# Removal of Abundant Myofilament Proteins From Rabbit Myocardium Using the ReadyPrep™ Protein Extraction Kit (Membrane I)

Melanie Y White[1,2] and Stuart J Cordwell[1], [1]Australian Proteome Analysis Facility, Macquarie University, Sydney, Australia 2109 and [2]Department of Medicine, The University of Sydney, Sydney, Australia 2006

## Introduction

The analysis of protein expression using the tools encompassed under the term "proteomics" is a powerful means of understanding how cells and tissues respond to changes in their environment. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) remains a technique of choice for separating complex mixtures of proteins (Görg et al. 2000, Rabilloud 2002), and is followed by high-resolution mass spectrometry (MS) for the identification and further characterization of specific proteins of interest. The major criticisms of 2-D PAGE are: 1) the poor representation of hydrophobic or membrane-associated proteins, and 2) the underrepresentation of lower-abundance proteins due to the dominance in gel patterns of abundant housekeeping proteins. The latter is especially apparent in samples where one or two individual protein species account for a significant fraction of the total protein present (for example, albumin in human plasma).

In recent times, the information that can be gained from 2-D PAGE experiments has been improved by targeting proteins from specific subcellular fractions, or those that can be enriched from among others in a complex mixture due to their physical, chemical, or functional properties. The ability to visualize lower-abundance proteins is now enabling detection of discrete changes in proteins that correlate with the onset of pathology. Targeting methods have included the specific isolation of proteins from membrane (Luche et al. 2003, Molloy 2000, Nouwens et al. 2000), nuclear, mitochondrial, and several other organellar fractions, as well as the use of prefractionating devices, based primarily on isoelectric focusing (IEF), to separate proteins into isoelectric point (pI) fractions (Davidsson and Nilsson 1999, Righetti et al. 2003, Shang et al. 2003). Additionally, several new methods have been described for the removal of abundant proteins from biological samples to enrich for lower-abundance proteins prior to 2-D PAGE. These have mainly been applied to the removal of abundant serum proteins, including serum albumin, IgG and IgA, transferrin, and α-1-antitrypsin, among others. Such strategies include Cibacron Blue affinity and immunoaffinity columns for the removal of specific proteins (Pieper et al. 2003, Steel et al. 2003). Where antibodies exist for other well-known abundant proteins (for example, RUBISCO in plants), such strategies can be applied to many different biological samples.

We have utilized the ReadyPrep protein extraction kit (membrane I) to isolate membrane-associated proteins from rabbit myocardial tissue. We have also determined that this method achieves the removal of abundant myofilament and contractile proteins into the insoluble pellet, allowing the visualization of lower-abundance proteins in the remaining fractions.

## Methods

### Isolation of Proteins From Rabbit Myocardium

For preparation of whole-tissue extracts, myocardial tissue (wet weight 300 mg) was homogenized using a tip-probe sonicator for 2 x 10 sec bursts in 3 volumes of ice-cold ReadyPrep sequential extraction kit reagent 3 (catalog #163-2104), supplemented with 65 mM dithiothreitol. The 2-D PAGE sample buffers were supplemented with phosphatase inhibitor (0.2% (v/v) okadaic acid), protease inhibitors (0.2% (v/v) protease cocktail inhibitor, Sigma), and endonuclease (300 U/ml).

Myocardial tissue (wet weight 300 mg) was also prefractionated using the ReadyPrep protein extraction kit (membrane I) (catalog #163-2088), which separates proteins via temperature-dependent phase partitioning in the presence of Triton X-114 (Santoni et al. 2000). The fractions derived from the aqueous and detergent-rich phases were treated using the ReadyPrep 2-D cleanup kit (catalog #163-2130). The protein-rich pellets generated from the cleanup kit, as well as the final insoluble pellet, were resolubilized in 1 ml of ice-cold ReadyPrep sequential extraction kit reagent 3.

