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Sample preparation protocol for bottom-up proteomic analysis of the secretome of the islets of Langerhans

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Abstract

We evaluate a set of protocols for preparation of the secretome from murine Islets of Langerhans for bottom-up proteomic analysis. Of the protocols evaluated, a filter-aided sample preparation based approach using sodium dodecyl sulfate as a detergent to solubilize proteins generated the most protein identifications. A total of 362 protein groups (average of 3.7 peptides/protein) were identified from the secretome using the SDS-FASP protocol; a combination of data from three protocols generated 413 protein group identifications. As expected, the identified proteins included insulin 1 and 2, somatostatin, and glucagon, the four main secreted components from islets. STRING network analysis classified the other proteins as being associated with extracellular exosomes, membrane-bounded vesicles, vesicles, and the extracellular region.

Introduction

Diabetes is a metabolic disease characterized by persistent high blood sugar.¹ Diabetes affects almost 400 million people worldwide with nearly 30 million Americans suffering from the disease.^{2,3} Blood sugar levels are regulated by the islets of Langerhans, the endocrine portion of the pancreas, via the secretion of hormones such as insulin, amylin, and glucagon.^{4–6} Diabetes occurs when islets release insufficient insulin to lower glucose due to islet dysfunction or excessive insulin resistance in target tissues. Studies have reported the proteome of healthy and diabetic islets,^{7–11} hormone levels, the proteome of the insulin secretory granule.^{12–13} To the best of our knowledge, there has been only one published study of the islet secretome;¹⁴ that publication did not compare sample preparation methods.

Secretomes, proteins secreted by a cell or organ, are of medium complexity, usually between 250–700 protein groups,¹⁵ and are of particular interest for disease and wellness markers.^{16–17} Secretomes pose challenges due to media contamination, low sample concentration, and presence of proteins from cell lysis.^{18–19} The islets of Langerhans pose an unusual challenge, because in addition to releasing vesicles and microparticles^{20–21} islets secrete insulin in large quantities^{22–23} making other protein identifications a challenge.

Secretomes are usually rich in membrane proteins^{24–25}, so that detergent selection is critical for optimization of the sample preparation protocol. In this manuscript, we investigated sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), Tergitol type NP-40 (NP-40),

and RapiGest, as well as urea and ammonium bicarbonate (NH_4HCO_3) for their ability to solubilize and denature islet secretome samples. In an initial study, insulin and other abundant soluble proteins were first removed from the sample via ultracentrifugation,²⁶ affording the opportunity to best determine which solution best solubilizes secreted proteins. Protein recovery was determined using a bicinchoninic acid (BCA) assay. In this study SDS, NP-40, and RapiGest proved to be the best and most reliable detergents.

An uncentrifuged secretome was used in a second study. Here, a filter aided sample preparation (FASP)^{26–29} was used to prepare tryptic peptides from SDS, NP-40, and RapiGest solubilized proteins. Proteins were loaded into a column, the denaturant was washed off, proteins were digested, and the resultant peptides are eluted via centrifugation. Previous work has shown that FASP is a robust and reliable technique for secretome studies.^{30–32} In addition, FASP reduced the background level of insulin by the use of comparatively large “cut-off” column.

Materials and methods

Reagents

Dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (NH_4HCO_3), sodium deoxycholate, SDS, urea, Tris(hydroxymethyl)aminomethane (Tris), formic acid (FA), Tergitol® Type NP-40 (NP40), Histopaque-1077, and fetal bovine serum (FBS) were from Sigma Aldrich (St. Louis MO USA). Pierce™ BCA Protein Assay Kit, collagenase, glucose, acetonitrile, and reagents required for Krebs-Ringer buffer and Hanks' balanced salt solution were obtained from Thermo Fischer Scientific (Waltham, MA USA). RapiGest SF™ Surfactant was from Promega (Madison WI USA). Sequencing grade trypsin was from Waters (Milford, MA USA). RPMI 1640 and penicillin/streptomycin were from Life Technologies (Carlsbad, CA USA). HCl was from VWR (Radnor, Pennsylvania).

