Impact of Temperature Dependent Sampling Procedures in Proteomics and Peptidomics – A Characterization of the Liver and Pancreas Post Mortem Degradome

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Little is known about the nature of post mortem degradation of proteins and peptides on a global level, the so-called degradome. This is especially true for nonneural tissues. Degradome properties in relation to sampling procedures on different tissues are of great importance for the studies of, for instance, post translational modifications and/or the establishment of clinical biobanks. Here, snap freezing of fresh (<2 min post mortem time) mouse liver and pancreas tissue is compared with rapid heat stabilization with regard to effects on the proteome (using two-dimensional differential in-gel electrophoresis) and peptidome (using label free liquid chromatography). We report several proteins and peptides that exhibit heightened degradation sensitivity, for instance superoxide dismutase in liver, and peptidyl-prolyl cis-trans isomerase and insulin C-peptides in pancreas. Tissue sampling based on snap freezing produces a greater amount of degradation products and lower levels of endogenous peptides than rapid heat stabilization. We also demonstrate that solely snap freezing related degradation can be attenuated by subsequent heat stabilization. We conclude that tissue sampling involving a rapid heat stabilization step is preferable to freezing with regard to proteomic and peptidomic sample quality. Molecular & Cellular Proteomics 10: 10.1074/mcp.M900229-MCP200, 1–15, 2011.

Published, MCP Papers in Press, January 28, 2010, DOI 10.1074/mcp.M900229-MCP200

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Received May 11, 2009, and in revised form, November 4, 2009

The evolving maturation of the field of proteomics has, in the same way as in genomics, highlighted the need of better sampling procedures and sample preparation methodologies to minimize the effect of post mortem alterations. The aspect of sample quality is not new in any way and is relevant in most biomedical fields but has only lately started to receive adequate attention. The main factors influencing sample quality is storage temperature of the body until tissue removal (foremost a problem in clinical settings and extraction of less accessible tissue samples from model organisms) and post mortem interval (PMI)1 (1–3). Post mortem degradation in during PMI is a well known compromising problem when studying endogenous peptides (2, 3) and has also been proven to affect the results of polypeptide (here defined as proteins larger than 10 kDa) studies (3–8). PMI degradation has mainly been studied on human or mouse brain tissue, using two-dimensional electrophoresis (2-DE), SDS-PAGE, and immunoblotting (1, 3–12). There are also a few proteomic studies on muscle tissue degradation in livestock (13–16).

We and others have previously explored the effect of focused microwave irradiation with regard to sample quality, demonstrating that this method is more reliable than snap freezing in liquid nitrogen, especially with regard to post-translational modification (PTM) stability (2, 3, 17–20). An alternative method based on cryostat dissection with subsequent heat treatment through boiling has also been reported to improve endogenous peptide sample quality (21). Besides focused microwave irradiation, which is specifically used for rodent brain tissue sampling, we have also demonstrated the efficiency of rapid heat stabilization through conductivity with

1 The abbreviations used are: PMI, post mortem interval; 2D-DIGE, two-dimensional differential in gel electrophoresis; 2-DE, two-dimensional electrophoresis; D, rapidly heat-stabilized samples; FD, rapidly frozen and subsequently heat stabilized samples; F, rapidly frozen samples; FvsD, pair-wise comparison between F and D; FvsFD, pair-wise comparison between F and FD; LC-MS, liquid chromatography mass spectrometry; PCA, principal component analysis; PTM, post-translational modification.

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regard to sample degradation (3, 22). Although somewhat constrained by its dependence on how quickly the tissue is harvested from the body, the latter procedure has the added advantage that it can be used on any type of tissue and species, fresh as well as frozen. This study will compare effects of sampling procedures on the liver and pancreas degradome following rapid heat stabilization, the more traditional snap freezing, or the combination of snap freezing with subsequent heat stabilization.

To summarize, this study investigated the effects of post mortem degradation in pancreas and liver. Both tissues are well studied because of their multiple functions in the body and their involvement in different diseases such as diabetes or hepatocarcinoma. Pancreas is especially interesting in this context as it displays endocrine secretion of peptides, and exocrine secretion of digestive enzymes, the later making it a protease rich tissue. We used both two-dimensional difference in gel electrophoresis (2D-DIGE) and label free liquid chromatography mass spectrometry (LC-MS) based differential peptide display (2, 18), the later to better investigate changes in small molecular fragment that are not easily detectable by gel-based methods. 2D-DIGE is an unrivaled methodology to characterize alterations in isoform patterns, which is an important aspect considering that post-translational modifications (PTMs) such as phosphorylations are especially sensitive to post mortem influence within a few min-

FIG. 1. Overview of general differences between sampling groups based on the proportion of proteins and peptides that is detected across all groups. A, Hierarchical clustering dendrograms based on significantly (F-test $p < 0.05$) differentially expressed spots with maximum number of observations for liver (54 spots), pancreas (191 spots), and peptide expression for pancreas (26 peaks). B, Two-dimensional principal component analysis (PCA) based on the same premises. C, Proportion of unaffected and affected (independent of level of fold change, minimum one missing observation per group, moderated t test $p < 0.05$ orange; $p < 0.01$ dark red) proteome and peptidome in different pairwise comparisons between sampling procedures for liver and pancreas.

Characterization of the Liver and Pancreas Post Mortem Degradome
utes PMI (3). The peptidomics approach has been used in several studies to point out early post mortem changes and protein degradation that tissue undergo following sampling and is therefore a well-suited method (3, 18, 22).

**EXPERIMENTAL PROCEDURES**

**Animals and Tissue Sampling**—A total of eight C57BL/6J mice were sacrificed by cervical dislocation. The animals were used to study protein degradation by following protein changes using 2D-DIGE (four animals) experiments, and peptide changes using LC-MS (four animals). The pancreas and liver were dissected from all animals (<2 min post mortem) and divided in three parts. Two pieces from each organ were snap frozen in liquid nitrogen. The third part was rapidly stabilized using the Stabilizor T1 system (Denator, Göteborg, Sweden) (22) and thereafter frozen. Before sample preparation one of the frozen pieces was rapidly stabilized directly from the frozen state. Samples snap frozen in liquid nitrogen were named “F,” rapidly heat inactivated “D,” and rapidly frozen and subsequently heat stabilized “FD.”