### 2-D PAGE

Proteins were separated using ReadyStrip™ 24 cm IPG strips covering the pH range 3–10. A total of 250 µg protein was loaded on each strip and the strips were allowed to rehydrate passively overnight at room temperature. IEF was performed using a PROTEAN® IEF cell for a total of 120 kV-hr at 20°C. Focused IPG strips were kept at –80°C until required and then reduced, alkylated, and detergent exchanged in buffer containing 6 M urea, 5 mM tributylphosphine, 2% (v/v) SDS, 20% (v/v) glycerol, and 2.5% acrylamide monomer. Second-dimension separation was performed using 24 cm precast PROTEAN® Plus 12.5%T slab gels on a PROTEAN Plus Dodeca™ cell. Gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid for 1–2 hr and then stained overnight in SYPRO Ruby protein gel stain. Gels were destained in 10% (v/v) methanol, 7% (v/v) acetic acid for a minimum of 2 hr before counterstaining with colloidal Coomassie Blue G-250 and destaining in 1% (v/v) acetic acid (Cordwell 2002).

### Mass Spectrometry (MS)

The protein spots isolated by 2-D PAGE were excised from the gels and a hierarchical approach taken for identification by MS. Protein spots were excised from the gel using the ProteomeWorks™ Plus spot cutter and placed into a 96-well plate. Gel pieces were washed with a 60:40 mixture of 50 mM ammonium bicarbonate (pH 7.8)/acetonitrile for 1 hr at room temperature and then vacuum-dried for 25 min. Gel spots were rehydrated in 8 µl of tryptic digest solution (12 ng/µl sequencing grade modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate) at 4°C for 1 hr, excess trypsin removed, and the gel pieces suspended in 20–30 µl 50 mM ammonium bicarbonate, then incubated overnight at 37°C. Peptides eluted from the gel pieces were concentrated and desalted using ZipTip C18 pipet tips (Millipore, Bedford, MA). For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) peptide mass mapping, bound peptides were eluted from the ZipTip tips onto a target plate using 0.8 µl matrix solution (10 mg/ml α-cyano-hydroxycinnamic acid (Sigma)).

The first round of MS consisted of MALDI-TOF MS peptide mass mapping using either a PerSeptive Biosystems Voyager-DE STR workstation (Framingham, MA) or an Applied Biosystems 4700 MALDI-TOF/TOF system. Parameters for the database search were as follows: 0.05 atomic mass accuracy, one missed cleavage, and cysteine-acrylamide and methionine sulfoxide modifications allowed. The quality of matches was defined by the number of matching peptide masses and the percentage of protein sequence covered by those masses in comparison to other potential matches. Generally, a sequence coverage of approximately 25% was required for match confidence; however, protein fragments and high-mass proteins may be identified with significantly lower coverage yet a high number of matching peptides, while low-mass proteins may not be identified with significantly higher coverage and few matching peptides. Peptide mixtures were also analyzed by tandem electrospray-ionization mass spectrometry (ESI MS) using a Q-Tof hybrid quadrupole/orthogonal-acceleration TOF mass spectrometer (Waters). Nanoelectrospray needles containing the sample were mounted in the source and stable flow obtained using capillary voltages of 900–1,200 V. The mass:charge (m/z) value for each peptide within the tryptic digest was first determined with a precursor ion scan. Subsequently, MS/MS was performed for each m/z value to obtain sequence information using collision energies of 18–30 eV. Fragment ions (corresponding to the loss of amino acids from the precursor peptide) were recorded and processed using MassLynx version 3.4 software (Waters). Amino acid sequences were deduced by the mass differences between y- and b-ion ladder series using the MassSeq program (Waters) and confirmed by manual interpretation. Peptide sequences were then used to search the SWISS-PROT and NCBI databases using the program BLASTP search for short nearly exact matches (Altschul et al. 1990).

