Simultaneous Quantification of Human Cardiac α- and β-Myosin Heavy Chain Proteins by MALDI-TOF Mass Spectrometry

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ABSTRACT

We have developed a novel method for quantifying protein isoforms, both in relative and absolute terms, based on MALDI-TOF mass spectrometry (MS). The utility of the

approach is demonstrated by quantifying the α and β protein isoforms of myosin heavy chain (MyHC) in human atrial tissue. α-MyHC (726-741) and β-MyHC (724-739) were identified as isoform specific tryptic peptides. A calibration curve was constructed by plotting ion current ratios against molar ratios of the two peptides prepared synthetically. MyHC was digested by trypsin and the ion current ratio determined for the two tryptic peptides. The ion current ratio was converted to the peptide ratio and hence the isoform ratio by reference to the standard curve. The accuracy of the method was confirmed by a comparison between these results and those determined by an established method of MyHC isoform ratio determination. So that the molar ratio could be converted to absolute values, a third peptide, an analog of the two peptides being measured, was synthesized for use as an internal standard (IS). The measured ion current ratios of synthetic α-MyHC (726-741), β-MyHC (724-739), and IS peptides were used to generate standard curves. A known quantity of the IS was added to the MyHC digests. The measured ion current ratios were converted to the actual quantities of the isoform specific peptides and hence the actual quantity of each protein isoform by reference to the standard curves. This method is of general applicability, especially when isoform quantification is required.

Keywords: MALDI-TOF MS, quantification, protein isoforms

INTRODUCTION

The sensitive, accurate and precise quantification of a particular protein in a complex biological sample can be challenging. Most existing methods rely on access to

an immunological reagent (antibody) that binds specifically with the target compound. This reagent can be employed in Western blots for semi-quantitative analysis, or in radio-immunoassays (RIAs) or ELISA assays for more accurate quantitative analysis. A specific antibody is not always available, and in these instances it must be prepared in what can be an expensive and time-consuming process. Further, the specificity of the reagent can be limiting in some situations, especially when closely related isoforms are measured. Alternatively, and in the absence of an antibody, relative protein levels can be determined following separation by 2D gel electrophoresis, staining and densitometry. This approach is typically used in the field of proteomics, and the proteins are subsequently identified by peptide mass fingerprinting. The stained-gel densitometry method is relatively imprecise and it is essentially impossible to determine absolute quantities and this approach is not useful for closely related isoforms that migrate to the same position on the gel.

Two closely related isoforms of myosin heavy chain (MyHC), α and β, are expressed in the human heart and changes in the ratio of their mRNAs have been implicated in human heart failure[1, 2] and a positive response to therapy[3]. A method to measure the protein isoforms is needed because there is not a precise correlation between mRNA and protein.[4-7] Previous attempts to quantify the proteins using immunologic reagents[8-10] or electrophoresis[11, 12] have produced inconsistent results. We have therefore developed a MALDI-TOF MS based method to measure the relative and absolute amounts of α- and β-MyHC.

MALDI-TOF MS has been shown to have considerable potential as a quantitative tool[13-19] and compares favorably with other quantitative MS techniques.[20, 21] However, its

utility for the direct quantification of large intact proteins is limited because of the observed peak width, which compromises specificity and reduces sensitivity. We are interested in accurate, precise and sensitive methods for protein quantification that are especially applicable to the independent assessment of closely related isoforms, and that are not bound by the limitations of antibody-based procedures. Here we describe a method of protein quantification, based on MALDI-TOF MS, that meets these demanding requirements. The approach relies on the generation of tryptic fragments of the two intact protein isoforms and determination of separate ion currents derived from peptides specific to each isoform. Given that the ionization efficiencies of the two peptides are comparable, then the ratio of the ion currents is in direct relation to the relative amounts of the two peptides, and hence the relative amounts of the protein isoforms. The accuracy of the technique has been confirmed by comparing the MS determinations with those derived by an established alternative approach. Relative amounts of the two isoforms can be converted to absolute values by incorporating an internal standard that is a synthetic peptide analog of the protein-specific peptides. Stable isotope labeled peptide analogs have been used in HPLC-ESI/MS to quantify specific tryptic peptides[22-24] and we now show that a similar approach is feasible with MALDI-TOF MS. A known amount of the internal standard is added to the protein digests and from the MALDI-TOF MS ion current ratio the amount of the protein specific peptide can be calculated using a standard curve (protein specific peptide to internal standard peptide). We have shown that the protein determinations are linear with sample load and consistent with the expected amount of myosin heavy chain.