Islet Isolation and Cell Culture

Islets of Langerhans were isolated from CD-1 mice 6–7 weeks old with a modified version of the protocol used by Carter *et al.*³³ Pancreata were injected with 2–3 mL of collagenase and placed in one mL of G solution before digestion. Histopaque-1077 and HBSS (5 mL each) were used for gradient separation, and islets were removed from the interface of the two layers. After isolation, groups of 50 islets were incubated overnight in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin. Islets were then rinsed in freshly prepared Krebs-Ringer buffer (KRB) with 11 mM glucose and incubated for 24-h in FBS free media. After 24-h incubation, media was collected.

Protein Extraction Efficiency

Islets were removed from media, which was pooled and vortexed. Membrane and vesicular proteins were pelleted by centrifuging 200 μL of media at $100,000 \times g$ (Beckman Coulter Optima Max-E Ultracentrifuge). Supernatant was removed and the pelleted proteins were resuspended in 30 μL of one of the following solutions: 100 mM NH_4HCO_3 , 8 M urea, 1% SDS, 1% SDC, 5% SDC, 1% Tergitol® type NP-40 (NP-40), or 1% RapiGest. The solution was repeatedly pipetted followed by sonication for 3 minutes. Protein was quantified using

the BCA protein assay. BCA standards were solubilized in the seven solutions. Protein extractions were performed in triplicate for each solution and quantified in duplicate.

FASP

Fresh (uncentrifuged) media from 50 islets was vacuum concentrated to 600 μL . The sample was split into three equal volumes; SDS, NP-40, or RapiGestTM was added to these aliquots to make a final concentration of 1% (w/v). Samples were sonicated for five minutes, reduced with 5 mM DTT at 60° C for thirty minutes, and then alkylated with the addition of 15 mM iodoacetamide. Alkylation was quenched with the addition of excess DTT after thirty minutes.

The NP-40 and SDS samples were first mixed with an equal volume of 8 M urea, 100 mM Tris-HCl pH 8.5 (UA) and loaded into a 30 kDa molecular weight cut-off spin column (Millipore) and centrifuged at 20,000 $\times$ G for twenty minutes. All subsequent centrifugation steps used these settings. SDS was washed five times and NP-40 three times with the addition of 100 μL of UA. Each of the three samples was subsequently washed by flushing the column with 25 mM NH_4HCO_3 (pH 8.0) three times. Samples were digested overnight at 37° C with 5 μg of mass spectrometry grade trypsin in 100 μL of NH_4HCO_3 . Peptides were eluted via centrifugation and an additional 100 μL of water was added to the column to ensure elution of all peptides. RapiGest peptides were acidified using HCl at 37° C for thirty minutes to cleave the detergent before centrifugation and supernatant recovery. Samples were then dried using a speed vacuum before being suspended in 30 μL of 2% acetonitrile (ACN) 0.1% FA.

LC-MS/MS

Liquid chromatography (LC) was performed with an ACQUITY UPLC BEH C18 column (100 μm i.d. $\times$ 100 mm length, 1.7 μm particles) on a nanoACQUITY (Waters) UPLC system. LC system solvents were 0.1% FA (A) and 0.1% FA in ACN (B). Two μL of sample peptides were loaded into the column with 2% solvent B at a flow rate of 0.7 $\mu\text{L}/\text{min}$ for 14 minutes followed by a three step gradient: 1 minute from 2–10% B with a flow rate of 0.600 $\mu\text{L}/\text{min}$, 10–30% B over 59 minutes with a flow rate of 0.600 $\mu\text{L}/\text{min}$, and 1 minute to 80% B at a flow rate of 0.700 $\mu\text{L}/\text{min}$. The column was equilibrated at 2% B for 15 minutes before the running the next sample. Each run was performed in triplicate.