**2D-DIGE**—Frozen pancreas or liver were crushed in a liquid nitrogen-cooled mortar and then homogenized with a glass/Teflon homogenizer in lysis buffer (8 M urea, 30 mM Tris, 5 mM magnesium acetate, and 4% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS)). The homogenates were shaken at room temperature for 30 min and then centrifuged at 40,000 × g for 30 min. The protein concentration in each supernatant was determined using the 2D-Quant kit (GE Healthcare). Protein supernatants were labeled by DIGE minimal labeling with Cy3 or Cy5 according to manufactures instruction (GE Healthcare). A total of 400 pmol of dye per 50 μg proteome was used. Equal amounts of protein from each sample were mixed to create a pool labeled with Cy2 as above. Thirty micrograms of protein from each dye were combined with rehydration buffer (8 M urea, 2% (w/v) CHAPS, 0.002% bromphenol blue, 18.2 mM dithiotreitol, 0.5% Phamalyte (pH 4–7)), left at room temperature for 30 min and centrifuged for 10 min before being applied to a 24-cm IPG 4–7 strip for overnight rehydration. Isoelectric focusing, strip equilibration, 12.5% SDS-PAGE, and gels fixation were performed as described in (23). The gels were scanned with Amersham Biosciences Typhoon 9400 variable imager as previously described (24).
The frozen pieces of pancreas (n = 4 for F and D, n = 3 for FD) were homogenized in sample buffer (0.25% acetic acid) using microtip sonication (Vibra cell 750, Sonics & materials, Inc., Newtown, CT). The homogenates were centrifuged (20,000 g for 40 min at 4 °C) and the supernatant were transferred to a centrifugal filter device with a molecular mass limit of 10,000 Da (Microcon YM-10, Millipore) and filtered through centrifugation (14,000 g for 45 min at 4 °C) (2). The filtrate was separated on a nano-LC system (Ettan MDLC, GE Healthcare) coupled to a mass spectrometer Q-Tof II (Waters, Denver, CO) for profiling or LTQ-FT (Thermo Electron) for identification. Five microliters filtrate was injected and desalted on a precolumn (PepMapT, LC Packings, Amsterdam, The Netherlands) at a flow rate of about 180 nanoL/min. The pancreatic peptide samples were separated during a 40-min gradient from 3–50% acetonitrile for the comparative studies. A 2-h gradient was used for peptide separation in the identification study. Profiling MS data was acquired on the Q-Tof MS instrument. The mass spectrometer was calibrated according to the manufacturer’s recommendations using a PEG solution (Fluka, Switzerland). MS data was collected in a continuous mode in the m/z range 300–1000 for 40 min. The tandem MS data analysis was performed on the LTQ-FT instrument in a data-dependent manner and set up to continuously switch between full MS scan (m/z 300–2000), zoom scan (most intense peak in full scan) and MS/MS scan in which the most intense peak can be picked twice in a time window of 40 s before placing on an exclusion list during a 150 s period.

2D-DIGE Data Normalization and Analysis—The 2D-DIGE images were analyzed using the DeCyder software suite (version 5.02; GE Healthcare). Following automatic matching, the majority of spots were also manually compared between all gels to minimize false spot matching. The gel with the largest number of spots was designated as