EXPERIMENTAL SECTION

Preparation of MyHC from tissue. For these studies seven archived patient samples of normal human right atrium were provided by the Donor Alliance Organ Recovery System from organ donor candidates whose hearts could not be placed. Normal human right atrium expresses substantial amounts of both α- and β-MyHC. Myofibrillar protein extracts were prepared from the tissue[11, 25] and assayed for protein concentration (Bio-Rad Protein Assay, Bio-Rad, CA). Aliquots containing 0.15 µg total protein were electrophoresed in triplicate on large format gels by the method of Reiser,[12, 26] then silver stained. For MS analysis, aliquots containing 3 µg total protein were electrophoresed in duplicate on NuPage Bis-Tris mini-gels and stained with colloidal Coomassie (Invitrogen). For determinations of assay linearity, aliquots of 0, 1, 2, 3, and 4 µg of protein were electrophoresed. Images of silver stained and colloidal Coomassie stained gels were captured on a PowerLook II scanner (UMAX) and analyzed by densitometry.

Preparation of MyHC peptides for MALDI-TOF MS. The MyHC band was excised from the Coomassie stained gels and placed in glass vials (0.3 ml) with Teflon caps (Alltech) in which all further processing was done. The glass vials had been washed with soap, rinsed with water, soaked in 10% TFA, extensively rinsed with resistivity >18 MΩ water, and dried prior to use. The gel pieces were washed twice with 50% acetonitrile (CH_3CN)/ 25 mM ammonium bicarbonate, once with 100% CH_3CN and dried in a vacuum centrifuge (Centrivap Concentrator, Labconco). The dried gel pieces were rehydrated with 20 µl of 50 mM ammonium bicarbonate, pH 8.0, containing 400 ng of sequencing grade trypsin (Promega) and incubated overnight at 37°C. A second aliquot of trypsin was added and the samples were again incubated overnight at 37°C. Tryptic

peptides were extracted by adding 200 µl of 50% CH_3CN/0.1% trifluoroacetic acid (TFA) and shaking vigorously for 4 hours. For absolute quantification a carefully measured aliquot containing 2 pmol of the internal standard peptide was added at this step to be carried through all further processing. The gel pieces were removed and the extract was taken to dryness in a vacuum centrifuge and resolubilized by adding 20 µl of 0.1% TFA and incubating overnight with gentle shaking. A C18 ZipTip (0.6 µl bed, Millipore), was wetted twice with 20 µl of 50% CH_3CN/0.1% TFA and equilibrated twice with 20 µl of 0.1% TFA. The resolubilized peptide extract was bound to the ZipTip by pipetting ten times through the bed. Three 20 µl aliquots of 0.1% TFA were pipetted through the bed to elute contaminants. The peptides were eluted into a second 0.3 ml glass vial containing 2 µl of 80% CH_3CN/0.1% TFA by pipetting this solution five times through the bed. The entire sample (2 µl) was spotted onto a stainless steel MALDI-TOF MS plate along with 1 µl of matrix solution. The matrix solution consisted of recrystallized α-cyano-4-hydroxy cinnamic acid (CHCA) dissolved in 80% CH_3CN/0.1% TFA at a concentration of 10 mg/ml. The peptide and matrix mixture was allowed to air dry and subjected to MALDI-TOF MS.