Mass data were measured using a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fischer Scientific). Full MS scans were collected with a range of 350–1800 m/z , a resolution of 60,000 (m/z 200), and an automatic gain control (AGC) of 3.00×10^6 . The ten most intense ions with a charged state ≥ 2 were subjected to a higher energy collisional induced dissociation (HCD). Normalized collisional energy was set to 28. MS/MS resolution was 60,000 (m/z 200), an isolation window of 1.4 m/z , and an AGC value was set to 1×10^5 . Dynamic exclusion was enabled ensuring peaks selected for fragmentation were excluded for 40 s.

Protein Group and Peptide Identification

MaxQuant³⁴ version 1.5.2.8 was used to process the raw spectra. Andromeda³⁵ searched the murine (*Mus musculus*) reference proteome (Uniprot: UP000000589) database, which contains 49,878 proteins. Our MS/MS data were searched against the tandem mass spectrometry database using default settings including a 20 ppm mass tolerance. Cysteine carbamidomethylation was the only fixed modification. Variable modifications were set to acetylation (N-terminus) and oxidation (M). Trypsin/P was selected as the protease with up to two missed cleavages allowed. Protein and peptide results were filtered using a 0.01 false discovery rate. The minimum peptide length was set to 7 residues. Label free quantification was enabled. Proteins and peptides were filtered to remove contaminants and reverse database identifications.

Statistical Analysis

One-way analysis of variance (ANOVA) tests were used to determine if any significant differences between the three FASP groups in regards to GRAVY score, pI, molecular weight, peptide length and the number of missed cleavages. Minimum significance was set at $p < 0.05$.

Protein Group Analysis

Cellular compartments of the identified proteins were determined using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database v10.0 (string-db.org).³⁶ GRAVY-scores were calculated according to the values from Kyte and Doolittle using the ProtParam tool available through Expasy (<http://web.expasy.org/protparam/>).³⁷

Results and discussion

100 mM NH_4HCO_3 , 8 M urea, 1% SDS, 1% SDC, 5% SDC, 1% NP-40, and 1% RapiGest were used to solubilize the membrane and vesicular proteins found in the secretome. SDS, NP-40, and RapiGest, generated the highest concentration of proteins (Table 1) and were used in a FASP based approach for the proteomics analysis.

Protein Extraction

Maintaining the solubility of secreted proteins throughout the preparative process can be challenging. Our initial evaluation of protein solubilization methods focused on measurement of the amount of solubilized proteins. In this initial evaluation, samples were ultracentrifuged to reduce the influence of insulin and other high abundance secreted products as well as to concentrate proteins. Pelleted proteins were then resuspended in solutions listed in Table 1. Protein concentrations were fairly consistent across all samples, with the exception of NH_4HCO_3 , which generated low protein concentrations. The 1% SDS sample yielded the highest protein concentration. Both 1% RapiGest and 1% NP-40 generated protein concentrations that were >80% of the protein concentration produced by SDS. 1% and 5% SDC generated lower protein concentrations than the other detergents and their results were significantly more variable.

FASP based secretome prep evaluation

We then evaluated the three top performing detergents (1% SDS, 1% NP-40, and 1% RapiGest) in a FASP based bottom-up proteomics approach (Figure 1 and supplemental materials); these samples were not ultracentrifuged and contained large amounts of insulin and other high abundance secreted products. SDS produced the most protein group identifications with 362, followed by NP-40 with 265, and RapiGest with 232 (Figure 2 and Table 2). The combined identifications from these three methods generated a total of 413 different protein groups. The peptide identifications were quite reproducible; triplicate analysis of the three extraction methods only generated ~8% more peptide identifications over the single run data presented in Figure 2. Roughly 40% of the protein groups were identified in common for the three extraction methods. MaxQuant showed that SDS extraction generated over twice the median protein intensity compared with the other extraction methods; it is not surprising that SDS also generated the most peptide identifications.

