminoterminal and carboxyterminal ends, respectively (Figure 4). In controls, both antibodies gave a similar signal corresponding to the native protein. In AP samples, a protein of reduced Mr , corresponding to a truncated form, was detected using the anti-aminoterminal antibody. A band corresponding to the native protein was also present in AP samples 2 and 3. In contrast, the anti-carboxyterminus antibody showed only the band corresponding to the native protein in AP samples 2 and 3. The truncated form was not detected using this antibody. Similar results were obtained with an antibody recognizing 14-3-3 θ carboxyterminus (data not shown). These data strongly support the hypothesis of a cleavage of 14-3-3 proteins at the C-terminus end. Western-blots were also performed for peroxiredoxins. Peroxiredoxin-3 and 6 were identified on 2-D gels as a single spot, which expression was decreased in AP samples (Figure 3 and Supporting Information 10). For peroxiredoxin-4, several spots were identified and total spot intensities suggested a reduced expression in AP samples. Immunoblots confirmed decreased expression of peroxiredoxin-3

and 4 in AP samples (Figure 5). However, for peroxiredoxin-6, only 2 controls out of 3 showed a higher level compare to AP samples. Another interesting finding on 2-D gels was the detection in AP samples, at two different locations, of strongly increased spots identified as alpha 1 inhibitor 3 (A1I3), a rat homolog of human alpha-2-macroglobulin (Supporting Information 6). Peptides identified in those spots suggest that they correspond to amino- and carboxyterminal fragments of A1I3, respectively (Supporting Information 12). Immunoblot validation was carried out using a polyclonal antibody raised against full-length A1I3 confirmed its cleavage in AP samples. Indeed, a strong signal around 75 kDa was detected, which corresponds to the expected size for amino- and carboxyterminal fragments of A1I3 (Figure 6). Similar bands were also detected in controls but at a very low level compare to AP samples. In addition, immunoblot analysis showed a very strong increase of full-length A1I3 in AP samples compared to healthy controls. This phenomenon was not visible on 2-D gels but this discrepancy could be explained by the poor recovery of high molecular weight proteins in 2-DE analysis.

4 Discussion:

Most proteomic studies published on the pancreas have been exploring pancreatic cancer or diabetes. To our knowledge, only three proteomic studies have been published that investigated AP pathobiology [11-13]. In these experiments, the authors used a pancreatic acinar cell line stimulated with cerulein. However, due to the complexity of the pathways involved in AP, we believe that much more relevant information could be obtained from proteomic analysis of tissue extracts from animal models. In the current study, we performed a 2-DE analysis of whole pancreatic tissue extracts from a rat model of AP characterized by pancreatic and systemic inflammation, apoptosis and mild necrosis [4]. The cerulein injection protocol we used was shown to induce a significant increase of different severity parameters, such as serum amylase, pancreatic water content, pancreatic myeloperoxidase and pancreatic necrosis, as early as 2 hours after the last injection [14]. Comparative 2-DE analysis of pancreatic tissue samples obtained from this model and healthy controls led to the identification of numerous proteins involved in key pathobiological pathways of AP.

4.1 Activation of digestive proteases

We identified in AP samples, but not in controls, strong spots corresponding to the active forms of carboxypeptidases A1, A2, and B. Activation of digestive proteases was shown to be a key and early event in the pathogenesis of AP [15, 16]. The ability to visualize such an important step of AP pathogenesis is therefore a good quality control for the validation of our approach. It also demonstrates the interest of a tissue-based workflow compared to previously published cell-based studies where such changes have not been detected [11-13]. Interestingly, we also identified in AP samples the carboxypeptidase B activation peptide, which was described as a reliable diagnosis and prognosis marker of AP [17, 18]. In addition to evidence of digestive proteases activation, we found in AP samples fragments of albumin and of several intracellular proteins, mainly cytoskeletal proteins and proteins involved in translation processes. We assumed that such fragments resulted from uncontrolled

intracellular proteases activation. Protective systems exist within the acinar cell to counteract the effects of digestive proteases, such as the pancreatic secretory trypsin inhibitor-I (SPINK-1) [19]. In this study, we detected in AP samples reduced levels of a new protease inhibitor, the leukocyte elastase inhibitor A. This protein, expressed in pancreas, belongs to the serpins family, whose members inhibit proteases by a suicide substrate inhibition mechanism [20]. Therefore, decreased levels observed in AP could be indicative a protective role by inactivation of digestive proteases, the resulting protease-inhibitor complexes being thereafter degraded.