Preparation of peptide standards for MALDI-TOF MS. Peptide standards consisted of α-MyHC (726-741), β-MyHC (724-739), and the internal standard (IS) peptide (Figure 2). These peptides were synthesized at the Molecular Resources Center of the National Jewish Hospital of Denver. The peptides were purified by 2 rounds of reverse phase HPLC using very shallow CH_3CN gradients to optimize purity. The masses were verified by MALDI-TOF MS and ESI-TOF MS and no contaminants were detected. Stock solutions of each peptide at approximately 0.4 mM were prepared in 5% CH_3CN to

prevent adsorption to glass vials and plastic pipet tips. Stock solutions and dilutions were always prepared in 5% CH_3CN in glass vials that had been cleaned as previously described. Glass vials were used because the peptides, especially at high dilution, bind to plastic vials reducing their concentration in solution. The exact concentrations of the stock solutions were determined by amino acid analysis of Asx, Glx, Pro, Gly, Ala, Val, Ile, Leu, and Phe using a Beckman 6300 High Performance Amino Acid Analyzer (triplicate analyses). Peptide mixtures for standard curves were spotted in 2 µl of 80% CH_3CN/0.1% TFA with 1 µl of matrix solution.

Acquisition of MALDI-TOF MS spectra and data analysis. All spectra were acquired on a Voyager-DE PRO mass spectrometer (Applied Biosystems) operating in reflector mode. This provides the highest mass resolution so that the signal from the peptides of interest would not be subject to interference from other components of the complex protein digests. A mixture of angiotensin I, glu1-fibrino-peptide B, and ACTH (18-39) in matrix was spotted adjacent to all samples and was used for external mass calibration. Data were accumulated over the limited mass window of *m/z* 1000-2500. All samples were prepared in duplicate and spotted, and spectra were acquired from five different regions of each spot to give 10 spectra for each sample. There were 10 spectra for each point on the standard curves, 10 spectra for each atrial MyHC sample, and 10 spectra for each atrial MyHC sample supplemented with IS peptide. Each spectrum was the result of averaging 100 separate laser shots. The laser power was carefully monitored so as to be high enough to have a good signal/noise ratio but low enough so as not to saturate the detector. (Excessive laser power resulted in a nonlinear response to higher concentrations of peptides.) All spectra from peptide standards and protein digests were

processed in the same manner. A macro was generated in the instrument DataExplorer (Applied Biosystems) software to truncate the spectra to an *m/z* range of 1735 to 1780, apply a noise filter with a correlation factor of 0.7, and baseline correct the spectra. The mass peak list was then exported and processed by a program written in the Java computer language.

The program identified the monoisotopic peak and the corresponding major isotope peak of α-MyHC (726-741), β-MyHC (724-739), and the IS peptide. This was done by searching the list of centroid masses for the values closest to the calculated masses of these peaks. An error limit of 0.5 Daltons was permitted because spectra were externally calibrated. Correct peak identification was verified by inspection of the spectra. The program extracted the peak height intensity data for the monoisotopic peak and the corresponding major isotope peak of each peptide. These were summed to give the ion current for the peptide of interest. The peak height intensities were found to be more reproducible than peak areas as has been previously shown.[19] (The peak area measurements were compromised by the unstable baseline characteristic of the MALDI process.) Across the mass range of these peptides the monoisotopic peak and the corresponding major isotope peak are of a similar intensity (Figure 3) so both were summed for ion current determinations. Other members of the isotope series were of much lower relative abundance so they were not incorporated in the calculations. Within the program, the peptide ion currents of each spectrum were converted to ion current ratios, which were used for regression analysis of standard curves and for the conversion of patient sample ion current ratios to peptide ratios and amounts by reference to the standard curves.