| Mspt | Protein                              | SwissProt | p-val (F-test) | FvD Log2 fc | FvD p-val | FvD Log2 fc | FvD p-val | FDvD Log2 fc | FDvD p-val |
|------|--------------------------------------|-----------|---------------|-------------|-----------|-------------|-----------|-------------|-----------|
| 1045 | Superoxide dismutase                 | P08228    | p<0.001       | -2.142      | p<0.001   | -2.131      | p<0.001   | -0.01       | 0.931     |
| 1057 | Superoxide dismutase                 | P08228    | p<0.001       | -1.798      | p<0.001   | -1.792      | p<0.001   | -0.006      | 0.958     |
| 1053 | Superoxide dismutase                 | P08228    | p<0.001       | -2.139      | p<0.001   | -2.222      | p<0.001   | 0.083       | 0.553     |
| 1051 | Superoxide dismutase                 | P08228    | p<0.001       | 3.693       | p<0.001   | 4.075       | p<0.001   | -0.382      | 0.109     |
| 616  | Sorbitol dehydrogenase               | Q64442    | 0.001         | -0.693      | 0.001     | -0.808      | p<0.001   | 0.115       | 0.335     |
| 614  | Sorbitol dehydrogenase               | Q64442    | 0.373         | -0.146      | 0.330     | -0.176      | 0.185     | 0.03        | 0.818     |
| 683  | Regucalcin                           | Q64374    | 0.002         | -0.663      | 0.002     | -0.833      | 0.001     | 0.17        | 0.195     |
| 784  | Regucalcin                           | Q64374    | 0.002         | -2.469      | 0.001     | -2.416      | 0.001     | -0.053      | 0.813     |
| 715  | Regucalcin                           | Q64374    | 0.064         | -0.399      | 0.057     | -0.478      | 0.032     | 0.079       | 0.65      |
| 922  | Regucalcin                           | Q64374    | 0.918         | 0.0189      | 0.914     | 0.067       | 0.704     | -0.048      | 0.782     |
| 609  | Acyl-CoA dehydrogenase               | Q70417    | 0.004         | -0.496      | 0.005     | -0.617      | 0.002     | 0.122       | 0.29      |
| 204  | Serum albumin precursor              | P07724    | 0.004         | -0.134      | 0.24      | -0.597      | 0.002     | 0.463       | 0.006     |
| 235  | Serum albumin precursor              | P38647    | 0.127         | -0.671      | 0.203     | -0.822      | 0.045     | 0.151       | 0.706     |
| 219  | 78 kDa glucose-regulated protein precursor | P20029    | 0.005         | -0.723      | 0.005     | -0.824      | 0.003     | 0.101       | 0.529     |
| 674  | 78 kDa glucose-regulated protein precursor | P20029    | 0.101         | -0.134      | 0.366     | -0.455      | 0.053     | 0.32        | 0.102     |
| 800  | GrpE protein homolog 1               | Q99LC5    | 0.007         | 0.585       | 0.008     | 0.617       | 0.003     | -0.032      | 0.787     |
| 1071 | 60S acidic ribosomal protein P2      | P99027    | 0.008         | -0.491      | 0.02      | -0.79       | 0.003     | 0.299       | 0.096     |
| 947  | Electron transfer flavoprotein alpha-subunit | Q99LP6    | 0.008         | -1.413      | 0.005     | -1.619      | 0.005     | 0.205       | 0.504     |
| 233  | Stress-70 protein                    | P38647    | 0.025         | -0.78       | 0.0182    | -0.829      | 0.015     | 0.049       | 0.639     |
| 685  | Stress-70 protein                    | P57776    | 0.253         | -0.184      | 0.143     | -0.166      | 0.178     | -0.018      | 0.872     |
| 625  | 60 kDa heat shock protein            | Q61176    | 0.027         | -0.361      | 0.048     | -0.493      | 0.01      | 0.132       | 0.331     |
| 193  | 60 kDa heat shock protein            | P07724    | 0.189         | 0.111       | 0.547     | 0.363       | 0.087     | -0.253      | 0.2      |
| 231  | Arginase-1                           | P63038    | 0.028         | -0.622      | 0.027     | -0.748      | 0.014     | 0.126       | 0.563     |
| 131  | Heat shock cognate 71 kDa protein    | P63017    | 0.041         | -0.496      | 0.028     | -0.528      | 0.024     | 0.033       | 0.852     |
| 128  | Heat shock cognate 71 kDa protein    | P63017    | 0.047         | -0.268      | 0.058     | -0.363      | 0.021     | 0.095       | 0.425     |
| 130  | Heat shock cognate 71 kDa protein    | P63017    | 0.051         | -0.794      | 0.035     | -0.822      | 0.032     | 0.029       | 0.904     |
| 134  | Heat shock cognate 71 kDa protein    | P63017    | 0.557         | 0.135       | 0.306     | 0.082       | 0.519     | 0.053       | 0.675     |
| 871  | Apolipoprotein A-I precursor         | Q00623    | 0.112         | -0.687      | 0.078     | 0.044       | 0.877     | -0.731      | 0.067     |
| Msot | Protein                                      | SwissProt | FvD  | FvFD | FvDvD |
|------|---------------------------------------------|-----------|------|------|-------|
|      |                                             | p-val (F-test) | Log2 fc | p-val | Log2 fc | p-val | Log2 fc | p-val |
| 1061 | Peptidyl-orotyl cis-trans isomerase B precursor | 0.001     | 0.001 | 0.001 | 0.001 |
| 680  | Alpha-enoilase                               | 0.001     | 0.001 | 0.001 | 0.001 |
| 664  | Alpha-enoilase                               | 0.001     | 0.001 | 0.001 | 0.001 |
| 552  | Alpha-enoilase                               | 0.001     | 0.001 | 0.001 | 0.001 |
| 444  | Alpha-enoilase                               | 0.001     | 0.001 | 0.001 | 0.001 |
| 499  | Keratin, type II cytoskeletal 8              | 0.001     | 0.001 | 0.001 | 0.001 |
| 482  | Keratin, type II cytoskeletal 8              | 0.001     | 0.001 | 0.001 | 0.001 |
| 498  | Keratin, type II cytoskeletal 8              | 0.001     | 0.001 | 0.001 | 0.001 |
| 411  | Keratin, type II cytoskeletal 8              | 0.001     | 0.001 | 0.001 | 0.001 |
| 903  | Electron transfer flavoprotein alpha-subunit | 0.001     | 0.001 | 0.001 | 0.001 |
| 661  | Apolipoprotein A-IV precursor                | 0.001     | 0.001 | 0.001 | 0.001 |
| 387  | Eukaryotic translation initiation factor 5A  | 0.001     | 0.001 | 0.001 | 0.001 |
| 623  | Lipomamide acyltransferase                   | 0.001     | 0.001 | 0.001 | 0.001 |
| 833  | Elongation factor 1-delta                   | 0.001     | 0.001 | 0.001 | 0.001 |
| 914  | Elongation factor 1-delta                   | 0.001     | 0.001 | 0.001 | 0.001 |
| 940  | Elongation factor 1-delta                   | 0.001     | 0.001 | 0.001 | 0.001 |
| 138  | Eukaryotic translation initiation factor 4B  | 0.001     | 0.001 | 0.001 | 0.001 |
| 893  | Annexin A4                                   | 0.001     | 0.001 | 0.001 | 0.001 |
| 546  | Dynamin subunit 2                           | 0.001     | 0.001 | 0.001 | 0.001 |
| 707  | Succinyl-CoA ligase                          | 0.001     | 0.001 | 0.001 | 0.001 |
| 619  | Keratin, type I cytoskeletal 18              | 0.001     | 0.001 | 0.001 | 0.001 |
| 618  | Keratin, type I cytoskeletal 18              | 0.001     | 0.001 | 0.001 | 0.001 |
| 929  | Keratin, type I cytoskeletal 18              | 0.001     | 0.001 | 0.001 | 0.001 |
| 617  | Dihydrolipoamide-residue succinyltransferase| 0.001     | 0.001 | 0.001 | 0.001 |
| 624  | Dihydrolipoamide-residue succinyltransferase| 0.001     | 0.001 | 0.001 | 0.001 |
| 705  | Keratin, type I cytoskeletal 19              | 0.001     | 0.001 | 0.001 | 0.001 |
| 617  | Dihydrolipoamide-residue succinyltransferase| 0.001     | 0.001 | 0.001 | 0.001 |
| 624  | Dihydrolipoamide-residue succinyltransferase| 0.001     | 0.001 | 0.001 | 0.001 |
| 705  | Keratin, type I cytoskeletal 19              | 0.001     | 0.001 | 0.001 | 0.001 |
| 440  | Protein disulfide-isomerase A3 precursor     | 0.001     | 0.001 | 0.001 | 0.001 |
| 459  | Protein disulfide-isomerase A3 precursor     | 0.001     | 0.001 | 0.001 | 0.001 |
| 444  | Protein disulfide-isomerase A3 precursor     | 0.001     | 0.001 | 0.001 | 0.001 |
| 428  | Protein disulfide-isomerase A3 precursor     | 0.001     | 0.001 | 0.001 | 0.001 |
| 16   | Protein disulfide-isomerase A3 precursor     | 0.001     | 0.001 | 0.001 | 0.001 |
| 15   | Protein disulfide-isomerase A3 precursor     | 0.001     | 0.001 | 0.001 | 0.001 |
| 924  | Elongation factor 1-beta                    | 0.001     | 0.001 | 0.001 | 0.001 |
| 927  | Prohibitin                                   | 0.001     | 0.001 | 0.001 | 0.001 |
| 749  | Protein disulfide-isomerase precursor        | 0.001     | 0.001 | 0.001 | 0.001 |
| 433  | Protein disulfide-isomerase precursor        | 0.001     | 0.001 | 0.001 | 0.001 |
| 443  | Protein disulfide-isomerase precursor        | 0.001     | 0.001 | 0.001 | 0.001 |
| 430  | Protein disulfide-isomerase precursor        | 0.001     | 0.001 | 0.001 | 0.001 |
| 757  | 26S protease regulatory subunit 6B           | 0.001     | 0.001 | 0.001 | 0.001 |
| 765  | Isovaleryl-CoA dehydrogenase                 | 0.001     | 0.001 | 0.001 | 0.001 |
| 825  | Transaldolase                                | 0.001     | 0.001 | 0.001 | 0.001 |
| 931  | Armadillo repeat protein deleted in velo-cardio-facial syndrome| 0.001 | 0.001 | 0.001 | 0.001 |
| 800  | Nucleophosmin                                | 0.001     | 0.001 | 0.001 | 0.001 |
| 791  | Nucleophosmin                                | 0.001     | 0.001 | 0.001 | 0.001 |
| 796  | Nucleophosmin                                | 0.001     | 0.001 | 0.001 | 0.001 |
| 1143 | Breast cancer metastasis-suppressor 1 homolog| 0.001     | 0.001 | 0.001 | 0.001 |
| 373  | Stress-70 protein, mitochondrial precursor   | 0.001     | 0.001 | 0.001 | 0.001 |
| 278  | 78 kDa glucose-regulated protein precursor   | 0.001     | 0.001 | 0.001 | 0.001 |
master gel. Unless otherwise stated, all proteomic and peptidomic data normalization and analysis were performed using the statistical software R (25). The 2D-DIGE data was normalized using the “2D loess” method which removes both intensity and spatial bias (26). To investigate general differences between experimental groups, moderated F-statistics using the eBayes function in Limma (27) was calculated for spots with no missing observations. F-statistics for spots with a significance $p < 0.05$ were considered statistically different.

Normalized volume intensity data for significant spots (log$_2$Cy5/Cy2 and log$_2$Cy3/Cy2 ratios) with a maximum number of samples/group was analyzed by principal component analysis (PCA), using the SIMCA-P software (version 12, Umetrics AB, Umeå, Sweden) and hierarchical clustering was performed on the median spot value in each group (F, FD, and D). The hierarchical clustering was done using the PermuteMatrix program (28) with complete linkage and Euclidian distance as the distance metric. To find spots differing between D, FD, and F, we used a linear model that takes into account dye-specific differences for the Cy3 and Cy5 channels and calculated moderated t and F-statistics using the eBayes function in Limma (27). For pair wise comparisons, (FvsD, FDvsD, and FvsFD) we made volcano plots displaying the statistical significance (lodsratios) versus the estimated log$_2$ fold change. Spots with $p < 0.05$ and log$_2$ fold change $> 1$ were considered statistically significant. Kernel density estimations were calculated to display the log$_2$ fold change distributions. The more general relationship between experimental groups was analyzed by hierarchical clustering using complete linkage and Euclidian distance as the distance metric and PCA. The hierarchical clustering was done on the proportion of the data sets that had a F-statistic significance of $p < 0.05$ and maximum samples/group (26 peaks) using the median value from the log$_2$ intensities for each peptide and group (F, FD, and D) subtracted with the total median intensity for each peptide. The PCA was based on the same data (F-statistics $p < 0.05$, maximum samples/group). To compare peak detection between the D, FD, and F treatments, all peaks were divided into three classes in each experimental group: (1) peptides detected in at least three LC-MS experiments, (2) 1–2 LC-MS experiments, and (3) not detected at all. Expression differences were then calculated irrespective of the number of available values. When possible, robust estimates of expression differences were calculated using Limma, otherwise arithmetic mean value differences were calculated.