## Results and Discussion

A whole-tissue protein extraction from rabbit myocardial tissue was separated in two dimensions (Figure 1A). We also solubilized proteins from the myocardial tissue using the ReadyPrep protein extraction kit (membrane I). The kit generates three fractions: one from the aqueous phase (containing mainly cytoplasmic proteins), one from the detergent-rich phase (containing mainly membrane proteins with one or two transmembrane spanning regions), and an insoluble pellet (containing more complex membrane proteins and poorly soluble proteins). Proteins from each of these fractions were separated by 2-D PAGE (Figure 1B–D).

**Table 1. Identification of proteins isolated on 2-D gels from an insoluble pellet using the ReadyPrep protein extraction kit (membrane I).** Spot #, as shown in Figure 1D; Acc. Code, SWISS-PROT accession code for closest matching homolog; # Peptides, number of matching peptide masses; % Seq, % of total protein sequence covered by those matching peptides.

| Spot # | Acc. Code | Protein Identification | # Peptides | % Seq | pI* | MW* |
|---|---|---|---|---|---|---|
| 1 | TPCC_MOUSE | Troponin C | 5 | 31.1 | 4.0 | 18,420 |
| 2 | MLRV_HUMAN | Myosin light chain 2 | 18 | 64.8 | 4.9 | 18,658 |
| 3 | MLEV_HUMAN | Myosin light chain | 16 | 31.1 | 5.0 | 21,800 |
| 4 | TPM1_RABIT | Tropomyosin 1 α | 22 | 56.3 | 4.7 | 32,680 |
| 5 | TRT2_RABIT | Cardiac troponin T | 12 | 31.2 | 6.1 | 31,482 |
| 6 | ACTA_HUMAN | Chain 1: actin (aortic smooth muscle) | 13 | 35.7 | 5.2 | 41,774 |
| 7 | ATPB_HUMAN | ATP synthase β chain | 18 | 54.4 | 5.0 | 51,769 |
| 8 | AAC2_HUMAN | α-actinin 2 | 24 | 31.1 | 5.3 | 103,853 |

* Theoretical.

