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Protein extraction and 2-DE of water- and lipid-soluble proteins from bovine pericardium, a low-cellularity tissue

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Abstract

Bovine pericardium (BP) is an important biomaterial used in the production of glutaraldehyde-fixed heart valves and tissue-engineering applications. The ability to perform proteomic analysis on BP is useful for a range of studies, including investigation of immune rejection after implantation. However, proteomic analysis of fibrous tissues such as BP is challenging due to their relative low-cellularity and abundance of extracellular matrix. A variety of methods for tissue treatment, protein extraction, and fractionation were investigated with the aim of producing high-quality 2-DE gels for both water- and lipid-soluble BP proteins. Extraction of water-soluble proteins with 3-(benzyltrimethylammonio)-propanesulfonate followed by *n*-dodecyl β -D-maltoside extraction and ethanol precipitation for lipid-soluble proteins provided the best combination of yield, spot number, and resolution on 2-DE gels (Protocol E2). ESI-quadrupole/ion trap or MALDI-TOF/TOF MS protein identifications were performed to confirm bovine origin and appropriate subcellular prefractionation of resolved proteins. Twenty-five unique, predominantly cytoplasmic bovine proteins were identified from the water-soluble fraction. Thirty-two unique, predominantly membrane bovine proteins were identified from the lipid-soluble fraction. These results demonstrated that the final protocol produced high-quality proteomic data from this important tissue for both cytoplasmic and membrane proteins.

Keywords

Biological scaffold; Bovine pericardium; 2-DE; Protein extraction

1 Introduction

Bovine pericardium (BP) is used in the fabrication of glutaraldehyde-fixed bioprosthetic heart valves [1–4] and may have an emerging application as an unfixed biological scaffold for tissue-engineering applications [5–8]. In the latter application, BP is treated by a process known as “decellularization” in an attempt to prevent immune rejection. [5–12]. Several groups have

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shown that the current generations of decellularized biological scaffolds are subject to immune-mediated rejection [1,13–17]. The fundamental concept of decellularization has thus come under scrutiny with the focus shifting away from removal of visible cells and toward identification and removal of antigenic proteins [11,12,18]. Any protein that differs in structure between the donor and recipient species is a potential antigen. As a result, the full spectrum of potential antigenic proteins when BP is implanted across species lines is not known [19–21]. In order to apply an immunoproteomic approach to identification of antigenic proteins in BP, a necessary first step is to generate high-quality 2-DE separations of BP proteins [22].

Development of a 2-DE protocol for BP presents difficulties due to its low-cellularity (high matrix-to-cell ratio) and relatively low abundance of soluble proteins compared with other tissues or organs [11,12,23]. The problem is exacerbated by highly abundant structural or matrix proteins that can overwhelm the remainder of the proteome [24–28]. Approaches to these problems in the proteomic analysis of other tissues have included prefractionation at the protein isolation stage or fractionation of the sample after protein extraction [24–29]. The analysis of lipid-soluble proteins in the tissue presents a further complication, as hydrophobic proteins in general are difficult to separate *via* 2-DE gels owing to their relative insolubility [30,31]. These difficulties make BP a challenging tissue from which to generate high-quality proteomics data [32].

Because both water- and lipid-soluble proteins from BP may have biologic interest, we were interested in methods for obtaining both fractions. The aim of this study is to develop and validate a protocol for extraction, prefractionation, and 2-DE separation of water- and lipid-soluble protein fractions from BP (Fig. 1). The methods reported here should have application for other low-cellularity high-matrix tissues.

2 Materials and methods

2.1 Tissue harvest

BP was harvested aseptically from adult cattle within 8 h of death. Animals showed no signs related to cardiovascular disease prior to death or evidence of cardiothoracic pathology at *postmortem*. BP was transported in pH 7.4 PBS, 0.1% w/v EDTA, 100 KIU/mL aprotinin, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. Pericardial fat and connective tissue were removed. Pericardium was sectioned into 1-cm squares (approximately 0.1–0.2 g), placed in separate 5-mL cryogenic vials with 85% DMEM and 15% DMSO, and stored at –70°C within 1 h of harvest.