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**TABLE II—continued**

| Msspot | Protein | SwissProt | FvD | FvFD | FDvD |
|--------|---------|-----------|-----|------|------|
| 301    | 78 kDa glucose-regulated protein precursor | P20029 | p<0.050 | -0.561 | p<0.010 | -0.171 | 0.277 | -0.380 | p<0.050 |
| 224    | 78 kDa glucose-regulated protein precursor | P20029 | p<0.050 | -0.324 | p<0.050 | -0.207 | -0.117 | 0.292 |
| 225    | 78 kDa glucose-regulated protein precursor | P20029 | 0.058 | 0.344 | p<0.050 | -0.311 | p<0.050 | -0.033 | 0.801 |
| 305    | 78 kDa glucose-regulated protein precursor | P20029 | 0.076 | -0.334 | p<0.050 | -0.044 | 0.740 | -0.290 | 0.084 |
| 7      | 78 kDa glucose-regulated protein precursor | P20029 | 0.151 | -0.370 | 0.066 | -0.298 | 0.151 | -0.072 | 0.720 |
| 220    | 78 kDa glucose-regulated protein precursor | P20029 | 0.282 | 0.082 | 0.688 | -0.250 | 0.247 | 0.332 | 0.139 |
| 200    | 78 kDa glucose-regulated protein precursor | P20029 | 0.302 | -0.222 | 0.128 | -0.133 | 0.374 | -0.089 | 0.569 |
| 253    | 78 kDa glucose-regulated protein precursor | P20029 | 0.311 | 0.270 | 0.151 | 0.167 | 0.337 | 0.103 | 0.538 |
| 199    | 78 kDa glucose-regulated protein precursor | P20029 | 0.361 | 0.083 | 0.505 | -0.099 | 0.430 | 0.183 | 0.170 |
| 122    | 78 kDa glucose-regulated protein precursor | P20029 | 0.469 | -0.189 | 0.221 | -0.162 | 0.439 | -0.027 | 0.908 |
| 223    | 78 kDa glucose-regulated protein precursor | P20029 | 0.646 | -0.066 | 0.654 | -0.137 | 0.388 | 0.070 | 0.635 |
| 230    | 78 kDa glucose-regulated protein precursor | P20029 | 0.707 | -0.059 | 0.781 | -0.170 | 0.430 | 0.112 | 0.599 |
| 378    | 60 kDa heat shock protein, mitochondrial | P63038 | p<0.050 | -0.634 | p<0.050 | -0.342 | 0.900 | -0.292 | 0.100 |
| 388    | Glutamate [NMDA] receptor subunit zeta 1 | P35438 | p<0.050 | 0.632 | p<0.050 | 0.311 | 0.454 | 0.843 | p<0.010 |
| 976    | Chymotrypsinogen | Q8CR35 | p<0.050 | 0.401 | p<0.050 | 0.501 | p<0.050 | -0.100 | 0.454 |
| 961    | Chymotrypsinogen | Q9CR35 | p<0.050 | 0.648 | p<0.010 | 0.487 | p<0.050 | 0.161 | 0.402 |
| 685    | Actin, cytoplasmic 1 | P60710 | p<0.050 | 0.627 | p<0.010 | 0.355 | 0.079 | 0.272 | 0.157 |
| 526    | Pancreatic lipase-related protein 1 precursor | Q8BK4 | p<0.050 | 0.269 | 0.088 | 0.493 | p<0.010 | -0.224 | 0.142 |
| 867    | Spermidine synthase | P97555 | p<0.050 | -0.145 | 0.311 | 0.324 | p<0.050 | -0.469 | p<0.050 |
| 852    | Inorganic pyrophosphatase | Q9D819 | p<0.050 | -0.306 | p<0.050 | -0.310 | p<0.050 | -0.057 | 0.640 |

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2D-DIGE Protein Identification

Preparative Gel—Seven hundred and fifty micrograms of protein combining all 16 samples were loaded onto a preparative gel to maximize the success rate of protein identification. Following the first and second dimension, the gel was stained in ruthenium bathophenanthroline disulfonate (RuBPS) with an improved staining and destaining method (30). The spot picking, destaining, protein digestion, extraction, sample preparation, and spotting on matrix-assisted laser desorption ionization (MALDI) target plates were carried out using a spot handling work station (Ettan Spot Handling Workstation, GE Healthcare) and a standard protocol provided by GE Healthcare. Briefly, protein spots were robotically excised using a Picker head with Ø 2.0 mm, and digested with modified, sequence-grade V5111 porcine trypsin, 20 μg/ml (Promega Biotech AB, Stockholm, Sweden). The extract was evaporated and dissolved in matrix (2.5 mg/ml of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile containing 0.05% trifluoroacetic acid) 2.5 μl and 2.0 μl is spotted on MALDI target plates. Preparative 2D-PAGE, spot excision, protein digestion, and MS were carried out at the SCIBLU proteomics resource center in Lund (http://www.lth.se/sciblu/).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS—The MS analysis were performed on a MALDI-time-of-flight MS (MALDI micro MX, Waters, Manchester, UK) instrument and each spectrum represents up to 400 laser shots. An external calibration was made with digested alcohol dehydrogenase from yeast. The raw data files were converted to dta files using Extract_msn through BioWorksBrowser (ThermoElectron) and further compiled into mgf files using an in-house developed script. MASCOT25 search was performed against NCBInr: 20070109 (4396331 sequences; 1512170332 residues) database with the following settings: species Mus musculus (108302 sequences); no enzyme specification; peptide mass tolerance of 1.5 Da; fragment mass tolerance of 0.7 Da; potential modifications N-terminal acetylation, N-terminal pyro-glutamic acid of glutamine and glutamic acid, C-terminal amidation, oxidation of methionine. Peptides with a MASCOT score (expectation value below 0.05) above the threshold were listed in supplementary file S3. Raw data (Pancreas and Liver PHF) associated with the manuscript may be downloaded from ProteomeCommons.org Tranche network (https://proteomecommons.org/index.jsp) using the following hash: https://proteomecommons.org/tranche/datadownloader.jsp?h=Va%2bVLAGXqvi4v3r9MLT1gGgCgK75TXdAki1mwb2rHlUJJd2ylH2sYOFuxFe8b1kB11KJyKbGw7S5YEIA6dE%2f%2bEAAAAAAA91Q%3d%3d.