RESULTS AND DISCUSSION

A. Measuring Protein Isoform Ratios by MALDI-TOF MS.

Selection of isoform specific quantification peptides. The presence of two isoforms in the MyHC gel band from Coomassie stained NuPage gels was confirmed by peptide mass fingerprinting. While approximately three quarters of the peptides matched both α- and β-MyHC, the remaining peptides were specific to one or the other isoform. This confirmed that the band contained a mixture of both isoforms. The sequences of α- and β-MyHC were examined to find a pair of tryptic peptides, one from each isoform, which would be suitable for MALDI-TOF MS quantification. Suitable peptides should be similar in sequence, be discriminated by mass, and should generate a strong MALDI-TOF ion current. Ideally the peptides should result from the cleavage of trypsin sites that are homologous between the isoforms so that one isoform is not cleaved in preference to the other isoform. It is important that the peptides be similar in sequence so that their recovery, crystallization with matrix, and ionization by MALDI would be equivalent. These requirements are readily achieved by a single conservative amino acid substitution. (Note, leucine for isoleucine was excluded because their masses are identical.) A search of the sequences revealed approximately ten pairs of tryptic peptides fitting these criteria. Inspection of the spectra revealed that one of these pairs gave a very strong ion current (Figure 1). The top panel shows a spectrum of a sample that is predominantly α-MyHC; the bottom sample is predominantly β-MyHC. The strongest signals in these spectra are α-MyHC (726-741), monoisotopic mass of 1768.96, and β-MyHC (724-739),

monoisotopic mass of 1740.93, and the sequences of these peptides and their flanking tryptic sites are shown in Figure 2.

