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Protocols

Optimized protocol for shotgun label-free proteomic analysis of pancreatic islets

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Abstract

Pancreatic islets are crucial in diabetes research. Consequently, this protocol aims at optimizing both the protein-extraction process and the proteomic analysis via shotgun methods for pancreatic islets. Six protocols were tested, combining three types of chemical extraction with two mechanical extraction methods. Furthermore, two protocols incorporated a surfactant to enhance enzymatic cleavage. The steps involved extraction and concentration of protein, protein quantification, reduction, alkylation, digestion, purification and desalination, sample concentration to ~1 μl, and proteomic analysis using the mass spectrometer. The most effective protocol involves either a milder chemical extraction paired with a more intensive mechanical process, or a more robust chemical extraction paired with a gentle mechanical process, tailored to the sample's characteristics. Additionally, it was observed that the use of a surfactant proved ineffective for these types of samples. Protocol 5 was recently used with success to examine metabolic changes in pancreatic islets of non-obese diabetic mice exposed to low doses of fluoride ions (F^-) and the primary pathways altered by the treatment.

Keywords: proteomic; shotgun; NOD islets; protocol

Introduction

Diabetes mellitus is a chronic disease of epidemic proportions worldwide, leading to multiple complications and significant mortality across all nations [1]. Type-1 Diabetes Mellitus (T1DM) is an autoimmune disease characterized by the destruction of pancreatic β-cells, leading to a reduced capacity to secrete insulin. Although insulin administration is the core of T1DM therapy [2], this treatment is often insufficient to prevent long-term complications [3]. T1DM is a major public health issue, potentially reducing life expectancy by 12 years due to long-term complications such as retinopathies, nephropathies, vasculopathies, and neuropathies [4–6].

Animal models of human disease play a vital role in scientific research, and they are essential in preclinical research for developing new therapies. Several animal models have been utilized to elucidate the mechanisms involved in T1DM development, offering insights into autoimmune damage to pancreatic islets. These models help with early detection, prevention, and treatment and have led to significant advancements in therapy [7].

The loss of β-cell function in T1DM typically unfolds over several years or decades, both before and after clinical diagnosis [8]. Insulin deficiency might be attributed to a specific loss of β-cells, resulting in a decrease in islet size, or an inability to develop a mass of β-cells sufficiently large to meet increased physiological demands [9, 10].

Given the significance of pancreatic islets in T1DM, further research into these structures could lead to new strategies for prevention and therapy. Moreover, exploring new biomarkers for T1DM opens up numerous opportunities for both prevention and intervention, which could enable the development of customized therapies by merging genetic susceptibility testing with islet-autoantibody detection, ideally before T1DM advances significantly [11]. Proteomics offers a powerful tool for screening for such disease biomarkers.

Proteomic analysis emerged from the synergy of protein-separation techniques and mass spectrometry. Currently, proteomic platforms are categorized based on their protein-separation techniques. These include gel-based methods such as 1D or 2D electrophoresis, high-performance liquid chromatography (HPLC or LC), and affinity media [12, 13]. Subsequent to separation, proteins can be both identified and quantified using mass spectrometry. Hence, proteomics aims to identify, quantify, and analyze a broad range of proteins (including their post-translational modifications) in various tissues, cells, or organisms [14, 15]. Crucially, it provides a powerful tool for uncovering new disease biomarkers, deciphering disease mechanisms, and formulating new therapies [12], as it allows for the discovery of previously unknown proteins or yields new insights into known proteins [12, 13, 15].

Our research group has been conducting several studies using proteomic analysis to uncover disease mechanisms and guide

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the development of new therapies [16–38]. Among these diseases is T1DM. Our findings include that treatment of non-obese diabetic (NOD) mice with water containing low fluoride concentrations not only reduces plasma glucose levels but also increases the percentage of β-cell function. Furthermore, we noted an increase in antioxidant enzymes in the liver, potentially playing a role in the protective mechanism of fluoride against the development of T1DM [18]. This is significant, as a reduced antioxidant defense in the islets is linked to the development of insulitis and the onset of diabetes in spontaneously diabetic lymphopenic biobreeding (BB) rats [39]. Therefore, to understand better the protective mechanism of fluoride in preventing T1DM, we opted to conduct shotgun label-free proteomic analysis of the islets. Shotgun proteomics involves the inferential analysis of proteoforms through the utilization of peptide proxies generated by the enzyme-catalyzed hydrolysis of entire proteomes. These peptides are commonly identified using nanoflow liquid chromatography coupled with tandem mass spectrometry analysis [40]. However, the amount of protein obtainable from mouse islets is extremely small, and most available studies utilize shotgun-labeled proteomics techniques [41, 42], rely on pooled samples from several animals [43], or use gel-based techniques [44]. In our current study, acknowledging extraction as the critical step in proteomic analysis [45], we assessed three chemical methods interlaced with two mechanical methods of protein extraction, culminating in the evaluation of six protocols for islet proteomic analysis. One of these protocols [5] was recently effectively used to examine metabolic changes in pancreatic islets of NOD mice exposed to low doses of fluoride ions and the primary pathways altered by this treatment [46].