Insulin 1 and 2, somatostatin, and glucagon, four of the main secreted products, were among the identified proteins. SDS produced the most unique protein group identifications (79), compared to 20 and 24 when using NP-40 and RapiGest, respectively. The filters used claimed a 30 kDa “cut-off”, but the molar mass of a large number of protein groups was <30 kDa. No significant differences between the three groups were found in molecular weights, GRAVY score, or pI (SI).

STRING network analysis (Table 3) revealed that the five most abundant enrichments were extracellular exosomes, membrane-bound vesicles, extracellular region part, vesicles, and extracellular region with p-values $<1 \times 10^{-100}$. The SDS sample was more enriched in extracellular and membrane bound proteins, which is explained by recent work showing that SDS is most efficient at breaking extracellular vesicles found in conditioned media.³⁸

As expected, there was less peptide overlap than protein group overlap between the three detergent datasets (Figure 2) because 30–50% of peptides were unique. SDS produced the most peptide IDs with 1,331 as well as the highest peptide/protein group ratio with 3.7. RapiGest once again performed the poorest with 540 peptide IDs and a 2.3 peptide/protein group ratio (Table 4). A one-way ANOVA test revealed a significant difference in peptide length between the three samples, Figure 3. A Tukey HSD test determined that there is a significant difference between the SDS and NP-40 samples ($p < 0.05$) and a significant ($p < 0.001$) difference between RapiGest and any other sample. This difference was particularly obvious for longer peptides, which were overrepresented in the RapiGest fraction. The cause of this overrepresentation is not clear; there was no significant difference in the number of missed cleavages by trypsin between the three groups (Table 4).

Intensity data of all shared protein groups were normalized to NP-40. RapiGest had the lowest overall intensity, explaining the lower overall protein and peptide identification, while SDS and NP-40 have similar intensities (Figure 4). To explain the much higher identification for the SDS sample the chromatograms of the SDS and NP-40 samples were compared. NP-40 was found to remain in the sample despite washing which led to ionization

suppression, unlike SDS. As the FASP method is necessary to reduce insulin to a manageable level for islet secretome analysis SDS outperforms NP-40.

We compared our data to the only published islet secretome dataset.²³ Our protocol generated fewer protein IDs with 413 to their 742. However, our starting sample was significantly smaller; we used conditioned media from 50 islets incubated for 24 hours compared to 1000 islets incubated for 48 hours. Despite having less starting material and fewer total protein IDs, we identified more membrane and extracellular protein IDs (Figure 5), which can be attributed to our use of detergent to lyse extracellular vesicles and solubilize the proteins in the sample workup.

Conclusions

Secretomes, in general, pose challenges to proteomic analysis. To address the challenges with the specific instance of the secretome of the islets of Langerhans, seven different solutions were tested for their ability to solubilize secreted proteins. SDS, NP-40, and RapiGest islet culture media generated the highest concentration of extracted proteins, and were analyzed using LC-MS/MS in a bottom up approach using a FASP protocol. SDS generated the cleanest spectra, had the highest number of protein and peptide identifications, best peptide/protein ratio, and was most significantly enriched in extracellular and vesicular proteins. Our use of detergent also generated more extracellular and membrane protein identification compared to the only other published islet secretome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generalized workflow of the FASP-based interrogation of the secretome of the islets of Langerhans. Islets were excised and then cultured, first in FBS-containing media and then in FBS-free media. Conditioned media was collected, concentrated, split into three equal groups, and mixed with SDS, NP-40, or RapiGest for a FASP based approach. SDS and NP-40 were washed with 8 M urea, 100 mM Tris-HCl (UA) five and three times respectively. All samples were then washed with 25 mM NH_4HCO_3 and digested overnight with trypsin. Peptides were eluted for MS analysis. Note that samples were not subjected to ultracentrifugation for the FASP- based analyses.

Figure 2.