2.2 Physical treatment of the tissue

Manual mincing, ultrasonication, and mechanical homogenization protocols were compared using water-soluble protein extraction solutions defined in Protocol A of Table 1. For manual treatment, samples of BP were snap frozen in liquid nitrogen, minced into pieces approximately 0.5–1 mm on a side using sterile surgical instruments, and placed in 1 mL of water-soluble extraction solution. For ultrasonic homogenization, minced samples of BP were subjected to an additional step of 2 min continuous ultrasonication at 30 kHz on ice (Artek Systems, Farmingdale, NY, USA). For mechanical homogenization, minced samples of BP were subjected to 2, 10, or 60 s of homogenization on ice at 15 000 rpm using a Powergen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). The remainder of the extraction protocol was performed as described in Section 2.3.

The potential for nucleic acid contamination was investigated by comparing physically processed samples with and without incubation with 2000 KIU/mL deoxyribonuclease I (Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/mL ribonuclease A (Sigma-Aldrich), prior

to 2-DE. Nucleic acid levels in samples were determined by measuring the 260/280 absorption ratio using a Gene-Quant II RNA/DNA calculator (Pharmacia Biotech, Buckinghamshire, UK) [33].

2.3 Protein extraction

A total of seven different extraction protocols were assessed. The standard extraction solution for all protocols contained 10 mM pH 8.0 Tris-HCl, 100 KIU/mL aprotinin, 1 mM DTT, 2 mM MgCl_2 , 10 mM KCl, and 0.5 mM Pefabloc (Sigma-Aldrich) in Nanopure water. Additives to this standard extraction solution were compared and are defined in Table 1.

Homogenized BP was placed in 1.5-mL cryogenic vials, containing 1 mL of a water-soluble extraction solution (Table 1). Tubes were shaken on ice for 1 h. Samples were centrifuged at 17 000g, 4°C for 25 min. The supernatant was collected and designated the water-soluble protein fraction (Fig. 1). The pellet was washed twice in 1 mL of water-soluble extraction solution by repeating the above extraction procedure. The supernatant from each wash was discarded. The remaining pellet was resuspended in 0.5 mL of various lipid-soluble extraction solutions (Tables 1 and 2) and shaken on ice for 2 h. Samples were centrifuged at 17 000g, 4°C for 25 min. The supernatant was designated the lipid-soluble protein fraction (Fig. 1).

The water-soluble fraction was concentrated with Centricon Ultracel YM-3 (cutoff 3000 Da) centrifugal filters (Millipore, Billerica, MA, USA) at 6500g, 4°C for 2h. The lipid-soluble fraction was concentrated with the same filters at 6500g, 4°C for 90 min. The final concentrated fractions were stored at -80°C until required (Fig. 1). Differences in protein yield between extraction protocols were determined by a one-way ANOVA. Values of $p < 0.05$ were considered significant. Where significant differences were found, *post hoc* analysis was performed using the Tukey-Kramer HSD test.

2.4 Protein precipitation

The effects of protein precipitation methods (TCA/acetone or ethanol precipitation) were investigated for the lipid-soluble fractions of Protocols A, E, and F (Fig. 1 and Table 2). TCA/acetone precipitation was performed using a modification of the technique described by Song *et al.* [28]. Briefly, lipid-soluble extracts were precipitated with three volumes of 10% w/v TCA in acetone. The sample was incubated at 4°C for 45 min and then centrifuged at 17 000g for 25 min at 4°C. The pellet was washed three times in ice-cold acetone, incubated for 45 min at 4°C, and centrifuged at 17 000g for 25 min at 4°C. The final pellet was air dried at room temperature and resuspended in one of several precipitate resuspension solutions detailed in Table 2.

Ethanol precipitation was performed as described previously [34]. Briefly, lipid-soluble extracts were precipitated with nine volumes of ice-cold 100% ethanol. Samples were incubated at 4°C for 60 min and then centrifuged at 17 000g for 25 min at 4°C. The supernatant was discarded. The pellet was air dried and resuspended in one of several precipitate resuspension solutions (Table 2).

2.5 Protein assays

Total protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (Sigma-Aldrich) was used to make standard solutions. Hydroxyproline content was determined using a modification of the procedure reported by Woessner [35]. Repeatability of both assays was within 1%.