Material and methods

Development of the protocol

This protocol was formulated by referencing previous protocols our group developed for extracting proteins from the liver [18, 37, 38], gut [22], or saliva [22], as well as a published protocol for analyzing the secretome of pancreatic islets [47]. We assessed three extraction solutions: 1% of surfactant RapiGest^{TM} SF (protocols 1 and 2) [47], 7 M urea, 2 M thiourea, and 40 mM dithiolthreitol (DTT), all diluted in 50 mM NH_4HCO_3 (Lysis Buffer A; protocols 3 and 4) [18, 22, 37, 38] or 6 M urea and 2 M thiourea, all diluted in 50 mM NH_4HCO_3 (Lysis Buffer B; protocols 5 and 6) [22]. For each of these three solutions, mechanical extraction was executed using vortexing, sonication, and centrifugation (total time of 75 min), as proposed by Ventura *et al.* [22] (protocols 1, 3, and 5; mechanical I) or sonication only (total time of 10 min), as detailed by Schmudlach *et al.* [47] (protocols 2, 4, and 6; mechanical II), respectively, as outlined in Table 1.

Among the protocols evaluated, Protocol 5 demonstrated the most effective performance, as shown in the Results section below (Fig. 1; Anticipated Results).

Ethics committee

The experimental protocol was approved by the Ethics Committee for Animal Experiments of Paulista State University (protocol: 1080/2019 vol.1). It was designed using six 20-week-old isogenic male A/J mice aged 21 days, allocating one for each protocol. The animals were sourced from the vivarium of Paulista State University. Euthanasia was performed using a CO_2 chamber followed by decapitation, and the pancreatic islets were isolated as detailed by Neuman *et al.* [48]. For each experimental protocol, approximately 100 islets were utilized.

Table 1. Protocols evaluated to extract proteins from pancreatic islets for shotgun label-free proteomic analysis.

Type of Protocol	Chemical extraction	Mechanical extraction
Protocol 1	1% RapiGest^{TM}	I
Protocol 2	1% RapiGest^{TM}	II
Protocol 3	Lysis buffer A	I
Protocol 4	Lysis buffer A	II
Protocol 5[a]	**Lysis buffer B**	**I**
Protocol 6	Lysis buffer B	II

Lysis buffer A: 7 M Urea, 2 M Thiourea and 40 mM DTT in 50 mM NH_4HCO_3, pH 7.8.
Lysis Buffer B: 6 M Urea, 2 M Thiourea in 50 mM NH_4HCO_3, pH 7.8.
Mechanical extraction I: Vortexing at 4°C for 10 min, sonication for 5 min and centrifugation for 20 817 *g* at 4°C for 10 min. These steps were repeated twice more. Total time: 75 min.
Mechanical extraction II: 1 min sonication, 1 min interval. Repeated 4 times more. Total time: 10 min.
[a] Provided the best results.
Bold is emphasizing the best protocol for extracting and identifying peptides in the islet.

Limitations of the protocol

There are no significant limitations to the protocol, as the reagents and equipment used are widely available in laboratory settings, and only a small amount of tissue is needed. It is feasible to analyze islets isolated from a single mouse. However, in the case of T1DM mice, where significant islet degradation has occurred, it might be necessary to pool samples from multiple mice.

Solutions, reagents, equipment, and softwares

Reagents

- PlusOne Urea (GE Healthcare Life Sciences, cat. #17131901).
- Thiourea ReagentPlus® (Sigma-Aldrich, cat. # T7875).
- Ammonium bicarbonate (AMBIC-NH_4HCO_3) (Fluka Analytic, cat. #40867).
- Dithiothreitol (DTT) (Bio-Rad, cat. #161-0611).
- Iodoacetamide (IAA) (GE Healthcare, cat. #RPN6302V).
- Pierce Trypsin Protease (Thermo Scientific, cat. #90057).
- Bradford Assay (Bio-Rad Bradford Assays, cat. #500-0205).
- Trifluoroacetic acid (TFA) (Sigma-Aldrich, cat. #91707).
- Acetonitrile (Fluka Analytical, cat. #34967).
- Formic acid (Sigma-Aldrich, cat. #F0507).
- RapiGest SF (Waters, cat. #186001861).
- Sodium Chloride (Synth, cat. #01C1060.01.AH).
- Potassium Chloride (Mallinckrodt, cat. #6858).
- Magnesium Sulfate Heptahydrate (Vetec, cat. #282).
- Sodium Phosphate Dibasic Dihydrate (Vetec, cat. #3309).
- Potassium Phosphate Monobasic (Synth, cat. #01F2002.01.AH).
- Calcium Chloride Dihydrate (Mallinckrodt, cat. #4160).
- Sodium Bicarbonate (Vetec, cat #306).
- Glucose (Vetec, cat #221).
- Bovine Serum Albumin (BSA) (Inlab, cat #1870).
- Collagenase Type V (Sigma-Aldrich, cat #C9263).
- Protease inhibitor: Cell lysis buffer (Cell Signalling, cat. #9803) with Phenylmethanesulfonyl Fluoride (PMSF).