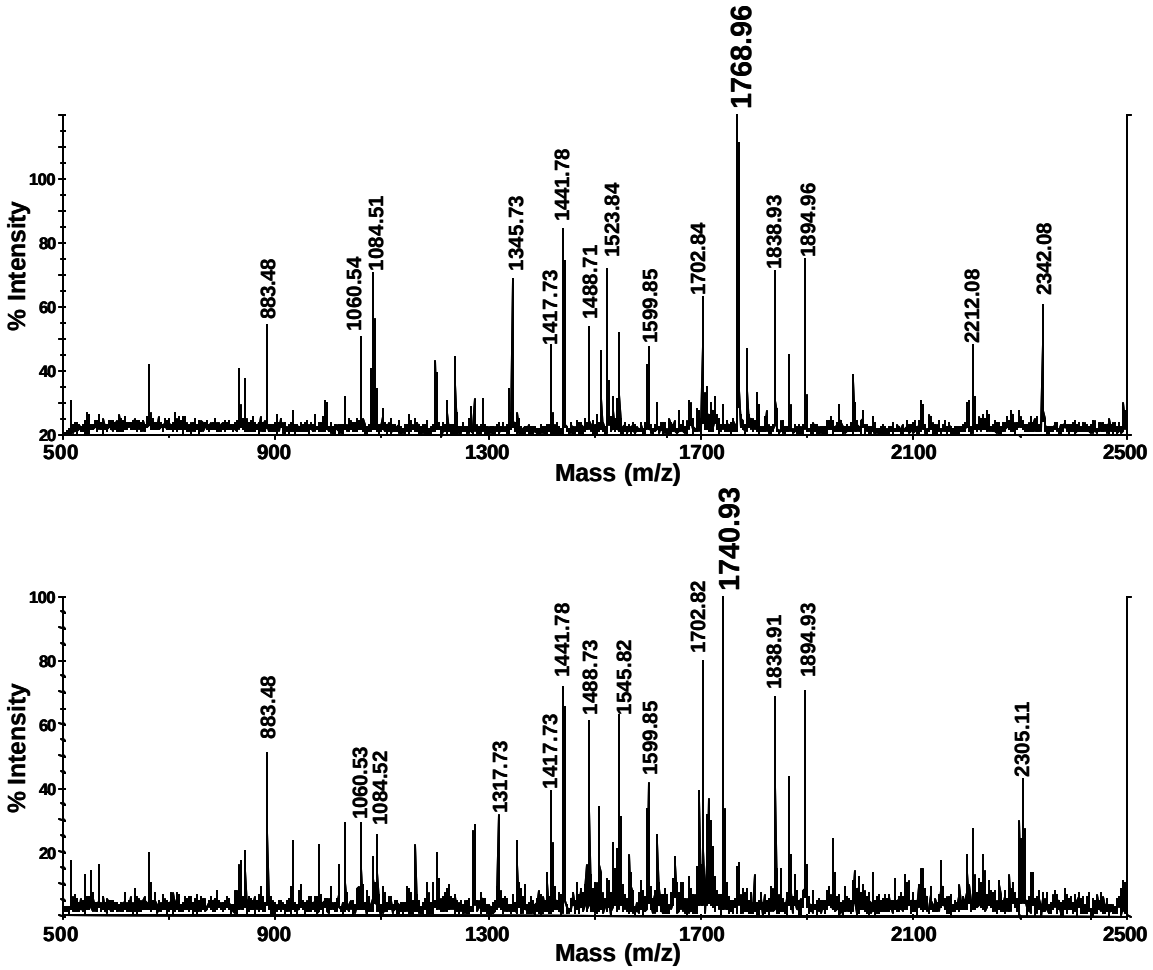

Figure 1. MALDI-TOF MS Spectra of Human Atrial MyHC. MyHC was prepared and subjected to MALDI-TOF MS using CHCA matrix as described in the experimental section. The peptide masses were used to search the SwissProt database with the MSFit program. The top panel was matched with 80 peptides and 42% sequence coverage to α-MyHC while the bottom panel was matched with 77 peptides and 36% sequence coverage to β-MyHC. In each case there were unmatched peptides that corresponded to the other isoform indicating that both samples were mixtures of the isoforms.

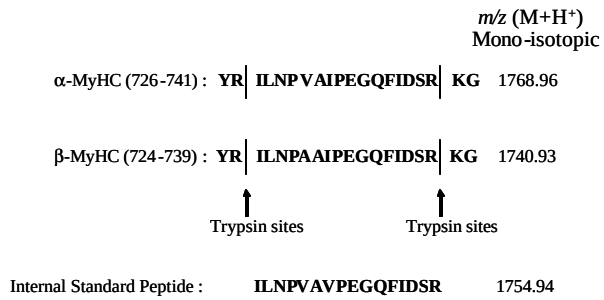

Figure 2. Peptides selected for MyHC quantification. The sequences of α-MyHC (726-741) and β-MyHC (724-739) and their surrounding tryptic cleavage sites are shown above. Also shown is an internal standard peptide that was designed to be highly homologous, but have a unique mass not found in either MyHC spectra. Amino acid residues that differ among the peptides are underlined.

Preparation of MyHC peptides for MALDI-TOF MS. Methods to quantify proteins by quantifying tryptic peptides rely on the efficient and essentially complete digestion of the target proteins so that the moles of peptide produced are equivalent to the moles of protein.[15, 22-24] When available, pure target proteins can be used as standards[15, 22] but when pure target protein standards are not available, conditions are used which give essentially complete digestion with a surrogate protein[23] or precursor peptide.[24] We could not rigorously validate our measurements by performing standard addition experiments because purified α-MyHC and β-MyHC were not available to us. The endogenous isoforms can not be resolved by conventional techniques and intact MyHC is very difficult to express.[27] Therefore we used conditions which are similar to those reported by others to give essentially complete digestion.[22-24] These included a large amount of trypsin (400 ng) relative to the amount of substrate (i.e., about 1 μg MyHC) and two rounds of digestion. When results from two rounds of trypsin digestion were compared to

those from a single round there was no additional production of peptides. (Data not shown) This indicates that the digestion had proceeded as far as possible with one round. However, two rounds were routinely employed to ensure maximum production of the desired tryptic peptides. A large volume of solvent (50% CH_3CN/0.1% TFA, 200 µl) was used to optimally extract peptides from gel pieces (vigorous shaking, 4 hours). Re-extracting gel pieces with a second aliquot of 50% CH_3CN/0.1% TFA did not yield any detectable peptides, indicating that the extraction was as complete as possible. (Data not shown) These experiments indicate that the conditions employed generate a reproducible sampling of peptides from the MyHC isoforms.