4.2. Inflammation

Comparative 2-DE analysis highlighted several proteins involved in inflammatory response, whose concentrations were elevated in rat pancreatic tissue following induction of AP: CRP, alpha-1-macroglobulin, and complement C3. A strong increase of A1I3, a rat acute phase protein highly homologous to human alpha-2-macroglobulin, was also detected by immunoblot analysis. In addition, we identified in AP samples increased levels of A1I3 amino- and carboxyterminal fragments. A1I3 inhibits endopeptidases through a trapping mechanism common to the members of the alpha-macroglobulin family [21]. This mechanism involves the reaction of the target protease with a single peptide bond within a region near the middle of the alpha-macroglobulin called the "bait region". This cleavage induces a conformational change of the alpha-macroglobulin that can, due to its large size, entrap the endopeptidase. Therefore, the observed fragments probably resulted from the interaction of A1I3 with activated digestive proteases. Similar fragments of other alpha-macroglobulins, murinoglobulins 1 and 2, were also identified. Taken together, these data suggest that alpha-macroglobulins play an important protective role in AP pathobiology. This assumption is supported by the fact that mice deficient in alpha-2-macroglobulin and murinoglobulin-1 develop more severe forms of AP than wild-type mice [22]. In addition, we observed elevated concentrations of several abundant plasma proteins - albumin, serotransferrin, apolipoprotein A1 - in tissue extracts from rats with experimental AP. This phenomenon can be explained by

the increased vascular permeability associated to inflammatory response.

4.3. Oxidative stress

Comparative 2-DE analysis suggested a decreased expression in AP samples of several peroxiredoxins, which are proteins playing an important role in the response to oxidative stress [23]. For peroxiredoxin-3 and 6, we observed a single spot whose intensity was reduced in AP. However, immunoblot validation clearly confirmed decreased expression only for peroxiredoxin-3. For peroxiredoxin-4, we identified three spots that probably represent the three possible oxidation states of the reactive cysteine [24]. In AP samples, the two most basic spots corresponding to the reduced cysteine and the lower oxidation state (e.g. sulfenic acid) were strongly decreased. In contrast, the intensity of the most acidic spot corresponding to overoxidation of the cysteine into cysteic acid was similar in both samples. Total peroxiredoxin-4 amount was reduced in AP samples. A possible explanation is that, under the strong oxidative stress associated with AP, most of the peroxiredoxin-4 was converted into the overoxidized form, which was afterwards degraded. Indeed, the oxidation of the cysteine into cysteic acid is irreversible and this acidic form must be considered as an inactive form of the peroxiredoxin [24]. The possibility of visualizing the consequences of oxidative stress using our 2-DE approach is very interesting since oxidative stress was shown to be involved in the development of inflammatory response in AP. Indeed, oxidative stress and pro-inflammatory cytokines trigger common signal transduction pathways that lead to amplification of the inflammatory cascade [25].

4.4. Stress response

We identified two stress proteins, pancreatitis-associated protein 2 and Lithostathine, which are known to be overexpressed in AP [26]. The detection of these proteins on 2D gels is of interest since they might play an important role in the course of AP by modulating the inflammatory response, as it was shown for pancreatitis-associated protein 1 [27]. It is noteworthy that overexpression of pancreatitis-associated protein 1 in AP tissue extracts was

detected using pH 3-10 IPG strips (data not shown). We also observed in AP samples a decreased expression of a heat shock protein (HSP) called endoplasmic reticulum chaperone protein or GRP94. In addition, a fragment of another HSP, Heat shock cognate 71 kDa protein, was detected in AP extracts but not in controls. The significance of these two last changes is unknown. Some HSPs, such as HSP70 and HSP 27, were shown to exert a protective role but they were increased in the pancreas after induction of AP [28, 29]. Finally, we did not observe the increase in HSP90 expression described by Yu and colleagues in cerulein-treated acinar cells [12]. However, this elevated HSP90 expression was not confirmed in a mice model of cerulein-induced AP [28].