Note: PMSF is an acutely toxic and corrosive compound. Toxic if swallowed; has a lethal dose of 50% of the population (LD-50 oral, mouse) of 200 mg/kg. It causes severe eye damage, including potential blindness. Causes severe skin burns, irritation, inflammation, and/or blistering.

- Protease inhibitor (Sigma-Aldrich, cat. #7626).

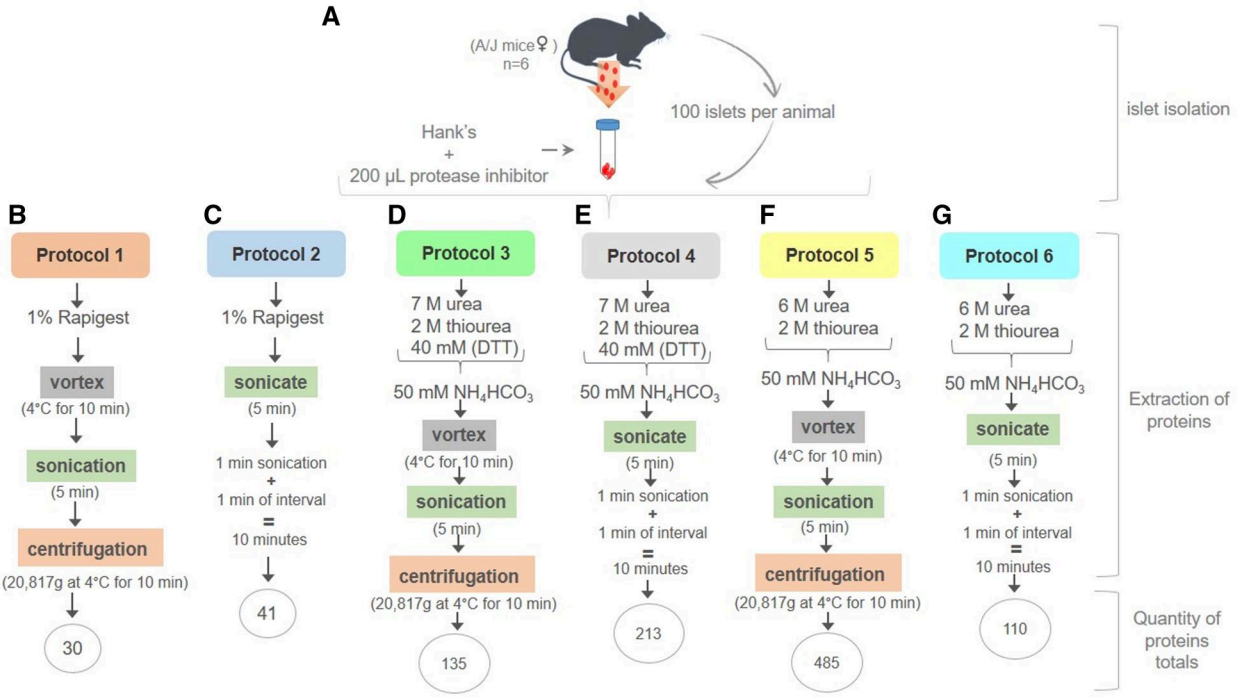

Figure 1. Experimental protocols tested for shotgun label-free proteomic analysis of pancreatic islets. (**A**) Isolation of islets from A/J female mice with Hank's solution and protease inhibitor. For protein extraction, the following protocols were evaluated, as displayed in the figure: (**B**) Protocol 1, (**C**) Protocol 2, (**D**) Protocol 3, (**E**) Protocol 4, (**F**) Protocol 5, and (**G**) Protocol 6, respectively. The numbers of proteins identified were 30, 41, 135, 213, 485, and 110 for protocols 1–6, respectively.