It was found that glass vials gave more reproducible preparations of tryptic peptides than plastic vials. Plastic vials tended to release components that interfered with matrix crystallization when they were exposed to solvent (50% CH_3CN/0.1% TFA, 200 µl, 4 hrs.) and the entire volume was concentrated and applied to a MALDI target. The interference was found to increase with larger solvent volumes, higher percentages of CH_3CN, and increasing extraction times. Our plastic vials may not have been of sufficient quality but we did not extensively test vials from various manufacturers. There were no interfering components extracted from glass, but these vials had to be washed with 10% TFA and rinsed with resistivity >18 MΩ water to remove sodium and potassium ions. Alkali metal ions caused adduct formation in the spectra. Extracts dried down in glass vials required gentle shaking overnight in 0.1 % TFA for complete solubilization. Clean-up with a C18 ZipTip (Millipore) was important to remove contaminants from the gel pieces that interfered with matrix crystallization. A sample of

MyHC from a normal human atrium was prepared and a narrow MS window containing the α- and β-MyHC quantification peptides is shown in Figure 3A.

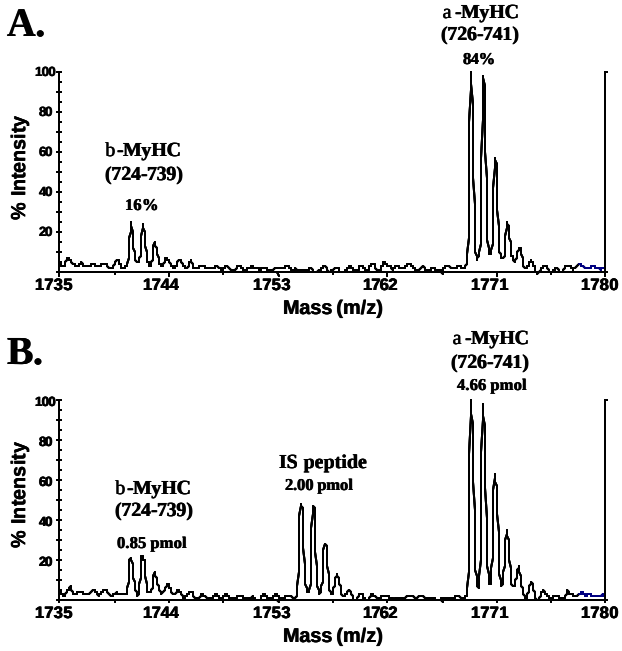

Figure 3. MALDI-TOF mass spectra of the quantification peptides. **A.** A relative quantification spectrum is shown from a human atrial sample (patient 1). The ratio of α-MyHC (726-741) to β-MyHC (724-739) was determined by reference to the standard curve (Figure 4). **B.** An absolute quantification spectrum is shown from a replica sample containing 2 pmol of the IS peptide. The pmol values of α-MyHC (726-741) and β-MyHC (724-739) were determined by reference to the standard curves (Figure 6).

Generation of the relative quantification standard curve. The standard curve for the relative isoform measurements was constructed using mixtures of synthetic α-MyHC (726-741) and β-MyHC (724-739) with a total of 4 pmol peptide (Figure 4). The pmol α-MyHC (726-741) was divided by the sum of the pmol α-MyHC (726-741) and pmol β-MyHC (724-739), and expressed as a percentage: the % α-MyHC (726-741) The ion

current derived from α-MyHC (726-741) was divided by the sum of the ion currents of α-MyHC (726-741) and β-MyHC (724-739), and this was converted to a percentage: the % α-MyHC (726-741) ion current. The % α-MyHC (726-741) ion current was plotted against the % α-MyHC (726-741) to generate the standard curve (Figure 4). Linear regression analysis utilized all ten values at each point to derive a line for the standard curve. Higher order analysis did not significantly improve the curve fit. This plot indicates that the ion current ratio is directly proportional to the peptide ratio.