2.6 2-DE

IEF was carried out using 18-cm pH 3–10 non-linear IPG ReadyStrips™ (Bio-Rad). For all samples, 100 µg of protein was loaded *per* gel. Three to nine replicate gels were performed for each protocol outlined in Tables 1 and 2. Samples were diluted (1:3 or greater dilution) with the rehydration buffer listed for each protocol (Tables 1 and 2) to a final volume of 408 µL. IEF rehydration was performed overnight using Immobiline™ DryStrip rehydration trays (GE Healthcare, Piscataway, NJ, USA). Protein IEF was performed using a Multiphor II electrophoresis system (GE Healthcare) at 20°C, with an initial 1 min linear increase in voltage to 500 V, followed by a linear increase in voltage to 3500 V over 5 h, and then a constant voltage of 3500 V for 17.5 h.

IPG strips were reduced by submersion in 2% w/v DTT, 6 M urea, 30% v/v glycerol, and 0.1% w/v SDS for 15 min and then alkylated by submersion in 2.5% iodoacetamide, 6 M urea, 30% v/v glycerol, 0.1% w/v SDS and a trace of bromophenol blue for 5 min. Strips were immediately loaded onto cast 12% polyacrylamide 2-D gels (18 cm × 20 cm × 1 mm) and electrophoresis performed at 40 mA *per* gel, 3000 V, 400 W for 3 h in a Protean II XL 2-D Multi-Cell (Bio-Rad) [36].

Gels were stained with a modified acidic silver staining protocol (Silver Stain PlusOne, AmershamPharmacia, Buckinghamshire, UK) [37,38]. Digital gel images were created using the UVP Bioimaging system. The number of gel spots was determined with ProteomWeaver software (Version 3.1, Definiens AG, Munich, Germany). Differences in spot number between protocols were compared with one-way ANOVA. Values of $p < 0.05$ were considered significant. Where significant differences were found, post hoc analysis was performed using the Tukey-Kramer HSD test.

2.7 Protein identification

To confirm the bovine origin of the proteins in the gel spots and to ascertain the effectiveness of the fractionation procedure (water-soluble *versus* lipid-soluble), a representative set of gel spots was selected for identification via mass spectrometry. A total of 75 spots, covering a range of *pI*, MW, and staining intensities, were excised from silver-stained 2-D gels generated using Protocol E2. Excised gel plugs were destained overnight with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, washed twice for 30 min in 200 mM ammonium bicarbonate (ABC) in 40% ACN, and dehydrated with 100% ACN for 5 min [39]. The supernatant was removed and gel pieces were dried using an SPD SpeedVac® (Thermo Electron, Waltham, MA, USA).

Tryptic digestion was accomplished by incubation of the gel spots with 40 µL of a 1 ng/µL solution of sequencing grade modified trypsin (Promega, Madison, WI, USA) in 40 mM ABC overnight at 37°C. Peptides were extracted from the gel spots by sequential incubation with 40 µL of 40 mM ABC for 15 min at room temperature, 5% formic acid for 15 min at 37°C, and 100% ACN for 15 min at 37°C [39]. The supernatant from the overnight tryptic digestion and those from all subsequent peptide extraction steps were collected and pooled. The pooled supernatants were dried using the Speedvac to a final volume of 5–10 µL. Peptides were desalted using ZipTip® C18 pipette tips (Millipore) prior to analysis by either ESI-quadrupole/ion trap (Q/Trap) or MALDI-TOF/TOF MS [40].

For ESI-Q/Trap analysis, extracted peptide samples were reconstituted in 10 µL of 0.5% formic acid with 2% ACN. Nanoflow liquid chromatography was carried out by an LC Packings UltiMate integrated capillary high-performance liquid chromatography system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA, USA). For each sample, 6.4 µL were injected using a Famos auto sampler onto a PepMap C18 trap column (5 µm, 300 µm 5 × mm,

Dionex) for on-line desalting and then separated on a PepMap C18 reverse-phase nanocolumn. Peptides eluted in a 15 min gradient of 5–40% ACN in 0.1% formic acid at 250 nL/min into a 4000 Q Trap (ABI/MDS Sciex, Mississauga, Ontario, Canada), a hybrid triple quadrupole linear ion trap mass spectrometer, that was equipped with a Micro Ion Spray Head II ion source. MS data acquisition was performed using Analyst 1.4.1 software (Applied Biosystems, Framingham, MA, USA) in positive ion mode for information-dependent acquisition analysis. The nanospray voltage was set to 2.0 kV for all experiments. Nitrogen was used as the curtain gas, set to 10, and as the collision gas, set to high, with a heated interface at 175°C. The declustering potential was set to 50 eV and Gas1 was set at 15 psi. After each survey scan between 400 and 1600 *m/z* and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for tandem MS (MS/MS) with rolling collision energy applied.