Equipment

- Axygen microcentrifuge tubes—Dnase and Rnase free (Axygen Scientific, cat. #311-03-051).
- Axygen pipette tips (Axygen Scientific, cat. #301-03-051).
- Pipettes (1000 µl; 200 µl; 10 µl; 2.5 µl) (Eppendorf Research Plus).
- Total Recovery Vial Kit (Waters, cat. #PTFE186000384C).
- Centrifuge (Eppendorf, model 5804 R).
- Vortex (Uniscience, model UNI-E0611).
- UltraCleaner 1600 A (Unique, model USC1600).
- Spin (Corning, Corning® LSE™ Mini Microcentrifuge, cat. # 6766).
- Balance (Mettler Toledo, model MS205DU).
- Dry water bath (Labnet International, model D1200-230V).
- Spectrophotometer (BioTek Synergy H1, microplate reader).
- Speedvac (Eppendorf—Vacufuge plus Vacuum Concentrator).
- Nano Liquid Chromatography Electron Spray Ionization Tandem Mass Spectrometer—nLC-ESI–MS/MS (Waters, model Xevo G2 Q-TOF).
- Falcon Amicon tubes (Amicon Ultra—15 Centrifugal Filter, Merck Millipore).
- Pierce C18 Spin columns (Thermo Scientific, cat. #89873), see https://assets.thermofisher.com/TFS-assets/LSG/manuals/MAN0011495_Pierce_C18_SpinCol_UG.pdf for use instructions.

Softwares

- ProteinLynx GlobalServer software (PLGS) version 3.03 (Waters Corporation).
- The download of the *Mus Musculus* database was obtained from the UniProt (Universal Protein Resource) catalog in June 2019 (http://www.uniprot.org).
- Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn).

Solutions

Hank's buffer solution

Wash and count the islets:

Hanks balanced saline solution (HBSS: 136.9 mM NaCl; 5.4 mM KCl; 0.81 mM $MgSO_47H_2O$; 0.34 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2.2H_2O$; 4.16 mM $NaHCO_3$; 0.06 mM glucose; and 15 mM BSA). Prepare HBSS in mixed gas (95% O_2 and 5% CO_2) per 10 min. Adjust to pH 7.4.

Isolate the islets:

HBSS (136.9 mM NaCl; 5.4 mM KCl; 0.81 mM $MgSO_47H_2O$; 0.34 mM $Na2HPO_4$; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2.2H_2O$; 4.16 mM $NaHCO_3$; 0.06 mM glucose; and 15 mM BSA, containing 0.1% Collagenase from *Clostridium histolyticum* Type V). Prepare HBSS in mixed gas (95% O_2 and 5% CO_2) per 10 min. Adjust to pH 7.4.

Lysis Buffer A

7 M urea, 2 M thiourea, and 40 mM DTT in 50 mM NH_4HCO_3, pH 7.8. Needs to be fresh. Prepare it on the day of the experiment.

Lysis Buffer B

6 M urea, 2 M thiourea in 50 mM NH_4HCO_3, pH 7.8. Needs to be fresh. Prepare it on the day of the experiment.

Resuspending solution

3% acetonitrile and 0.1% formic acid.

Mobile phase A

0.1% formic acid in water.

Mobile phase B

0.1% formic acid in acetonitrile.

Procedure

Islet samples preparation

1. Centrifuge the microtubes containing the islets in Hank's buffer containing protease inhibitor (10% v/v) for 5000 *g*, 4°C for 5 min.
2. After sediment formation, discard the supernatant and use the pellet for analysis.

Note: If no granule formation occurs, centrifuge again.

Optimized proteins extraction

3. Protocol 1: For the extraction of proteins, add RapiGest SF (Waters) at 1% v/v. Vortex the samples at 4°C for 10 min, sonicate for 5 min (under ice), and centrifuge for 20 817 g at 4°C for 10 min. Repeat this step twice more.
4. Protocol 2: For the extraction of proteins, add RapiGest SF (Waters) 1% v/v and sonicate the samples under the ice for 5 min (1 min of sonication and 1 min of interval), totaling 10 min.
5. Protocol 3: For the extraction of proteins, use the extraction solution containing 7 M urea, 2 M thiourea, and 40 mM DTT in 50 mM NH_4HCO_3, pH 7.8. Use an equal volume of sample and extraction solution (1:1 v/v). Vortex the samples at 4°C for 10 min, sonicate for 5 min (under ice), and centrifuge at 20 817 *g* at 4°C for 10 min. Repeat this step twice more.
6. Protocol 4: For the extraction of proteins, use the extraction solution containing 7 M urea, 2 M thiourea, and 40 mM DTT in 50 mM NH_4HCO_3, pH 7.8. Use an equal volume of sample and extraction solution (1:1 v/v). Sonicate the samples under the ice for 5 min (1 min of sonication and 1 min of interval), totaling 10 min.
7. Protocol 5: For the extraction of proteins, use the extraction solution containing 6 M urea, and 2 M thiourea prepared in 50 mM NH_4HCO_3, pH 7.8. Use an equal volume of sample and extraction solution (1:1 v/v). Vortex the samples at 4°C for 10 min, sonicate for 5 min (under ice), and centrifuge at 20 817 *g* at 4°C for 10 min. Repeat this step twice more.
8. Protocol 6: For the extraction of proteins, use the extraction solution containing 6 M urea, and 2 M thiourea prepared in 50 mM NH_4HCO_3, pH 7.8. Use an equal volume of sample and extraction solution (1:1 v/v). Sonicate the samples under the ice for 5 min (1 min of sonication and 1 min of interval), totaling 10 min.