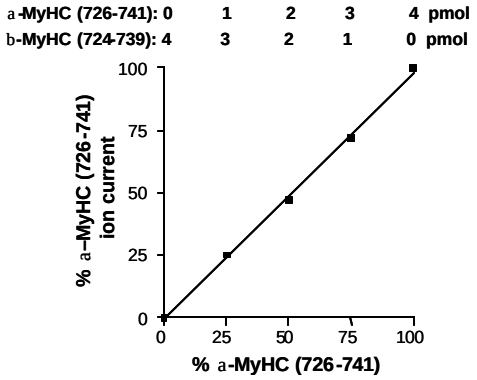

Figure 4. The α-MyHC (726-741)/β-MyHC (724-739) relative quantification standard curve. Mixtures of α-MyHC (726-741) and β-MyHC (724-739) were used to generate the standard curve. Each point is the average (+/- SD) of 10 measurements (Because the SDs are less than 1.2% they are difficult to visualize). Regression analysis indicated a linear relationship between ion current ratio and peptide ratio (r^2 = 0.998 and slope = 0.99).

Comparison of Methods to Quantify Myosin Isoform Ratios. Myofibrillar protein extracts were prepared as described from a set of normal human right atria samples. Triplicate aliquots were analyzed using the gel system of Reiser[12, 26] which can resolve very small amounts of the isoforms. Densitometry of the silver-stained α- and β-MyHC bands was then performed to determine the proportion of the α- and β-MyHC isoforms.[11,

[12] Aliquots of the same samples were then resolved on NuPage gels and the MyHC band processed as described in the experimental section. A narrow window of a representative spectrum is shown in Figure 3A. The % α ion current was converted by reference to the standard curve to the % α-MyHC (726-741) which is equivalent to the % α-MyHC. The % α-MyHC as determined by MALDI-TOF MS for the samples is graphed against the % α-MyHC as determined by silver stained gels (Figure 5). The silver stained gel method of Reiser is currently the best available method to measure human α- and β-MyHC isoform ratios. As shown in Figure 5 the two methods return equivalent values.

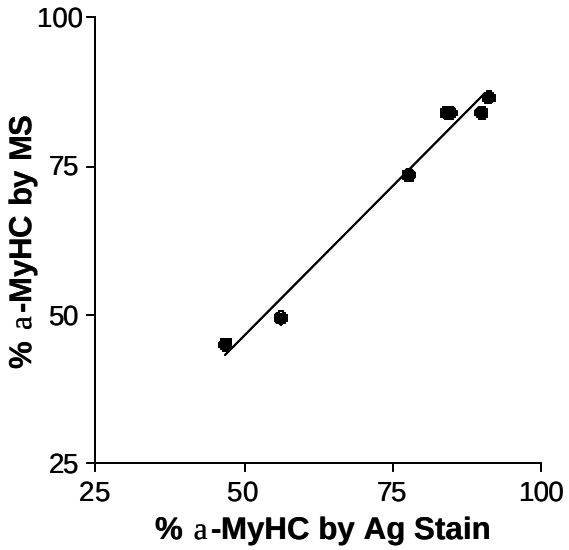

Figure 5. Comparison of the Silver Stained Gel Method and the MALDI-TOF MS Method. Regression analysis was performed on a comparison of the % α-MyHC values determined by the silver stained gel method and by the MALDI-TOF MS method. There was excellent agreement between the methods as shown by a linear relationship (r^2 = 0.979 and slope = 1.01).