MS/MS spectra generated from ESI-Q/Trap analysis were interrogated using Mascot 2.2 (Matrix Science, London, UK) and searched against the mammal taxonomy of the NCBI database (downloaded July 2007). The search parameters were set to allow for one missed cleavage, two variable modifications (methionine oxidation and cysteine carboxyamidomethylation), a peptide tolerance of 1.2 Da, and an MS/MS tolerance of 0.6 Da. Only peptides defined by a Mascot probability analysis (www.matrixscience.com/help/scoring_help.html#PBM) to be better than “identity” were considered and used for protein identifications. For protein identifications based on a single peptide match, the identity score criterion was made more stringent by also requiring a confidence interval of $\geq 95\%$.

For MALDI-MS/MS, desalted digests were spotted onto target plates with 5 mg/mL of α -cyano-4-hydroxycinnamic acid and 1 mg/mL of ammonium phosphate in 50% ACN/0.1% TFA. MALDI-MS/MS was performed on a 4800 Proteomics Analyzer running v2.0 software (Applied Biosystems). MS was performed in positive ion reflector mode over the 800–4000 *m/z* mass range, with 1250 laser shots *per* spot and internal calibration. Up to four of the most intense peaks, excluding trypsin autolysis peaks, were selected from each MS spectrum for MS/MS analysis. Tandem MS was performed in positive ion mode with 4200 laser shots, 2 kV collision energy, air at 1E^{-6} torr as the collision gas, and default calibration. GPS Explorer (v3.6 Applied Biosystems) was used as an interface between the raw data from the mass spectrometer and a local copy of Mascot search engine (v2.1.04 Matrix Science). A combined MS and MS/MS search was performed against a local copy of NCBI nr (downloaded 8/15/06). Mascot searches were restricted to mammalian taxonomy with 50 ppm MS and 0.3 Da MS/MS mass tolerances, trypsin specificity allowing for one missed cleavage and the following three variable modifications: methionine oxidation and cysteine modifications by iodoacetamide and acrylamide. The criterion used to determine protein identification was a GPS Explorer protein score confidence interval $>99\%$ [36,41].

Cellular location (membrane, cytoplasmic, or nuclear) of identified proteins was determined by a combination of peer-reviewed literature search, NCBI protein database search [42], PSORT subcellular localization prediction [43], and prediction of trans-membrane protein segments using HMMTOP [42–44].

3 Results

3.1 Physical treatment of the tissue

The method of physical treatment of the tissue resulted in a significant effect on the yield and 2-DE gel quality for water-soluble proteins. Mechanical homogenization increased protein yield but resulted in smearing in the high MW, low pH region of the 2-DE gel, which worsened with increasing duration of homogenization. Based on nuclease digestion prior to 2-DE, and

260/280 nm absorption, nucleic acid contamination was not the source of the gel smearing. Similarly, hydroxyproline analysis of mechanically homogenized samples eliminated fragmented collagen as the cause of smearing. Mechanical homogenization was abandoned as a viable protocol due to poor spot number, intensity, and significant smearing. Manual mincing gave the best combination of yield, spot number, and resolution on 2-DE gels and was used for subsequent comparisons among protein extraction protocols.

3.2 Protein extraction

Protein extraction protocols (Table 1) were not different for total or water-soluble protein yield (Fig. 2). However, Protocol A (1.25% SDS) provided a significantly higher yield of lipid-soluble proteins (Fig. 2).