4.5. Apoptosis

We identified on 2-D gels three 14-3-3 proteins, which are known negative regulators of apoptosis [30]. Interestingly, we observed in AP samples the apparition of new 14-3-3 spots having a more acidic *pI* and a slightly reduced *Mr*. This shift could be related with a pro-apoptotic process. Indeed, 14-3-3 proteins bind to pro-apoptotic factors, including BAD and BAX, thereby preventing them to exert their pro-apoptotic functions. Following stimulation of the cell by an apoptotic signal, 14-3-3 proteins were shown to be the target of a limited proteolysis by caspases resulting into the release of the pro-apoptotic factors and the induction of apoptosis [9, 10]. Interestingly, the cleavage of 14-3-3 proteins by caspases removes a small carboxyterminal fragment matching the modification of *Mr* and *pI* observed on 2-DE gels. This carboxyterminal cleavage was confirmed by immunoblot analysis for 14-3-3 ϵ . To our knowledge, this is the first description of a possible involvement of 14-3-3 proteins in AP. Moreover, the hypothesis of a role in the regulation of apoptosis is interesting since the balance between apoptosis and necrosis is a key element in the course of AP [31]. Indeed, the extent of pancreatic necrosis was identified as a crucial factor determining the severity of the disease [32]. However, further investigations will obviously be necessary to better characterize the possible role of 14-3-3 proteins in AP.

4.6 Other changes

We detected in AP samples a reduced expression of several enzymes that could be explained by an alteration of cellular metabolism following acinar cell injury. In particular, we found a decreased expression of ATP synthase beta subunit, which was in contrast with the results published by Yu and colleagues [11, 12]. This discrepancy could result from the fact that they worked with cells in culture instead of an animal model. The significance of the other changes observed, of which several were described for the first time in AP, remains unclear and will need further investigation.

Taken together, our results showed that using a proteomic approach we were able to detect a significant number of changes in protein expression that were related to the main pathobiological pathways of AP. Furthermore, several of these changes have, to our knowledge, never been described in the context of AP. Therefore, this study demonstrated the potential interest of applying proteomic techniques to the investigation of experimental models of AP.

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Legends

Figure 1. 2-DE map of pancreatic tissue extract from a control rat. One hundred fifty μg of proteins was loaded on an IPG strip (pH 4-7, 18 cm). The second dimension was performed on a 12.5% T SDS-PAGE gel. Gel was stained with ammoniacal silver. Complete names and UniPROTKB accession numbers are given in Supplemental Information 1. CBPB1a: activated carboxypeptidase B; (fgt): fragment; //: 2 proteins identified in the same spot; /: identified peptides matched several homologous proteins.

Figure 2. 2-DE map of pancreatic tissue extract from a rat with experimental acute pancreatitis. One hundred fifty μg of proteins was loaded on an IPG strip (pH 4-7, 18 cm). The second dimension was performed on a 12.5% T SDS-PAGE gel. Gel was stained with ammoniacal silver. Complete names and UniPROTKB accession numbers are given in Supplemental Information 1. CBPA1a, CBPA2a, CBPB1a: activated carboxypeptidase A1, A2, and B; (fgt): fragment; //: 2 proteins identified in the same spot; /: identified peptides matched several homologous proteins.

Figure 3. Gel enlargements showing differentially expressed proteins between pancreatic tissue extracts from control and acute pancreatitis rats. One hundred fifty μg of proteins was loaded on IPG strips (pH 4-7, 18 cm). The second dimension was performed on 12.5% T SDS-PAGE gels. Gels were stained with ammoniacal silver. Complete names and UniPROTKB accession numbers are given in Table 2. Color indicates change in protein expression in acute pancreatitis relative to control: black, decrease; red, increase; green, probable post-translational modification. CBPA1a, CBPA2a, CBPB1a: activated carboxypeptidase A1, A2, and B; (fgt): fragment; //: 2 proteins identified in the same spot.

Figure 4. Immunoblot of 14-3-3 protein ϵ . Ten μg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane essentially as described by Towbin *et al.* [8]. Immunodetection was performed as described in Methods section using polyclonal antibodies against 14-3-3 ϵ amino- and carboxyterminus and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland). Ctl: control, AP: acute pancreatitis.