RESULTS

The aim of this study was to evaluate how sampling procedures affect post mortem proteome degradation in mouse liver and pancreatic tissue. We used three different sampling
Characterization of the Liver and Pancreas Post Mortem Degradome

Table III

“2-DEgradome” proteins also found in brain and muscle. Notice that the different cited studies use different sampling procedures and PMI, whereof many substantially longer than 2 min, and should therefore not be taken to be identical to the experimental design in this study. L is liver, P is pancreas, Mm is mouse musculus and HS is human.

| Protein                                      | Mm L/P | Mm brain | Hs brain | Bovine muscle | Pig muscle | Ref |
|----------------------------------------------|--------|----------|----------|--------------|------------|-----|
| HSP60                                        | L/P    | √        | √        | √            |            | (3,13) |
| Actin, cytoplasmic 1                         | P      | √        | √        |              |            | (3,11,14) |
| Alpha enolase                                 | P      | √        |          |              |            | (1,16) |
| Apolipoprotein A-1**                         | L      | √        |          |              |            | (3,11) |
| Dihydrolipoyl-sine-residue succinyltransferase| P      |          | √        |              |            | (14) |
| Elongation factor 1-beta                     | P      |          | √        |              |            | (3) |
| Heat shock cognate 71 kDa protein            | L/P    | √        | √        |              |            | (4,11,12) |
| Isocitrate dehydrogenase [NAD] subunit alpha | P      |          | √        |              |            | (13) |
| Protein disulfide-isomerase precursor         | P      |          | √        |              |            | (11) |
| Serum albumin precursor                      | L      | √        |          |              |            | (4,11) |
| Stress-70 protein, mitochondrial precursor    | L/P    | √        |          |              |            | (11) |

** Not significantly affected in liver (p < 0.15).

procedures; the most commonly used application of snap freezing the tissue (F), rapid heat stabilization using the Stabilizor T1 instrument (D), and snap freezing followed by rapid heat stabilization (FD). The effects of the sampling procedures were analyzed on a proteomic (2D-DIGE) and peptidomic (label-free LC-MS) level.

2D-DIGE of Liver and Pancreas—To better estimate global proteome effects in the 2D-DIGE experiments we normalized the data as previously described (26). The liver 2D-DIGE gels (six 2D-DIGE gels, 12 samples, n = 4 for the F, D, and FD groups) had a total of 1151 spots, whereof 841 spots were matched in at least four out of six 2D-DIGE gels and therefore used for effect estimation. In the pancreas 2D-DIGE study (six 2D-DIGE gels, 12 samples, n = 4 for the F, D, and FD groups), the corresponding numbers were 1148 spots in total, with 831 matched in at least four out of the six 2D-DIGE gels. Hierarchical clustering and PCA was performed on significantly affected spots (F-statistics, p < 0.05; liver n = 54, pancreas n = 191). In both tissues, the D and FD groups are more similar to each other than F (Fig. 1A, 1B). The first component (liver and pancreas PC1) explained ~59% of the variation in the data and separated rapid freezing sample preparations (F) from rapid heat stabilization groups (D and FD). Pair wise comparisons (FvsD, FDvsD, and FvsFD) were performed on spots matched in at least four gels (Fig. 1C). Irrespective of fold change differences, the liver tissue was not as affected by the post mortem sampling procedure as the protease rich pancreas, 86–87% of the spots being unaffected between snap freezing (F) and preparations involving rapid heat stabilization (D, FD), compared with 66–72% in pancreas (Fig. 1C). That the pancreas is more subjected to degradation than liver is also seen by the fact that there were virtually no differences between liver FD and D (FD versus D) samples, whereas 11% is differed in pancreas. No particular bias regarding increase or decrease of protein intensities between sampling procedures was detected. The fold-change distributions for all 2D-DIGE pair wise comparisons for liver and pancreas were mainly contained within log2 < 1 (Fig. 2D). Statistically significant different proteins (p < 0.05, log2 fold change > 1) are listed in Table I and II. Although there was a somewhat greater variation in the liver 2D-DIGE data compared with the pancreas data, the levels were comparable between all sample preparation groups (Fig. 3A, 3B).

General proteome degradation that affects molecular weight can be hypothesized to increase the expression of small proteins spots in the lower part of the gels as larger proteins are degraded into smaller fragments. A spatial analysis of increase and decrease in expression levels in liver and pancreas gels did not discover any such effect in any pair wise comparison (supplemental file S1).

Sampling Procedure Sensitive Proteins—Following 2D-DIGE image and data analysis, several proteins were picked from preparative liver and pancreas 2-DE gels for protein identification. A total of 32 liver spots were identified (six significantly affected fold change log2 > 1, F-test p < 0.05), the main candidates being Superoxide dismutase [Cu-Zn]
(P08228), Sorbitol dehydrogenase (Q64442), and Regucalcin (Q64374) (Table I). Sample preparation effects on superoxide dismutase demonstrated by 2D-DIGE (three out of four identified 2D-DIGE isospots were more highly expressed in D and FD compared with F liver tissue) were confirmed by immunoblotting (supplemental file S4).

All three top candidate proteins were detected as differentially regulated isoforms, Sorbitol dehydrogenase and Regucalcin being found as both affected and unaffected isoforms. In pancreas, a total of 88 spots were identified (20 significantly affected, fold change log2 > 1 , F-test p < 0.05). The top pancreas candidates include Peptidyl-prolyl cis-trans isomerase (Q9DCY1), α-enolase (P17182), type II cytoskeletal keratin 8 (P11679) and type I cytoskeletal keratin 18 (P05784), and several protein translation regulation-related proteins (P63242, P57776, Q8BGD9 and O70251) (Table II). In both liver and pancreas, the top candidates exhibit the greatest differences in the F and D/FD group comparisons. Several proteins affected both in pancreas and liver are involved in chaperone and stress functions. These include 78 kDa glucose-regulated protein precursor (GRP78; P20029), 60 kDa heat shock protein (P63038), heat shock cognate 71 kDa protein (P63017), and the mitochondrial Stress-70 protein precursor (P38647). Only three out of 13 identified GRP78 isoforms were significantly affected (F-test p < 0.05) in pancreas and one out of two detected GRP78 isoforms in liver, indicating degradation modulations of post-translational modifications rather than a breakdown of total protein levels.

**Differential Peptide Display of Pancreas**—Considering the level of pancreatic peptide hormone production and its im-

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**Fig. 4. Presence of endogenous pancreatic peptides following different sampling procedures.** A, Endogenous peptides derived from known protein precursors. Green letters show amino acids surrounding the identified peptides. Peptides present in at least three samples/group are marked green, between one and two samples/group are blue, and not detected at all are black. Log2 fold change differences of F or FD compared with D (centered at log2 fold change = 0) are also shown. F-statistics significance: * p < 0.05, *** p < 0.001, NS is nonsignificant and NE is not estimable. Green letters in the sequences designate surrounding amino acids.
Importance for metabolic syndrome diseases such as diabetes, it is especially interesting to study the effect of sampling procedures on the pancreatic peptidome (enabling more mechanistic analysis). Out of 3175 peaks detected over all samples ($n=4$ for F and $n=3$ for FD), only 123 were present following normalization in at least eight of the 11 samples analyzed and of those 26 peaks were differentially expressed ($F$-test $p<0.05$, maximum number of samples/group). A hierarchical clustering and PCA based on these 26 peaks shows the same profile as in the 2D-DIGE data (Fig. 1A, 1B), i.e. D and DF groups being similar to each other and separate from the F group. The general variation in the LC-MS data set is comparable between the different sample preparation groups (Fig. 3C).