3.3 2-DE

For the water-soluble protein extracts, only protocols using 0.1% SDS (Protocols A, F, and G) or 134 mM 3-(benzyltrimethylammonio)-propanesulfonate (NDSB-256) (Protocol E) produced good-quality gels with a large number of well-defined spots. Other protocols produced gels with significant horizontal smearing, low spot number (mean <50 spots/gel), and/or poor spot resolution. The mean spot number *per* gel was 294.3 ± 48.3 for 0.1% SDS (Protocols A, F, and G) and 305.5 ± 30.4 for 134 mM NDSB-256 (Protocol E) and was not significantly different among these protocols ($p = 0.77$).

Owing to the previously reported success of chaotropic agents in extracting highly hydrophobic proteins [45], 7 M urea/2 M thiourea extraction of lipid-soluble proteins was assessed. However, this protocol resulted in horizontal smearing with no discernable spots (data not shown).

For the lipid-soluble extracts, only protocols incorporating 1% *n*-dodecyl β -D-maltoside (Protocols E, F, and G) or 1.25% SDS (Protocol A) yielded resolved spots. Extraction protocols with 1% *n*-dodecyl β -D-maltoside (Protocols E, F, and G) produced a higher number and better-resolved spots (248.8 ± 47.5 *per* gel) than protocols containing 1.25% SDS (Protocol A) (153.5 ± 21.9 spots/gel).

Spot number ($p = 0.02$) and combined spot intensity (subjective assessment) were less for lipid-soluble extracts compared with water-soluble extracts with equal protein loading. Owing to this finding, the effects of a precipitation step were assessed for the lipid-soluble fraction. No combination of precipitation solution, resuspension buffer, or running buffer (Protocols A1–A4 in Table 2) improved gels produced by 1.25% SDS lipid-soluble extraction. Only ethanol precipitation (Protocols E1, E2, F1, and F2 in Table 2) increased spot number and intensity for 1% *n*-dodecyl β -D-maltoside extractions. Protocol E2 yielded the highest number (472 ± 52.3 spots *per* gel) and best resolution of spots with the least amount of smearing (Figs. 3 and 4).

3.4 Protein identification

A total 75 proteins, from 2-DE gel spots generated using protein extraction Protocol E2, were identified and all were confirmed to be of bovine origin (Supporting Information Tables 1 and 2). Thirty-seven proteins were identified from water-soluble protein gels, yielding 25 unique proteins (Supporting Information Table 1). The subcellular location was confirmed or predicted to be cytoplasmic or secreted in 84% of these proteins [42, 46–49]. Thirty-eight protein identifications, representing 32 unique proteins, were obtained from lipid-soluble protein gels (Supporting Information Table 2). The subcellular location was confirmed or predicted to be nuclear or membrane in 66% of the proteins identified from lipid-soluble gels [42, 46–56].

4 Discussion

Fibrous tissues such as BP have a large extracellular matrix component and low-cellularity compared with other tissues. Type 1 collagen alone accounts for as much as 76% of the protein content of BP [16,23,57]. Proteomic analysis of fibrous and other tissues with high extracellular matrix content such as bone or cartilage can prove challenging, particularly when the proteins of interest are relatively low-abundance cellular proteins [24–28,58–60]. Because extracellular matrix proteins such as collagen are large relatively insoluble polymers, the problem they present is fundamentally different from tissues such as serum with highly abundant soluble proteins (*e.g.* albumin) [59]. Our approach was to minimize sample contamination with high-abundance extracellular matrix proteins by avoiding homogenization methods that could fragment and solubilize matrix proteins. In addition, the extraction solution composition was chosen to exploit differences in protein solubility between matrix and cellular proteins. Protein extraction methods and solutions are known to affect 2-DE gel spot number and resolution in ways that can be difficult to predict [27,28,61–63]. We thus adopted a systematic approach to protein extraction and fractionation with the ultimate endpoints of generating the best combination of yield, spot number, and resolution on 2-DE gels for both water- and lipid-soluble protein fractions (Fig. 1).

In this study, simple manual mincing of the tissue prior to protein extraction produced the best 2-DE gels. Both mechanical and ultrasonic homogenization methods introduced significant horizontal and vertical smearing in the high MW, low pH region of the gel. Such smearing has been variously reported to result from high ionic detergent concentration, high salt concentration, or contamination with nucleic acids, lipids, polysaccharides, proteoglycans, and glycosaminoglycans [58,64,65]. We eliminated nucleic acids, lipids, high ionic detergent, high salt concentration, and fragmented collagen as causes of the observed smearing, but ultimately did not determine the cause of the smearing. Because smearing could be prevented by avoiding aggressive methods of tissue homogenization, the cause was not pursued further.