Figure 5. Immunoblot of peroxiredoxins. Ten μg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane essentially as described by Towbin *et al.* [8]. Immunodetection was performed as described in Methods section using antibodies against peroxiredoxin-3, 4 or 6 and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland) was used for detection. Ctl: control, AP: acute pancreatitis.

Figure 6. Immunoblot of alpha 1 inhibitor 3. Ten μg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane essentially as described by Towbin *et al.* [8]. Immunodetection was performed as described in Methods section using a polyclonal antibody against full-length A1I3 and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland). Ctl: control, AP: acute pancreatitis.

Table 1. Serum amylase and lipase activities in control and AP rats. AP was induced in rats by two intraperitoneal injections of cerulein, 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). AP: acute pancreatitis.

Table 2. List of the differentially expressed proteins between control and acute pancreatitis samples. Differences in spot intensities between control (n=6) and AP (n=6) 2-DE gels were detected using ImageMaster 2D Platinum software (GE healthcare). Only changes with a minimum %volume ratio of 1.8 and consistently detected between the six AP and the six control gels were considered. Putative links with pathological processes are indicated. Increase: increased expression in AP samples, Decrease: decreased expression in AP samples, PTM: post-translational modification.

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Table 1

Rats	Amylase (U/L)	Lipase (IU/L)
Control 1	1219	7
Control 2	1506	10
Control 3	1791	8
AP 1	5885	902
AP 2	9680	3738
AP 3	7293	1650

Table 2

Accession	UniProtKB	Name	AP/CTL	Pathobiology
P10719	ATPB_RAT	ATP synthase subunit beta	Decrease	Alteration of cellular metabolism
P35738	ODBB_RAT	Branched-chain alpha-keto acid dehydrogenase E1 component beta chain	Decrease	Alteration of cellular metabolism
Q4QQV4	Q4QQV4_RAT	Dead end homolog 1 (Homolog to SYHC_MOUSE)	Decrease	Alteration of cellular metabolism
Q99PU6	Q99PU6_RAT	Branched-chain alpha-keto acid dihydrolipoyl acyltransferase (homolog to ODB2_MOUSE)	Decrease	Alteration of cellular metabolism
Q6P799	SYSC_RAT	Seryl-tRNA synthetase	Decrease	Alteration of cellular metabolism
P02770	ALBU_RAT	Serum albumin	Increase	Inflammatory response
P04639	APOA1_RAT	Apolipoprotein A-I	Increase	Inflammatory response
P12346	TRFE_RAT	Serotransferrin [fragment]	Increase	Inflammatory response
P01026	CO3_RAT	Complement C3c alpha' chain fragment 2	Increase	Inflammatory response
P48199	CRP_RAT	C-reactive protein	Increase	Inflammatory response
P14046	A1I3_RAT	Alpha-1-inhibitor 3 (C-terminus)	Increase	Inflammatory response
P14046	A1I3_RAT	Alpha-1-inhibitor 3 (N-terminus)	Increase	Inflammatory response
Q63041	A1M_RAT	Alpha-1-macroglobulin (45 kDa subunit)	Increase	Inflammatory response
P00732	CBPA1_RAT	Carboxypeptidase A1 (activated form)	Increase	Protease activation
P19222	CBPA2_RATa	Carboxypeptidase A2 (activated form)	Increase	Protease activation
P19223	CBPB1_RATa	Carboxypeptidase B (activated form)	Increase	Protease activation
Q4G075	ILEUA_RAT	Leukocyte elastase inhibitor A	Decrease	Proteases inhibition
Q68FR6	EF1G_RAT	Elongation factor 1-gamma	Decrease	Proteolysis
P60711	ACTB_RAT	Beta-actin [fragment]	Increase	Proteolysis
P63259	ACTG_RAT	Gamma-actin [fragment]	Increase	Proteolysis
P02770	ALBU_RAT	Serum albumin [Fragments]	Increase	Proteolysis