Protein quantification and concentration

9. Remove an aliquot of 1 µl from each sample and perform protein quantification using the Bradford method [49] (Bio-Rad Bradford Assays, USA).
   Note: Photosensitive, so it is necessary to prepare in the absence of light.
10. After protein extraction, add a volume corresponding to 1.5× the sample volume of 50 mM NH_4HCO_3, pH 7.8 solution to the samples to dilute the urea and thiourea.
11. Transfer the samples to Falcon Amicon tubes (Amicon Ultra—15 Centrifugal Filter—Merck Millipore®, Tallagreen, Ireland), centrifuge at 4500 *g* at 4°C until a volume of approximately 150 µl is reached.

Enzymatic digestion of proteins (only for protocols 3 and 4)

12. Add 5 µl of RapiGest SF (Waters) and incubate at 37°C for 30 min.
    Protein reduction, alkylation, and digestion (this step was the same for protocols 1–6).
13. Reduce samples by adding 5 mM DTT followed by incubation for 40 min at 37°C.
    Note: Prepare with 50 mM NH_4HCO_3, pH 7.8.
14. Alkylate samples by adding 10 mM iodacetamide (IAA) and incubate in the dark for 30 min.
    Note: Prepare with 50 mM NH_4HCO_3, pH 7.8.
15. After this procedure, add 5 µl of 50 mM NH_4HCO_3, pH 7.8.
16. For protein digestion, add 2% (w/w) trypsin (Thermo Scientific, Rockford, USA, cat. #90057) and incubate for 840 min (14 h) at 37°C.
    Note: Trypsin was diluted in 50 mM NH_4HCO_3.
    Note: Trypsin should be thawed at the time of analysis and should be performed only under up-down movement (do not vortex).
    Peptides purification
17. After incubation, add 10 µl of 5% (v/v) TFA to stop the activity of trypsin.
18. Only for protocols 3 and 4, incubate at 37°C for 90 min.
19. Only for protocols 3 and 4, after 90 min, centrifuge at 20 817 *g* at 4°C for 30 min
20. Purify and desalinate the samples using C18 Spin columns (Thermo Scientific®, Rockford, Illinois, USA).
    Note: Follow exactly the manufacturer's instructions.
21. Dry the samples on Speedvac (Eppendorf—Vacufuge plus Vacuum Concentrator, Germany). Resuspend in a solution containing 3% Acetonitrile (ACN; v/v) and 0.1% formic acid (v/v) to each 50 µg protein, add 108 µl of the ACN/formic acid solution and 12 µl of the internal standard (enolase).

Acquisition of proteomic analysis using the nano liquid chromatography tandem mass spectrometry (nanoLC-ESI-MS/MS).

22. Use the Xevo G2 (Waters) mass spectrometer coupled to the nanoACQUITY (Waters) system for the peptide analysis. Inject all samples (1 µl each) in triplicate. The nanoACQUITY Ultra Performance Liquid Chromatography (UPLC) system should be equipped with a Trap Column 100Å, 5 µm, 180 µm × 200 mm) previously equilibrated with 99.9% phase A (0.1% formic acid in water) at a flow of 5 µl/min and an HSS T3 M-Class type column (analytical column; Acquity UPLC HSS T3 M-Class column 75 µm × 150mm; 1.8 µm) (Waters, Milford, MA, USA), previously equilibrated with 93% mobile phase A and mobile phase B (0.1% formic acid in ACN). Peptides should be separated by a linear gradient of 7–85% mobile phase B for 70min with 0.35 µl/min flow rate; the column temperature must be maintained at 45°C. The Xevo G2 Q-TOF mass spectrometer must be operated in positive nano-electrospray ion mode and data must be collected using the MSE method in elevated energy (19-45V). Source optimal conditions will include capillary voltage, 2.8kV; sample cone, 40V; extraction cone, 3.0V and source temperature 100°C. However, source conditions may vary due to detector and lockspray voltage setups. Data acquisition occurred over 70 min, and the scan range was 50–1500 Da. The lockspray was run with a [Glu1] fibrinopeptide solution (1pmol/µl) at a flow rate of 0.3 µl/min.
23. Use the PLGS version 3.03 software to process and search for continuous data with label-free liquid chromatography mass spectrometry in data-independent analysis mode ($LC–MS^E$).