Protein yields were predictably low compared with yields reported for more cellular tissues such as myocardium and skeletal muscle [59,61,62]. Yields were comparable to those reported for other relatively low-cellularity tissues and all protocols produced similar total protein yield, and we thus concluded that extraction efficiency was acceptable [62].

Several extraction protocols studied here were shown to be incompatible with subsequent 2-DE despite the fact that they have been used successfully for other tissues [25,27,61,64,65]. Since the extraction detergent was the only variable among these protocols, incomplete sample solubilization in the extraction buffer or poor solubility in the rehydration buffer were the most likely explanations for the poor 2-DE results with Protocols B, C, and D [65,66]. One extraction protocol (Protocol E, NDSB-256) produced clearly superior overall results for water-soluble proteins based on the combination of yield, spot number, and resolution on 2-D SDS-PAGE gels. NDSB-256 is a zwitterionic molecule that has previously been shown to be useful in increasing protein yield during extraction [65–67]. Twenty-five unique bovine proteins were identified from gels generated using this protocol. Eighty-four percent of these proteins were known or predicted to be cytoplasmic or secretory in origin, suggesting that prefractionation to the water-soluble fraction was appropriate [42,46–49].

Lipid-soluble proteins are difficult to display on 2-DE gels because of their relative insolubility in non-ionic or zwitterionic detergents and tendency to precipitate at pH values close to their isoelectric point [30,64,66]. Only protocols employing n-dodecyl β -D-maltoside in the extraction solution produced resolved gel spots with the lipid-soluble fraction. This non-ionic detergent has previously been used successfully for analysis of lipid-soluble proteins [30,31,65]. However, spot number ($p = 0.02$) and combined spot intensity (subjective assessment) were

less for lipid-soluble extracts compared with water-soluble extracts with equal protein loading. Contamination of samples with lipid has been shown to cause low spot number and intensity on 2-DE gels by forming protein-lipid complexes or protein precipitation during IEF [30,64, 66,68]. This possibility was investigated by performing a TCA/acetone or ethanol precipitation (delipidation) step after extraction [60]. Precipitation significantly increased the number of spots resolved, with one protocol (E2) producing gels with the highest number of spots. Thirty-two unique bovine proteins were identified from gels produced from Protocol E2. Sixty-four percent of these proteins were known or predicted to be membrane or nuclear in origin, suggesting that prefractionation to the lipid-soluble fraction was appropriate [42,46–55].

The GRAVY index values for proteins identified from the lipid-soluble fraction were not found to be significantly different from those for the water-soluble fraction. Previous reports have noted that not all membrane proteins are hydrophobic and thus may have a negative GRAVY index value [69,70]. Such proteins may contain only a single transmembrane domain with the remainder of the protein being hydrophilic [69,70]. Our findings suggest that GRAVY index may not be a reliable validation of appropriate fractionation of water- and lipid-soluble proteins in all cases.

In conclusion, we found that extraction of water- and lipid-soluble protein fractions is possible from relatively lowcellularity fibrous tissues such as BP. Protocol E2 produced the best combination of protein yield, spot number, and resolution of 2-DE gels. Protein yields and spot numbers for both water- and lipid-soluble fractions were comparable to those reported for other relatively low-cellularity tissues [60,62]. Other proteomic methodologies (*e.g.* shotgun, 16-BAC/SDS-PAGE, agarose gel IEF) may provide data complementary to those presented here. Such approaches may have particular merit in resolving additional membrane, soluble matrix, and high molecular weight proteins [69,71,72]. However, 2-DE remains a valuable method in proteomics, providing the ability to zoom in on small *pI* ranges and to focus efforts only on differentially expressed proteins [64]. The results presented here demonstrate that high-quality 2-DE gels can be generated and appropriate protein identifications made from both water-and lipid-soluble protein fractions. To our knowledge, the methods developed here are the first to be successfully applied to pericardium for 2-DE gel proteomic analysis. These methods should have application to other fibrous tissues such as tendon, ligament, fascia, and cardiac valves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ABC	ammonium bicarbonate
BP	bovine pericardium
NDSB-256	3-(benzyltrimethylammonio)-propanesulfonate
Q/Trap	quadrupole/ion trap

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Figure 1.
Schematic of the workflow involved in testing protocols for Tables 1 and 2.