Q68FR6	EF1G_RAT	Elongation factor 1-gamma [fragment]	Increase	Proteolysis
Q10758	K2C8_RAT	Cytokeratin-8 [fragment]	Increase	Proteolysis
P38983	RSSA_RAT	40S ribosomal protein SA	Decrease	Proteolysis
P62260	1433E_RAT	14-3-3 protein ϵ	Limited proteolysis	Regulation of apoptosis?
P68255	1433T_RAT	14-3-3 protein θ	Limited proteolysis	Regulation of apoptosis?
P63102	1433Z_RAT	14-3-3 protein ζ	Limited proteolysis	Regulation of apoptosis?
Q66HD0	Q66HD0_RAT	Tumor rejection antigen gp96 (Homolog to ENPL_MOUSE)	Decrease	Response to cellular stress
P10758	LITH_RAT	Lithostathine	Increase	Response to cellular stress
P35231	REG3A_RAT	Pancreatitis-associated protein 2	Increase	Response to cellular stress
Q9Z0V6	PRDX3_RAT	Peroxiredoxin-3	Decrease	Response to oxidative stress
O35244	PRDX6_RAT	Peroxiredoxin-6	Decrease	Response to oxidative stress
Q9Z0V5	Q9Z0V5_RAT	Peroxiredoxin 4	Probable oxidation	Response to oxidative stress
P61980	HNRPK_RAT	Heterogeneous nuclear ribonucleoprotein K [fragment]	Decrease	Unknown
Q5XI38	Q5XI38_RAT	Lymphocyte cytosolic protein 1 (homolog to PLSL_MOUSE)	Decrease	Unknown
P12788 / P60901	TRY4_RAT / PSA6_RAT	Trypsin-4 and/or Proteasome subunit alpha type 6	Increase	Unknown
P02767	TTHY_RAT	Transthyretin	Increase	Unknown
P10760	SAHH_RAT	Adenosylhomocysteinase	Probable PTM	Unknown
P31044	PEBP1_RAT	Phosphatidylethanolamine-binding protein 1	Decrease	Unknown
Q794E4	HNRPF_RAT	Heterogeneous nuclear ribonucleoprotein F [fragment]	Increase	Unknown
Q63081	PDIA6_RAT	Protein disulfide-isomerase A6	Probable PTM	Unknown

P63018	HSP7C_RAT	Heat shock cognate 71 kDa protein	Increase	Unknown
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For Peer Review