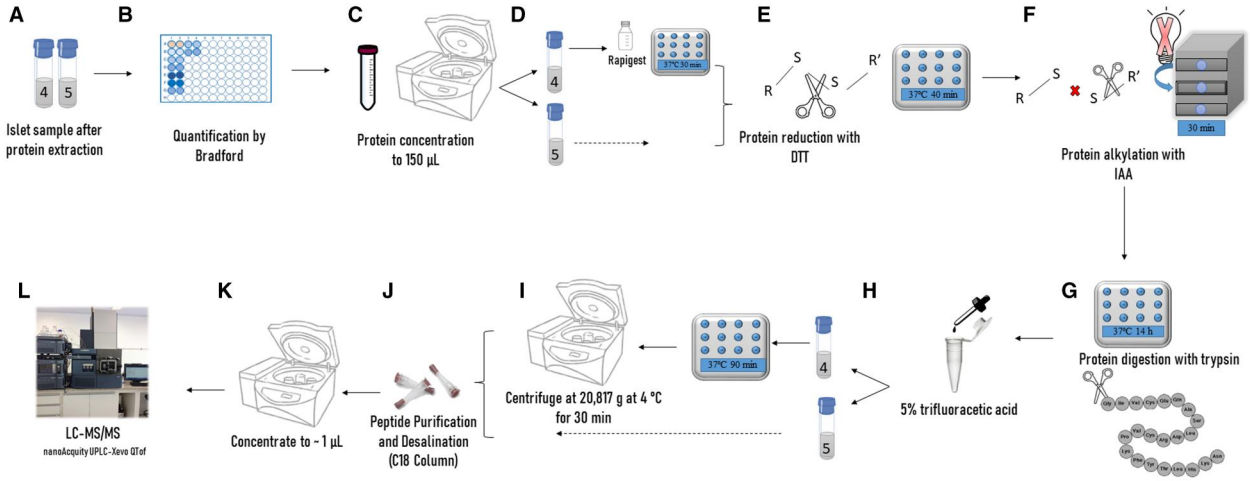

Figure 2. Steps involved in optimized protocols 4 and 5 for shotgun label-free proteomic analysis of pancreatic islets. (**A**) Islet sample after protein extraction, (**B**) quantification by Bradford, (**C**) protein concentration to 150 µl with Amicon (AmiconUltra—15 Centrifugal Filter—Merck Millipore®, Tallagreen, Ireland), (**D**) add Rapigest in sample number 4 and sample number 5 not add rapigest, (**E**) protein reduction with DTT, (**F**) protein alkylation with IAA in the absence of light, (**G**) protein digestion with trypsin, (**H**) stop action of trypsin with 5% trifluoroacetic acid, (**I**) sample number 4 should be incubated and then centrifuged to counteract the rapigest and sample number 5 not incubated and centrifuged, (**J**) peptide purification and desalination (C18 column), (**K**) concentrate to ~1µl to be later resuspended, and (**L**) mass spectrometer.

24. Identify the peptides using the software's ion counting algorithm and a search on the updated *Mus musculus* database (UniProtKB).

Figure 2 illustrates the steps involved in proteomic analysis for protocols 4 and 5 that showed the best results.

Timing

- Islet samples preparation •Timing ~5 min.
- Proteins extraction •Timing ~10 min for Protocols 2, 4, and 6; and ~75 min.
- Protein concentration (this step was the same for protocols 1–6) •Timing ~120 min.
- Enzymatic digestion of proteins (only for protocols 3 and 4) •Timing ~35 min.
- Protein reduction, alkylation, and digestion (this step was the same for protocols 1–6) •Timing ~910 min.
- Peptides purification •Timing ~180 min.
- Acquisition nanoLC-ESI-MS/MS •Timing ~1260 min (21 h).

Troubleshooting

Problems that were found in the protocol and were solved.