The peptide data demonstrates that F samples contain significantly ($p<0.05$, pair wise $t$ test) more degradation peptides compared with D or FD (Fig. 3D, supplemental file S5). The 176 peaks in common for all groups represent only a small fraction of the number of peaks found in the respective experimental groups (Fig. 3D, 3E, 3F). The number of peptides shared by D and FD (demonstrated for $\geq 2$ samples/group and maximum samples/group) is greater than between F and FD or F and D, again indicating that D and FD are more similar to each other than F. F on the other hand exhibits many more unique degradation peaks than the other two groups. If the sums of the mean in each group in steps of 100 Da are compared between the groups there is no difference between the D and the FD samples but the intensity of F sample peaks are higher especially at the low molecular area (Fig. 3G). If peaks that could be matched between samples ($\geq 2$ samples/group) are analyzed in the same way it displays an increased intensity among the D and FD sample peaks compared with F-sample peaks (Fig. 3H).

Interestingly, if only identified peaks that originate from precursors known to contain endogenous peptides are compared between groups the intensity is higher in the D and FD compared with the F group 15 (Fig. 3I). The relationship is reversed if only the identities of the peptides originate from proteins not known to contain any endogenous peptides are compared (Fig. 3J).

**Sampling Procedure for Sensitive Peptides**—The differential peptide display analysis identified 218 peptides, 36 of them being present in at least eight of the 11 samples analyzed (Table IV, supplemental file S5, S6, S7, S8). Forty-six
percent of the peptides were derivates from the insulin-I and II C-peptides. No other insulin precursor-derived peptides were detected. Besides the C-peptides, we also detected endogenous peptides derived from chromogranin I, II, and III, glucagon, islet amyloid polypeptide (IAPP), neuroendocrine convertase II, somatostatin, and thymosin β-4 (Fig. 4, Table III). The majority of these endogenous peptides were not estimable for statistical significance because of low coverage between samples. Among the C-peptides, most estimable peptides, in particular the longest, were significantly less expressed (\(t\) and F-statistics \(p < 0.05\)) following the F-procedure (Figs. 5 and 6). The full length insulin 1 and 2 C-peptides are four times more prevalent in D and FD compared with F. There were several peptides that were only found in certain sample procedures. The D procedure resulted in only a few D-specific peptides, with five degradation fragments from larger proteins (four nonredundant identities). The F-group on the other hand, had up to 33 degradation fragments from larger proteins (21 nonredundant identities) (see supplemental file S5). There is furthermore a pattern of oxidized methionine (M) among the F-peptides that is not present or less expressed among D and FD samples (Fig. 4). The oxidized form of a somatostatin-28 derived peptide (SANSN-PAMAPRE) shows both an F and FD sensitive pattern as it is least four more times more expressed in FD than D whereas the unoxidized form is six times less expressed in F than D.

**DISCUSSION**

This is the first study to analyze how sampling procedures affect the proteome in liver and pancreas tissues; liver being...
one of the most common tissues analyzed in many types of proteomics experiments whereas the pancreas is a more specialized tissue with small islands of hormone producing endocrine cells (Islets of Langerhans) embedded within the larger structure of digestive enzyme producing exocrine cells. The overall results demonstrate that rapid heat stabilization (D) is very similar to snap freezing followed by rapid heat stabilization (FD) whereas single snap freezing (F) differs. As stated in the introduction, the methodology of measuring the proteins/peptides that are in common between all the samples following a very short PMI (<2 min) and using total lysate 2D-DIGE is potentially more likely to reflect regulated protease driven activity and/or PTM alterations than stochastic post mortem degradation. The more sensitive LC-MS analysis of endogenous 10 kDa peptides is on the other hand based on an individual sample approach and demonstrates (see Venn diagrams in Fig. 3) that the F procedure displays two to three times more peptides/peak, indicating increased degradation.

The liver proteome is less affected by the different sample procedures than the proteome in the protease rich pancreas (Fig. 1). In 2D-DIGE, based on total lysate sample preparations, the spots analyzed are mainly of multicopy housekeeping and tissue-specific nature and therefore relatively easy to match between different individuals. It is unlikely that specific protein spots are completely absent at this level of resolution, although this of course is very much correlated with the properties of the image analysis software used. On the plus side, 2-DE in general and 2D-DIGE in particular is unrivaled as methodology to characterize alterations in isoform patterns, which is an important aspect when one considers that PTMs, such as phosphorylations are especially sensitive to post mortem influence already within minutes post mortem (3, 17). The properties of the usual 2-DE based approach therefore clearly influence the degradome data, making it tempting to define these results as "2-DEgradomes." It should also be noticed that the PMI PTM knowledge is mainly based on protein phosphorylation, with different proteins displaying varying levels of PMI sensitivity within the first 10h (1, 3, 9, 17) but there are also reports on PMI sensitivity among acetylated lysines (9). The more proteolytic nature of the pancreas is probably responsible for the fact that ~99% of the liver proteome is similar between D and FD whereas this is only the case for ~89% in pancreas. Our analysis to see if the potential degradation of large proteins would affect the occurrence

| Peptide | UniProt | F-test | FvD | FvFD | FDvD | Relative StDev |
|---------|---------|--------|-----|------|------|---------------|
| Chromogranin B [R.SFARAPQLDLK] | P16014 | 0.367 | 0.319 | 0.425 | 0.554 | 0.202 | -0.235 | 0.867 | 4.097 | 7.443 | 3.670 |
| Glucagon [Q.HALQDTENPR.S] | P55095 | 0.291 | -0.601 | 0.137 | 0.612 | 0.352 | -0.195 | 0.929 | 6.560 | 6.037 | 9.902 |
| Glucagon [A.LQDTENPR.S] | P55095 | p<0.050 | 1.649 | p<0.010 | 0.279 | 0.638 | 1.371 | 0.113 | 2.857 | 5.999 | 12.957 |
| Islet amyloid polypeptide [A.TPVRSGSNPQMD.K] | P12968 | 0.100 | -1.385 | p<0.050 | -0.725 | 0.304 | -0.660 | 0.692 | 9.441 | 8.458 | 4.549 |
| Neuroendocrine convertase 2 [R.IKMALQQEGFD.R] | P21661 | p<0.050 | -2.619 | p<0.050 | -3.117 | p<0.050 | 0.300 | 0.923 | 18.18 | 6.270 | 8.925 |
| Somatostatin [R.SANSNPAMPRE.R] | P60041 | p<0.001 | -3.394 | p<0.001 | -2.533 | p<0.001 | -0.861 | 0.296 | 7.994 | 9.269 | 2.677 |
| Somatostatin [R.SANSNPAMPRE.R] | P60041 | 0.107 | -1.068 | 0.105 | -0.910 | 0.218 | -0.160 | 0.929 | 1.305 | 8.043 | 6.795 |
| Somatostatin [R.SANSNPAMPRE.R] + OX (M) | P60041 | p<0.001 | 0.616 | 0.062 | -2.439 | p<0.001 | 3.055 | p<0.001 | 2.352 | 2.720 | 1.385 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQLTALAEVARO.K] | P10325 | p<0.010 | -2.484 | p<0.010 | -2.774 | p<0.010 | 0.290 | 0.923 | 8.940 | 15.327 | 22.875 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQLTAL.E] | P10325 | p<0.010 | -2.318 | p<0.010 | -2.555 | p<0.010 | 0.237 | 0.923 | 9.421 | 3.670 | 7.153 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQLTAL.L] | P10325 | p<0.010 | -2.425 | p<0.010 | -2.799 | p<0.010 | 0.371 | 0.730 | 6.949 | 3.373 | 2.833 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQLTAL.E] | P10325 | p<0.010 | -3.104 | p<0.010 | -3.153 | p<0.010 | 0.050 | 0.989 | 9.334 | 6.628 | 9.093 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQLTAL.L] | P10325 | p<0.010 | -1.385 | p<0.010 | -1.478 | p<0.010 | 0.113 | 0.923 | 2.555 | 3.102 | 2.547 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQLT.L] | P10325 | p<0.010 | -1.551 | p<0.010 | -1.687 | p<0.010 | 0.136 | 0.929 | 2.303 | 3.705 | 3.968 |
| C-peptide, Inst1 [R.EVEDPQVEQLEGLGSPDGQL.Q] | P10325 | p<0.050 | 1.561 | p<0.010 | 1.364 | p<0.010 | 0.197 | 0.885 | 2.544 | 5.063 | 7.008 |
| C-peptide, Inst1 [T.LALEVARO.K] | P10325 | p<0.010 | -0.264 | 0.209 | 0.471 | 0.109 | -0.735 | 0.985 | 2.104 | 10.253 | 1.041 |
| C-peptide, Inst1 [E.VEDPQVEQLEGLGSPDGQLTAL.L] | P10325 | p<0.001 | -3.075 | p<0.010 | -3.410 | p<0.010 | 0.335 | 0.867 | 11.910 | 6.329 | 4.037 |
| C-peptide, Inst1 [E.VEDPQVEQLEGLGSPDGQL.T] | P10325 | p<0.001 | -2.130 | p<0.001 | -2.313 | p<0.001 | 0.183 | 0.865 | 0.034 | 5.213 | 1.880 |
| C-peptide, Inst1 [E.DPOVEQLEGLGSPDGQL.T] | P10325 | p<0.001 | -2.050 | p<0.001 | -2.056 | p<0.001 | 0.006 | 0.969 | 0.683 | 5.950 | 1.691 |
| C-peptide, Inst1 [E.VEDPQVEQLEGLGSPDG.Q] | P10325 | 0.7358 | 0.310 | 0.503 | 0.291 | 0.602 | 0.019 | 0.989 | 5.154 | 4.831 | 3.669 |