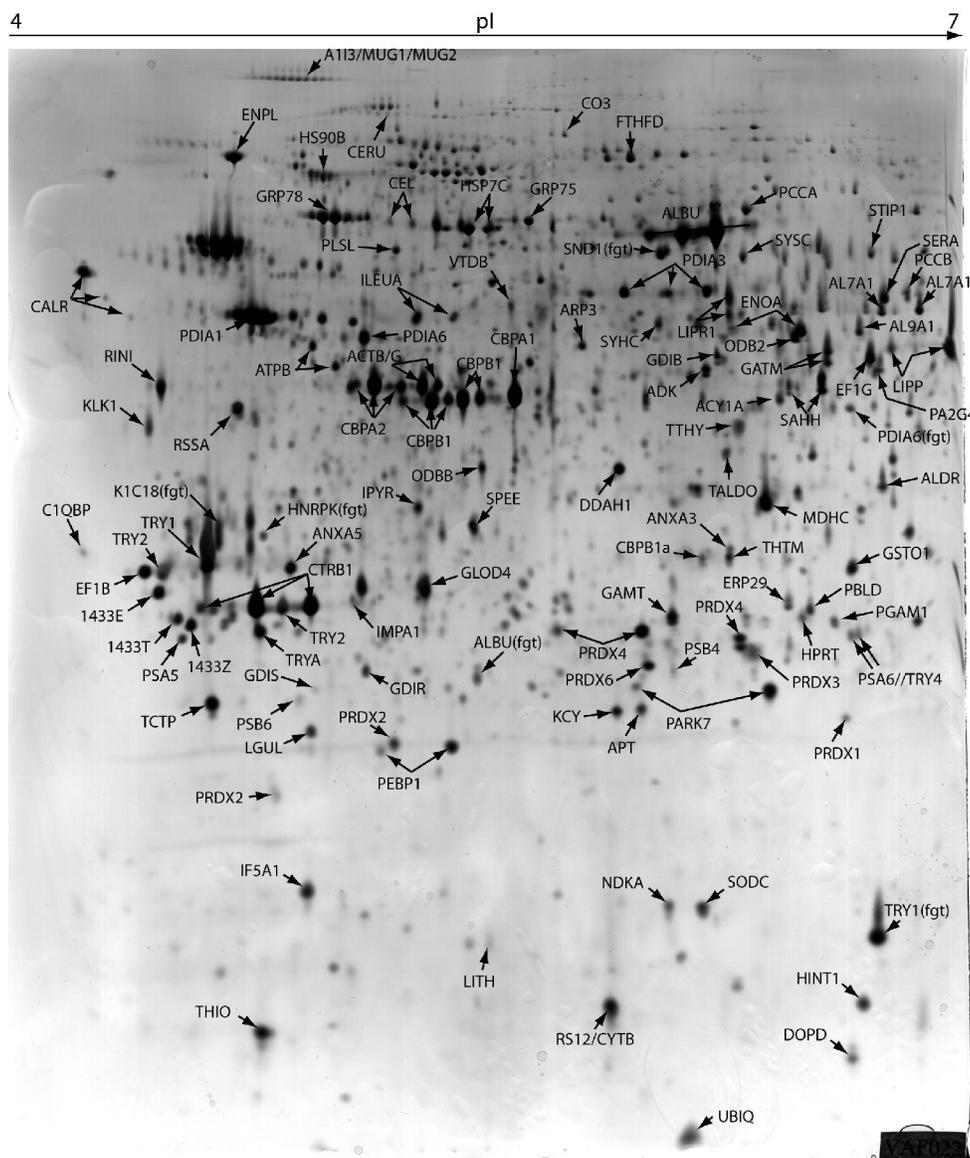


Figure 1. 2-DE map of pancreatic tissue extract from a control rat. One hundred fifty μ g of proteins was loaded on an IPG strip (pH 4-7, 18 cm). The second dimension was performed on a 12.5% T SDS-PAGE gel. Gel was stained with ammoniacal silver. Complete names and UniPROTKB accession numbers are given in Supplemental Information 1. CBPB1a: activated carboxypeptidase B; (fgt): fragment; //: 2 proteins identified in the same spot; /: identified peptides matched several homologous proteins. 179x221mm (300 x 300 DPI)