Step	Problem	Possible reason	Solution
1	Low protein concentration	Protein degradation	Add protease inhibitor in Hank's buffer
2	Absence of pellet	Centrifugation was not effective	Centrifuge again
3–8	Low protein concentration	Protein degradation	Sonicate under ice
9	Sample loss	Centrifuge Amicon more than necessary	Concentrate until 150 µl. Centrifuge for short periods, stopping from time to time
12 and 13	Unstable pH during reduction and alkylation	Preparation of DTT and IAA in deionized water	Prepare in 50 mM NH_4HCO_3, pH 7.8
9 and 14	Low peptide identification	High urea concentration in the sample	Dilute the sample using 50 mM NH_4HCO_3, pH 7.8 to reduce the urea concentration
15	Low peptide identification	Inefficient digestion	Do not vortex trypsin; perform only up-down movement with the aid of a pipette
18	Obstruction of the HPLC column	Suspended residues in the samples	Follow the protocol of the C18 column correctly

Results and discussion

One hundred islets were used for testing each protocol. After purification (step 18), the total amounts of protein obtained for the different protocols are displayed in Table 2.

In protocols 1 and 2, RapiGest™ SF was used in protein extraction, mirroring the approach used by Schmudlach *et al.* [47] in their proteomic analysis of the secretome of the pancreatic islets. This surfactant enhances the enzymatic digestion of proteins, aiding their solubilization and increasing their susceptibility to trypsin cleavage, without inhibiting enzyme activity [50–52]. However, among all the protocols, these yielded the lowest amount of proteins, suggesting that RapiGest™ SF is not well suited to protein extraction from the islets. This could be due to the high content of hydrophobic proteins in the pancreatic islets

[53], which might require a higher concentration of RapiGest™ SF.

A total of 1.014 proteins were identified in the islets across all protocols (Table 2). After identification, using the UNIPROT database, we excluded the unreviewed proteins (Supplementary Tables S1–S6), resulting in 756 proteins. Notably, the lowest numbers of identified proteins were obtained for protocols 1 and 2. Although the amount of protein obtained in protocols 1 and 2 (around 10 μg) should be adequate for protein identification, given that typically only 0.25–0.50 μg protein is injected using the column (HSS T3 column 75 μm × 150 mm; 1.8 μm), the use of RapiGest™ SF may have led to the exclusion of hydrophobic proteins, thereby reducing the number of identified proteins. The highest number of identified proteins was achieved with protocol 5, followed by protocol 4 (Table 2). In these protocols, a weaker chemical extraction is paired with a stronger mechanical extraction (protocol 5) or vice versa (protocol 4). Conversely, protocols employing either strong chemical and mechanical extraction (Protocol 3) or weak chemical and mechanical extraction (Protocol 6) also yielded fewer identified proteins (Table 2). These results indicate that both chemical and mechanical procedures are critical for the extraction of islet proteins, and a weak chemical extraction combined with a strong mechanical extraction leads to more favorable outcomes in terms of protein numbers identified. Another key difference to note between protocols 4 and 5 is that RapiGest™ SF was also used in protocol 4 after protein extraction and concentration. Additionally, following the addition of TFA to stop the action of trypsin, samples in protocol 4 were incubated at 37°C for 90 min, as per the manufacturer's instructions. It is possible that, after the addition of TFA, an acid degradation by-product might co-precipitate with highly hydrophobic peptides or proteins, which could have reduced the number of proteins identified when protocol 4 was employed (Table 3).

Table 2. Total amount of protein obtained for the different protocols evaluated.

Protocols	Total amount of protein (μg)
Protocol 1	10.6
Protocol 2	11.9
Protocol 3	21.1
Protocol 4	19.9
Protocol 5	19.2
Protocol 6	20.5

Note: Reading was done in duplicate.

Table 3. Numbers of reviewed (Swiss-Prot database), unreviewed (TrEMBL), and total proteins identified in the islets of A/J mice for the distinct extraction protocols evaluated.

Protocols	Reviewed	Unreviewed	Total
1	17	13	30
2	25	16	41
3	89	46	135
4	166	47	213
5	387	98	485
6	72	38	110

Figure 3 shows the number of proteins common to the different groups, as well as the number of proteins found in only one of the groups.

Some important proteins related to the islet function were found, but observed variations depended on the protocol used. This suggests that the extraction method may be influencing these differences among the groups. Beta enolase (P21550) was detected in protocols 1, 2, 3, 4, and 5, and gamma-enolase (P17183) was found in protocols 1, 3, 4, 5, and 6. These proteins play roles in the glycolytic pathway (UNIPROT) (Supplementary Tables S1–S5; Fig. 1). Proteins such as ATP synthase subunit beta_ mitochondrial (P56480), Pancreatic alpha-amylase (P00688), Endoplasmic reticulum chaperone BiP (P20029), Elongation factor 2 (P58252), 60S ribosomal protein L12 (P35979), 40S ribosomal protein S7 (P62082), and Alpha-amylase 1 (P00687) (Supplementary Tables S3–S6; Fig. 1) were only identified in the protocols 3, 4, 5, and 6, so extraction with RapiGest™ SF might possibly have excluded these proteins.