| TABLE IV |
| Identified peptides with effect estimations. Yellow fields show peptides with log2 fold change > 1 and p value <0.001. Grey fields indicate nonsignificance (p value >0.05) |

10.1074/mcp.M900229-MCP200–12
of small proteins in the lower part of the gels did not detect any such trend (supplemental file S1). Instead, whole spot families were affected in the pancreas with no similar pattern in liver except possibly among the more acidic low molecular weight proteins. The sensitivity of total lysate 2D-DIGE is too low and too few proteins were identified in order to be certain that these spot families encompass all isoforms of any given protein, which seems unlikely anyway. It seems more reasonable that the spot families represent an altered PTM status such as phosphorylations, as described for dynamin-1 in post mortem mouse brain tissue (12). The general level of sample procedure induced differential intensity between the samples, with the exception of some few proteins, lies within ±100% for the proteomes (Fig. 2). The pancreatic peptidome on the other hand shows a larger impact, differences in ranging to at least ±400% when comparing snap freezing with rapid heat stabilization procedures (F versus D, F versus FD). This is especially noticeable for the full length insulin 1 and 2 C-peptides, which were four times more prevalent in D or FD groups than F (Figs. 5 and 6). Muscle and brain are the most characterized tissues with regard to post mortem degradation, with most proteomics studies focused on brain tissue (1, 3–5, 11–16). Several proteins found in the brain degradome were also detected in the liver or pancreas degradome (Table III, IV). The absence of cytoskeletal associated proteins, with the exception of cytoplasmic actin in liver and pancreas is noticeable considering their general presence in the brain degradome (3–5, 8, 11). These data emphasize the impact of tissue specificity on degradation properties and the relation to the chosen sampling procedure.

Among the pancreatic peptides, there was a clear overrepresentation (46%) of insulin C-peptide derived fragments. The insulin C-peptides have graduated from being considered as merely insulin byproducts to becoming bioactive peptides in their own right (32).
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does not seem to possess any secondary structure in aqueous solutions (33), possibly making it more accessible to degradation and explaining the large number of C-peptide derivates detected whereas no other insulin peptides were detected. In rat, the mid-third region of the insulin-I C-peptide and the C-terminal penta-peptide (Fig. 5) are biological functional fragments with regard to stimulating Na\(^+\), K\(^+\)-ATPase activity, at least when de novo synthesized and tested in vitro (34). It is not clear if the C-peptide derivates posses any biological function of their own, but it is intriguing that the majority retain the mid third region previously mentioned.

Overall, more peaks/degradation peptide were found in F-samples (Fig. 3D, 3E, 3F). This together with a greater presence of oxidized peptides clearly marks solely snap freezing as a poor sampling procedure with regard to sample quality. We also detected a general trend among estimable pancreatic peptides (i.e. peptides that are present in the majority of samples from all groups) to have a slightly higher ion intensity when derived from D or FD samples compared with F-samples and vice versa (Fig. 3H). Peptides derived from peptide precursors have a somewhat higher intensity in D and FD samples than F whereas the patterns was the reverse for peptides derived from probable degradation of larger proteins (Figs. 3I and 3J). It remains to be seen if these patterns also occur when using other LC-MS systems and in other tissues. The purpose of the sampling (for instance searching for clinical biomarkers, peptides, proteins, and PTMs) together with the sensitivity of the technique (such as gel-based versus LC-based proteomics) need to be taken into account, but it is still striking that such a large extent (around 40% in the pancreas) of the measured 2-DEgradome is affected by the sample preparation procedure. Some of us previously demonstrated the use of the instrument Stabilizor T1 (Denator AB), for rapid heat stabilization of fresh or frozen samples (22). The close similarity between D and FD compared with F proteomes together with the greater amount of degradation products in F samples in this study further proves that rapid heat stabilization is superior to snap freezing and that frozen tissues practically can become “rescued” by subsequent rapid heat stabilization.

Finally, on a more general level, it should be noted that any experiment that studies biology on an omics level needs to use adequate data normalization tools. This is especially true for 2-DE based methods (26) and should be even more relevant when studying a system in degradation. A more comprehensive approach to the field of degradomics is therefore needed. There are without doubt numerous proteins, whose degradation (or cleavage) products may have biological properties of their own, the C-peptide being just one case in point. A better understanding of the properties (biochemical, protease cleavage sites and otherwise) that promote or inhibit degradation in different systems, post-translational status and the differences between more regulated and random degradation in vivo and ex vivo, has therefore the potential to lead to the discovery of several new bioactive protein isoforms and peptides.

CONCLUSIONS

This is the first in-depth study of sampling procedure-related proteomic degradation in liver and pancreatic tissue. We show that both mouse liver and pancreas are very sensitive to the choice of sampling procedure, the protease rich pancreas especially so. We present several liver and pancreatic proteins and peptides that are especially sensitive to post mortem degradation and choice of sampling procedure. A comparison between rapid heat stabilization and snap freezing of fresh tissue samples demonstrates that rapid heat stabilization is better for sample quality, with snap frozen samples displaying a greater amount of degradation products. We also demonstrate on a systemic proteomic and peptidomic level that snap frozen samples can be substantially improved by subsequent heat stabilization, making them very similar to directly heat stabilized samples.

This article contains supplemental Files S1 to S8.