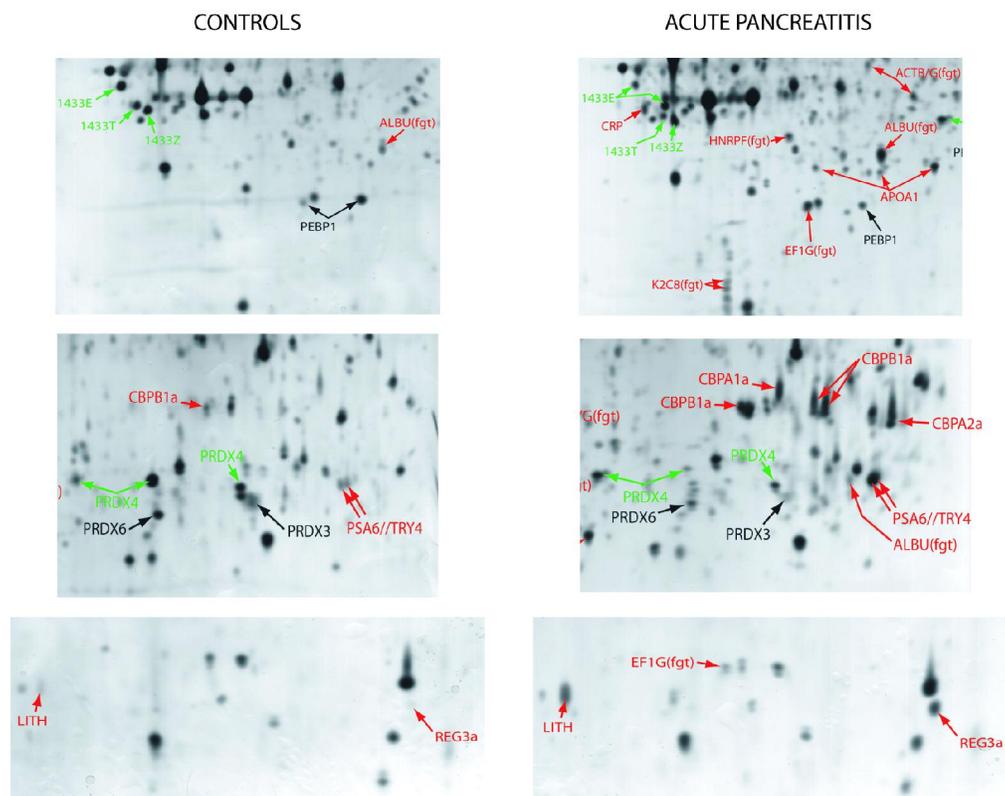


Figure 3. Gel enlargements showing differentially expressed proteins between pancreatic tissue extracts from control and acute pancreatitis rats. One hundred fifty μ g of proteins was loaded on IPG strips (pH 4-7, 18 cm). The second dimension was performed on 12.5% T SDS-PAGE gels. Gels were stained with ammoniacal silver. Complete names and UniPROTKB accession numbers are given in Table 2. Color indicates change in protein expression in acute pancreatitis relative to control: black, decrease; red, increase; green, probable post-translational modification. CBPA1a, CBPA2a, CBPB1a: activated carboxypeptidase A1, A2, and B; (fgt): fragment; //: 2 proteins identified in the same spot.

149x119mm (300 x 300 DPI)

Figure 4

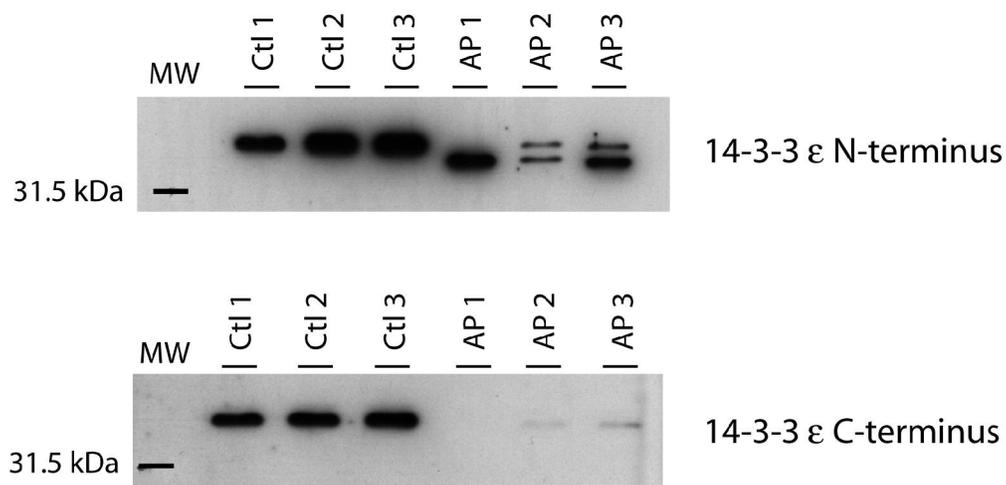


Figure 4. Immunoblot of 14-3-3 protein ϵ . Ten μg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane essentially as described by Towbin et al. [8]. Immunodetection was performed as described in Methods section using polyclonal antibodies against 14-3-3 ϵ amino- and carboxyterminus and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland). Ctl: control, AP: acute pancreatitis.

135x86mm (300 x 300 DPI)