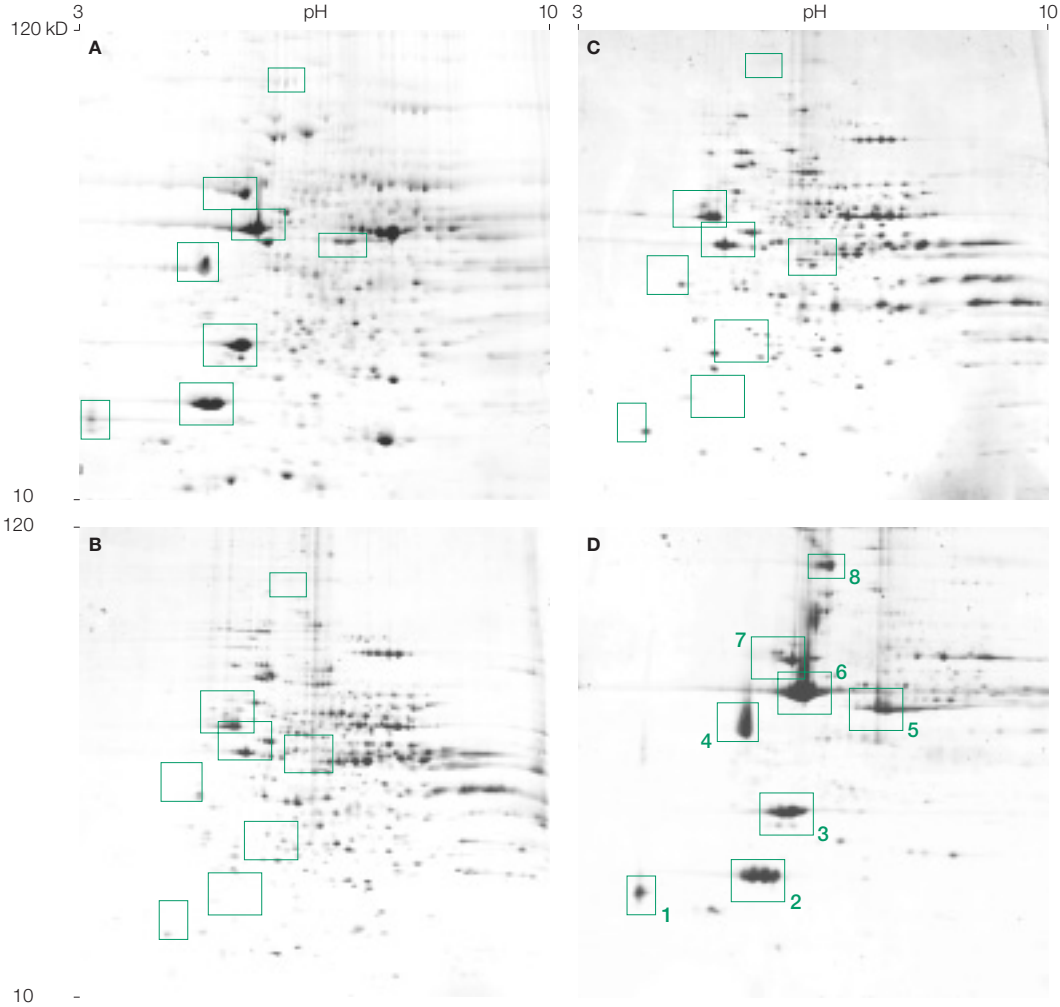


**Fig. 1. Separation of rabbit myocardial proteins.** A, whole-tissue proteins extracted using ReadyPrep sequential extraction kit reagent 3; B–D, proteins extracted following prefractionation using ReadyPrep protein extraction kit (membrane I). B, aqueous-phase proteins; C, detergent-rich-phase proteins; D, proteins from insoluble pellet. Numbers represent proteins identified by MS and shown in Table 1. Boxes represent the location of these proteins on the other 2-D gels.

Several abundant proteins with approximate molecular masses of 20, 25, 30, 35, 40, 50, and 100 kD dominated the overall spot pattern in the 2-D gel derived from whole-tissue solubilization, but were found only in the insoluble pellet fraction when tissue was prefractionated using the membrane I kit. These proteins were identified using a combination of MALDI-TOF and ESI MS (Figure 1 and Table 1). Seven of the eight abundant proteins that were extracted into the insoluble pellet were identified as components of the muscle myofilament. The myofilament complex is made up of thick filaments (myosin heavy chain and the regulatory proteins myosin light chains 1 and 2) and thin filaments (double helix of actin monomers, the troponin complex, and a continuous strand of tropomyosin). The troponin complex consists of three subunits (TnI, TnC, and TnT). Of the proteins that make up the myofilament, only myosin heavy chain and TnI could not be found in the insoluble pellet fraction. Myosin heavy chain is a 220 kD protein that is most likely too large to enter either the IPG strip or the 12.5%T SDS-PAGE slab gel, while TnI is a basic protein with an approximate pI of 10.0, and hence may be too alkaline to be visualized here.

The removal of these abundant myofilament-associated proteins allowed a relatively higher concentration of protein sample to be applied to the 2-D PAGE gels for each of the aqueous and detergent-rich fractions and hence allowed the visualization of lower-abundance proteins. We saw very little contamination derived from the myofilament proteins in these fractions (Figure 1).

Prefractionation in proteomics studies is essential for providing more information about a particular tissue or cell type. Such fractionation is routinely based on subcellular localization or physicochemical properties of individual proteins or protein functional groups. The ReadyPrep protein extraction kit (membrane I) is routinely used to separate fractions of proteins based on their relative solubilities in Triton X-114 and therefore their transmembrane-spanning regions. We have determined that for rabbit heart tissue (and most likely other samples containing contractile proteins), this kit is capable of selectively removing high-abundance proteins associated with the muscle myofilament, thus allowing the visualization of lower-abundance proteins in the remaining fractions, which may be important for determining subtle modifications to the myocardial proteome that occur during various stages of pathology.

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