Venn Diagrams for peptide and protein identifications using the three detergents. (A) Protein group overlap. (B) Peptide overlap. There exists significant overlap at the protein group level, but much less so at the peptide level. SDS produced the largest number of identified protein groups, largest number of identified peptides, and the best peptide/protein group ratio. NP-40 and RapiGest had similar protein group identifications, but NP-40 identified more peptides.

Detergent	SDS	NP-40	RapiGest
Mean peptide length (residues)	12.0	12.4	14.8

Figure 3.

Comparison of peptide lengths for SDS, NP-40 and RapiGest samples. A significant difference exists between the lengths of peptides for the three treatment methods as determined by a one-way ANOVA test. RapiGest is significantly different at the $p < 0.001$ when compared to both NP-40 and SDS. SDS and NP-40 were significantly different at $p < 0.05$.

Figure 4.

Mass spectral intensity comparison for SDS, NP-40, and RapiGest. A. Box plot of intensity ratios of shared identified proteins. SDS values are comparable to NP-40 (SDS/NP-40). SDS intensities divided by RapiGest intensities illustrate the markedly lower intensities of RapiGest. Comparison of total ion chromatograms from NP-40 (B) and SDS (C) show an overall higher intensity for SDS. A periodic dip in intensity is observable for NP-40 indicating residual detergent after FASP due to ion suppression from residual detergent in the sample.

Figure 5. Comparison of total protein group IDs, membrane (GO: 0016020), and extracellular region (GO: 0005576) between Stützer et al and our data.¹⁴ Despite fewer total protein IDs, our SDS-FASP method generated more membrane and extracellular protein IDs.

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Table 1

Protein concentration after ultracentrifugation and resuspension

Resuspension material	8 M Urea	1% SDS	1% SDC	5% SDC	1% NP-40	1% RapiGest	100 mM NH ₄ HCO ₃
Mean Concentration (µg/mL)	100	163	115	90	131	145	23
Standard deviation of distribution (µg/mL)	30	17	39	70	12	37	11

All detergent based solutions also contained 50 mM NH₄HCO₃. Samples were prepared in triplicate and measured in technical duplicate. SDS had the largest amount of protein recovered and 100 mM NH₄HCO₃ had the least. SDC produced the most variable results. SDS, NP-40, and RapiGest were used in subsequent experiments.

Table 2

Characteristics of identifications using three efficient solubilization reagents.

Detergent	Protein Groups	Peptides	Average # Peptides/Protein
SDS	362	1331	3.7
NP-40	265	814	3.1
RapiGest	232	540	2.3

Table 3

Top five enriched cellular component by STRING analysis of all identified protein groups.

Cellular Component	SDS		NP-40		RapiGest	
	# of Genes	p-value (Bonferroni)	# of Genes	p-value (Bonferroni)	# of Genes	p-value (Bonferroni)
extracellular exosome	224	1.65×10^{-127}	165	8.02×10^{-94}	156	2.47×10^{-82}
membrane-bounded vesicle	236	1.12×10^{-123}	177	5.02×10^{-95}	155	1.24×10^{-74}
extracellular region part	240	2.45×10^{-118}	174	4.75×10^{-84}	144	1.16×10^{-79}
vesicle	235	3.71×10^{-116}	177	2.80×10^{-90}	154	7.96×10^{-76}
extracellular region	228	9.29×10^{-107}	164	2.24×10^{-74}	147	5.19×10^{-67}

Comparison of the number of missed cleavages as detected by MaxQuant when searched against the murine UniProt database using Andromeda

Table 4

Missed Cleavages	SDS		NP-40		RapiGest	
	Peptides	Percent	Peptides	Percent	Peptides	Percent
0	1161	87	725	89	457	85
1	166	12	87	11	81	15
2	4	0.3	2	0.2	2	0.4

A one-way ANOVA test determined there to be no significant difference (significance level for the test was set to 0.05) between the groups in terms of missed cleavages.