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REFERENCES

1. Ferrer, I., Santpere, G., Arzberger, T., Bell, J., Blanco, R., Boluda, S., Budka, H., Carmona, M., Giacone, G., Krebs, B., Limido, L., Parchi, P., Puig, B., Strammello, R., Ströbel, T., and Kretzschmar, H. (2007) Brain protein preservation largely depends on the postmortem storage temperature: implications for study of proteins in human neurologic diseases and management of brain banks: a BrainNet Europe Study. J. Neuropathol. Exp. Neurol. 66, 35–46

2. Sköld, K., Svensson, M., Kaplan, A., Björksten, L., Aström, J., and Andreén, P. E. (2002) A neuroproteomic approach to targeting neuropeptides in the brain. Proteomics 2, 447–454

3. Sköld, K., Svensson, M., Normann, M., Sjögren, B., Svenningsson, P., and Andrén, P. E. (2007) The significance of biochemical and molecular sample integrity in brain proteomics and peptidomics: stathmin 2–20 and peptides as sample quality indicators. Proteomics 7, 4445–4456

4. Fountoulakis, M., Hardmeier, R., Höger, H., and Lubic, G. (2001) Postmortem changes in the level of brain proteins. Exp. Neurol. 167, 86–94

5. Franzen, B., Yang, Y., Sunnemark, D., Wickman, M., Ottervald, J., Oppermann, M., and Sandberg, K. (2003) Dihydropyrimidinidase related protein-2 as a biomarker for temperature and time dependent post-mortem changes in the mouse brain proteome. Proteomics 3, 1920–1929

6. Siew, L. K., Love, S., Dawbarn, D., Wilcock, G. K., and Allen, S. J. (2004) Measurement of pre- and post-synaptic proteins in cerebral cortex: effects of post-mortem delay. J. Neurosci. Methods 139, 153–159

7. Li, X., Greenwood, A. F., Powers, R., and Jope, R. S. (1996) Effects of postmortem interval, age, and Alzheimer’s disease on G-proteins in human brain. Neurobiol. Aging 17, 115–122

8. Schwab, C., Bondada, V., Sparks, D. L., Cahay, L. D., and Geddes, J. W. (1994) Postmortem changes in the levels and localization of microtubule-associated proteins (tau, MAP2 and MAP1B) in the rat and human hippocampus. Hippocampus 4, 210–225

9. Li, J., Gould, T. D., Yuan, P., Manji, H. K., and Chen, G. (2003) Post-mortem interval effects on the phosphorylation of signaling proteins. Neuropsychopharmacology 28, 1017–1025

10. Taniguchi, S., Fujita, Y., Hayashi, S., Kakita, A., Takahashi, H., Murayama, S., Saito, T. C., Hisanaga, S., Iwasubu, T., and Hasegawa, M. (2001) Calpain-mediated degradation of p35 to p25 in postmortem human and rat brains. FEBS Lett. 489, 46–50

11. Crecéllius, A., Götz, A., Arzberger, T., Fröhlich, T., Arnold, G. J., Ferrer, I.,
13. Jia, X., Hildrum, K. I., Westad, F., Kummen, E., Aass, L., and Hollung, K. (2006) Molecular & Cellular Proteomics 10.3, 10.1074/mcp.M900229-MCP200–15

15. Jia, X., Hollung, K., Therkildsen, M., Hildrum, K., and Bendixen, E. (2006) J. Proteome Res. 5, 1763–1769

16. Lametsch, R., Karlsson, A., Rosenvold, K., Andersen, H. J., Roepstorff, P., Svensson, M., Skoold, K., Svenningsson, P., and Andren, P. E. (2003) J. Neurosci. Methods 1276–1291

18. Svensson, M., Boren, M., Skoold, K., Fälth, M., Sjögren, B., Andersson, M., Faélth, M., Sjögren, B., Andersson, M., Boren, M., Skoold, K., Fälth, M., Sjögren, B., Andersson, M., and Bendixen, E. (2003) Postmortem Proteome Changes of Porcine Muscle Related to Tenderness. J. Agri. Food Chem. 51, 5992–5997

17. O’Callaghan, J. P., and Striram, K. (2004) Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins. J. Neurosci. Methods 135, 159–168

19. Scharf, M. T., Mackiewicz, M., Naidoo, N., O’Callaghan, J. P., and Pack, A. I. (2008) AMP-activated protein kinase phosphorylation in brain is dependent on method of killing and tissue preparation. J. Neurochem. 105, 833–841

20. Che, F. Y., Lim, J., Pan, H., Biswas, R., and Fricker, L. D. (2005) Quantitative neuroepitopeomics of microwave-irradiated mouse brain and pituitary. Mol. Cell Proteomics 4, 1391–1405

21. Dowell, J. A., Heyden, W. V., and Li, L. (2006) Rat neuroepitopeomics by LC-MS/MS and MALDI-FTMS: Enhanced dissection and extraction techniques coupled with 2D RP-RP HPLC. J. Proteome Res. 5, 3368–3375

22. Svensson, M., Boren, M., Sköld, K., Flath, M., Sjögren, B., Andersson, M., Svenningsson, P., and Andren, P. E. (2009) Heat Stabilization of the Tissue Proteome: A New Technology for Improved Proteomics. J. Proteome Res. 8, 974–981

23. Krogh, M., Fernandez, C., Teilum, M., Bengtsson, S., and James, P. (2007) A probabilistic treatment of the missing spot problem in 2D gel electrophoresis experiments. J. Proteome Res. 6, 3335–3343

24. Bäck, P., Nägård, F., Bolmsjö, G., Bengtsson, S., and James, P. (2003) Automating gel image acquisition. J. Proteome Res. 2, 662–664

25. R Development Core Team: (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, http://www.R-project.org, Vienna, Austria.

26. Kultima, K., Scholz, B., Alm, H., Sköld, K., Svensson, M., Crosaman, A. R., Bezard, E., André, P. E., and Lönnstedt, I. (2006) Normalization and expression changes in predefined sets of proteins using 2D gel electrophoresis: a proteomic study of L-DOPA induced dyskinesia in an animal model of Parkinson’s disease using DIGE. BMC Bioinformatics 7, 475

27. Smyth, G. K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3

28. Caraux, G., and Pinloche, S. (2005) PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. Bioinformatics 21, 1280–1281

29. Rossbach, U., Nilsson, A., Flith, M., Kultima, K., Zhou, Q., Hallberg, M., Gorth, T., André, P. E., and Nyberg, F. (2009) A quantitative peptidomomic analysis of peptides related to the endogenous opioid and tachykinin systems in nucleus accumbens of rats following naloxone-precipitated morphine withdrawal. J. Proteome Res. 8, 1091–1098

30. Lamanda, A., Zahn, A., Röder, D., and Langen, H. (2004) Improved Ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. Proteomics 4, 599–608

31. Samuelsson, J., Dalevi, D., Levander, F., and Rögnvaldsson, T. (2004) Modular, scriptable and automated analysis tools for high-throughput peptide mass fingerprinting. Bioinformatics 20, 3628–3635

32. Johansson, J., Ekberg, K., Shafqat, J., Henriksson, M., Chibalai, A., Wahren, J., and Jörnvall, H. (2002) Molecular effects of proinsulin C-peptide. Biochem. Biophys. Res. Commun. 295, 1035–1040

33. Henriksson, M., Shafqat, J., Liepinsh, E., Tally, M., Wahren, J., Jörnvall, H., and Johansson, J. (2000) Unordered structured of proinsulin C-peptide in aqueous solution and in the presence of lipid vesicles. Cell Mol. Life Sci. 57, 337–342

34. Ötomo, Y., Bergman, T., Johansson, B. L., Jörnvall, H., and Wahren, J. (1998) Differential effects of proinsulin C-peptide fragments on Na+, K+-ATPase activity of renal tubule segments. Diabetologia 41, 287–291