Figure 2.

Protein yield for water- and lipid-soluble fractions and total protein yield for each extraction protocol. Error bars indicate one standard deviation. No significant differences between protocols were found in total or water-soluble protein yield. For the lipid-soluble fraction, protocols not connected by the same letter are significantly different.

Figure 3. Modified acidic silver-stained 2-DE gel of water-soluble proteins prepared with Protocol E2 (305.5 ± 30.4 spots *per* gel). Circled spots were excised and subjected to tryptic digestion, ESI-Q/Trap or MALDI-TOF/TOF MS/MS analysis, and database searching for protein identification (Supporting Information Table 1).

Figure 4. Modified acidic silver-stained 2-DE gel of lipid-soluble proteins prepared with Protocol E2 (472 ± 52.3 spots *per* gel). Circled spots were excised and subjected to tryptic digestion, ESI-Q/Trap or MALDI-TOF/TOF MS/MS analysis, and database searching for protein identification (Supporting Information Table 2).

Table 1Protein extraction protocols evaluated in this study^{a)}

Protocol	Water-soluble extraction solution	Lipid-soluble extraction solution	IEF rehydration buffer
A	0.1% SDS	1.25% SDS	A
B	0.4% CHAPS	4% CHAPS	A
C	0.01% Triton X-100	0.1% Triton X-100	A
D	0.005% sodium deoxycholate	0.05% sodium deoxycholate	A
E	134 mM NDSB-256	1% <i>n</i> -dodecyl β -D-maltoside	B
F	0.1% SDS	1% <i>n</i> -dodecyl β -D-maltoside	A
G	0.1% SDS	1% <i>n</i> -dodecyl β -D-maltoside	B

^{a)}The reagents listed in the second and third columns were added to the standard extraction solution (see Section 2.3). IEF rehydration buffer A consisted of 8 M urea, 2% CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL. IEF rehydration buffer B consisted of 8 M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% amidosulfobetaine-14, 2% Ampholyte 3-10NL.

Table 2

Subprotocols for lipid-soluble extraction precipitation experiments^{a)}

Protocol	Water-soluble extraction solution	Lipid-soluble extraction solution	Lipid-soluble fraction precipitation method	Precipitate resuspension solution	IEF rehydration buffer
A1	0.1% SDS	1.25% SDS	10% w/v TCA in acetone	1.25% SDS in standard solution	A
A2	0.1% SDS	1.25% SDS	100% ethanol	1.25% SDS in standard solution	A
A3	0.1% SDS	1.25% SDS	10% w/v TCA in acetone	9 M urea, 3% CHAPS, 1% DTT, 1% TritonB	
A4	0.1% SDS	1.25% SDS	100% ethanol	X-100, 1% NDSB-256	9 M urea, 3% CHAPS, 1% DTT, 1% TritonB
E1	134 mM NDSB-256	1% <i>n</i> -dodecyl β-D-maltoside	10% w/v TCA in acetone	9 M urea, 3% CHAPS, 1% DTT, 1% TritonB	
E2	134 mM NDSB-256	1% <i>n</i> -dodecyl β-D-maltoside	100% ethanol	X-100, 1% NDSB-256	9 M urea, 3% CHAPS, 1% DTT, 1% TritonB
F1	0.1% SDS	1% <i>n</i> -dodecyl β-D-maltoside	10% w/v TCA in acetone	X-100, 1% NDSB-256	9 M urea, 3% CHAPS, 1% DTT, 1% TritonB
F2	0.1% SDS	1% <i>n</i> -dodecyl β-D-maltoside	100% ethanol	9 M urea, 3% CHAPS, 1% DTT, 1% TritonB	

^{a)}The basic components added to the standard solution (see Section 2.3) for each of the water- and lipid-soluble extraction steps are shown for each protocol. The precipitation method, resuspension solution, and IEF rehydration buffer for each protocol are detailed. For IEF rehydration buffer composition, see Table 1.