Figure 5

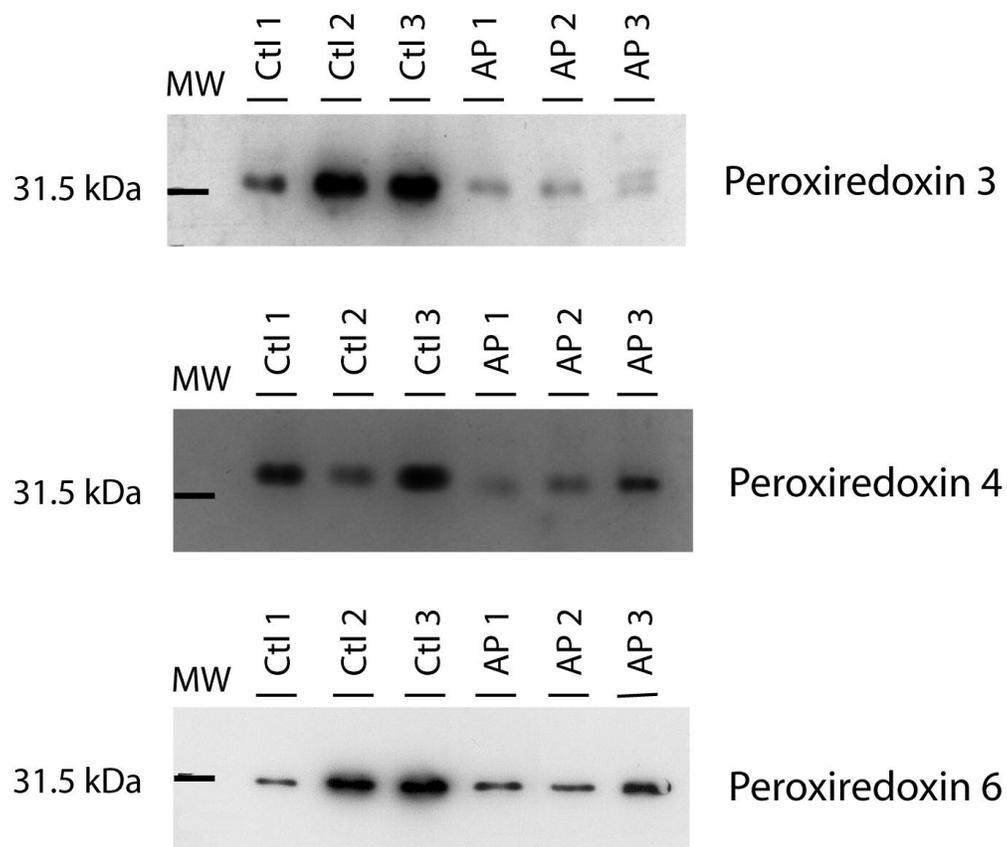


Figure 5. Immunoblot of peroxiredoxins. Ten μ g of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane essentially as described by Towbin et al. [8]. Immunodetection was performed as described in Methods section using antibodies against peroxiredoxin-3, 4 or 6 and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland) was used for detection. Ctl: control, AP: acute pancreatitis.

115x120mm (300 x 300 DPI)

Figure 6

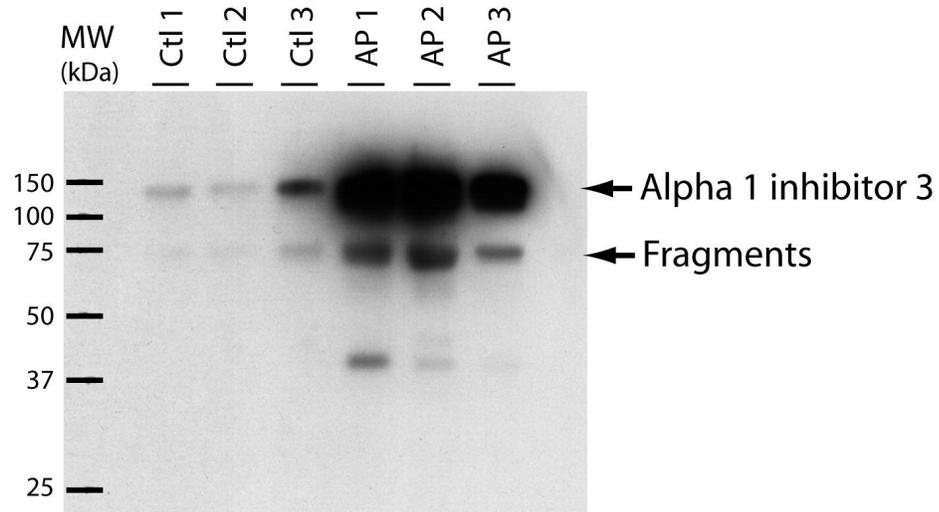


Figure 6. Immunoblot of alpha 1 inhibitor 3. Ten μg of pancreatic protein extracts were separated by SDS-PAGE on homemade Tris-Glycine gels (12.5% T). Proteins separated by 1-DE were electroblotted onto a nitrocellulose membrane essentially as described by Towbin et al. [8]. Immunodetection was performed as described in Methods section using a polyclonal antibody against full-length A1I3 and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland). Ctl: control, AP: acute pancreatitis.

127x88mm (300 x 300 DPI)