Twenty-three common proteins were identified across protocols 3, 4, and 5. However, only one is associated with the glycolytic pathway (Alpha-enolase; P17182), while the remainder primarily belongs to the histone family and/or are ribosomal proteins (Supplementary Tables S3–S5; Fig. 1).

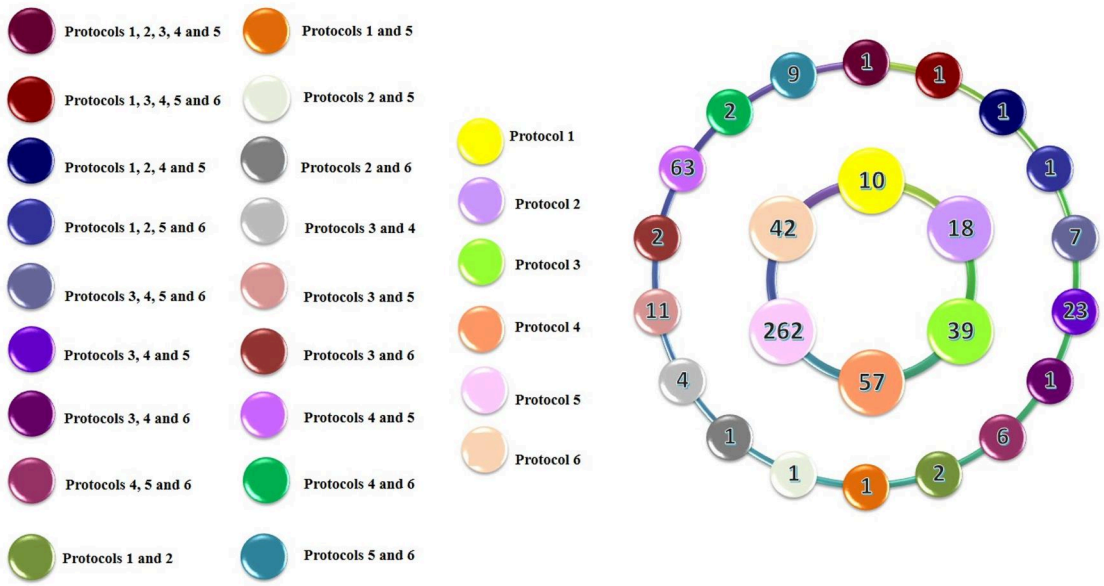

Figure 3. Organogram showing the number of proteins identified in the pancreatic islets in the different protocols of proteomics analysis.

Notably, a significant finding is that 63 common proteins were detected in both protocols 4 and 5. Among them are two key proteins secreted by the pancreatic islets: Glucagon (P55095) and Insulin-2 (P01326) [54]. Additionally, important proteins involved in energy flux were also identified under protocols 4 and 5, such as: Phosphoenolpyruvate carboxykinase [GTP]_ mitochondrial (Q8BH04), Glyceraldehyde-3-phosphate dehydrogenase (P16858), Protein ERGIC-53 (Q9D0F3), Pancreatic triacylglycerol lipase (Q6P8U6), and Cytochrome c oxidase subunit 5A_ mitochondrial (P12787) (Supplementary Tables S4 and S5; Fig. 1). As previously mentioned, the shared feature of protocols 4 and 5 is the integration of a strong with a weak extraction procedure (either chemical or mechanical). We hypothesize that this variation in intensity for these two protocols is crucial for a more effective protein extraction, thus enhancing not only the quantity but also the quality of identified proteins (Supplementary Tables S4 and S5; Fig. 1).

Author contributions

Juliana SanchesTrevizol (Conceptualization [equal], Data curation [equal], Methodology [equal], Project administration [equal], Resources [equal], Software [equal], Visualization [equal], Writing—original draft [equal]), Aline Dionizio (Data curation [supporting], Formal analysis [supporting], Methodology [equal], Writing—original draft [equal]), Aislan Quintiliano Delgado (Data curation [supporting], Methodology [supporting]), Talita Mendes Oliveira Ventura (Methodology [supporting]), Caroline Fernanda da Silva Ribeiro (Methodology [equal]), Nathalia Rabelo Buzalaf (Investigation [equal], Methodology [equal]), José Roberto Bosqueiro (Conceptualization [equal], Formal analysis [equal], Supervision [equal], Validation [equal]), and Marília Afonso Rabelo Buzalaf (Conceptualization [equal], Data curation [equal], Methodology [equal], Supervision [equal], Writing—original draft [equal]).

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

Conflict of interest statement

None declared.

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Data availability

All the data that are presented are included in the article materials or supplementary material and further inquiries can be directed to the corresponding author.

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