B. Measuring Protein Amounts by MALDI-TOF MS

Design of an internal standard peptide. The relative amounts of the α- and β-MyHC protein isoforms can be determined from the relative amounts of α-MyHC (726-741) and β-MyHC (724-739) peptides, but the quantification of the absolute amounts requires the incorporation of an internal standard (IS) peptide. A known quantity of the IS peptide is added to tryptic digest peptides and carried through the processing steps. The ratio of α-MyHC (726-741) and β-MyHC (724-739) to the IS peptide can be determined from appropriate standard curves. From this ratio, and the amount of the IS peptide added, the absolute amounts of α-MyHC (726-741) and β-MyHC (724-739) can be determined. Design of the internal standard peptide should take into account the same issues described previously for the selection of the isoform specific peptides. The internal standard peptide should be very similar to the isoform specific peptides yet be discriminated by mass, and it should generate a strong MALDI-TOF ion current. The amino acid sequence should be very similar so that recovery, crystallization, and ionization closely mimic α-MyHC (726-741) and β-MyHC (724-739). This is most readily achieved by a conservative amino acid substitution. The region where α-MyHC (726-741) and β-MyHC (724-739) differ was examined to find a suitable residue to mutate. The rationale was to maintain the regions where α-MyHC (726-741) and β-MyHC (724-739) are the same so that the internal standard peptide could be used to measure both peptides. The internal standard peptide should have a mass that is not found in the samples so that its signal is not subject to interference by endogenous peptides. The mass range between the peptides was free of signal and therefore the internal standard was designed to appear in this region (Figure 3A). α-MyHC (726-741) was chosen as the starting point. A conservative hydrophobic amino acid substitution, isoleucine-7 to valine

(Figure 2), was selected because this produces little change in chemical properties and yields a peptide with a mass intermediate between the isoform peptides.

Generation of the absolute quantification standard curves. The internal standard (IS) peptide was mixed with the synthetic α-MyHC (726-741) and β-MyHC (724-739) to generate standard curves. Each spot contained 2 pmol of IS and either 0-6 pmol of the synthetic α-MyHC (726-741) or 0-4 pmol of the synthetic β-MyHC (724-739). The ion current ratio of α-MyHC (726-741) /IS peptide (α/IS) was graphed against the pmol of α-MyHC (726-741) in Figure 6A. Likewise, the ion current ratio for the β-MyHC (724-739)/IS peptide, (β/IS), was graphed against the pmol of β-MyHC (724-739) in Figure 6B. Linear regression analysis utilized all ten values at each point. In both cases the ion current ratios were linear with the amount of peptide. Higher order analysis did not significantly improve the curve fit of either standard curve.

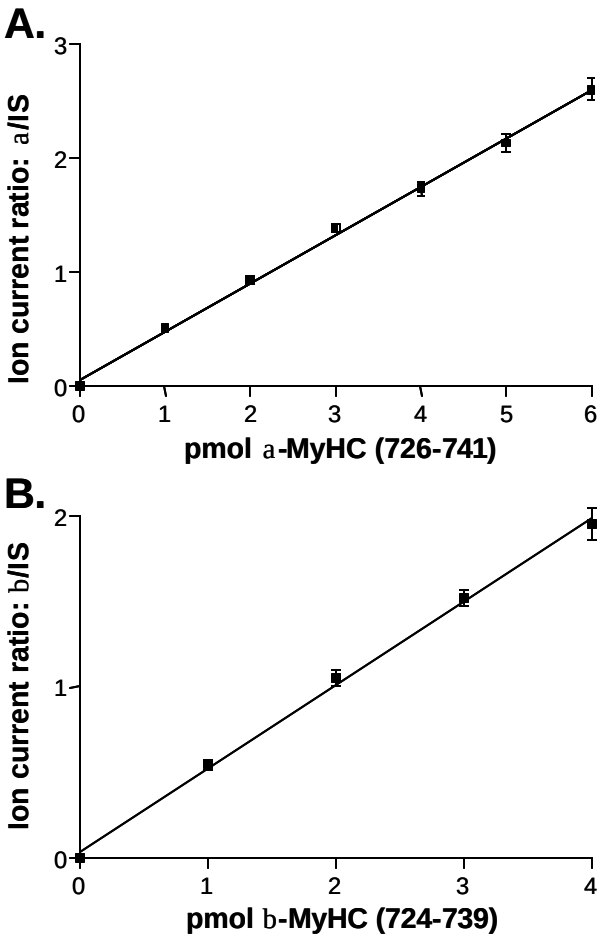

Figure 6. A. α-MyHC (726-741) Standard Curve. α-MyHC (726-741) was mixed with 2 pmol IS peptide and the ion current ratio, α/IS, was measured. Each point is the average (+/- SD) of 10 measurements. The ion current ratio, α/IS, was linear (r^2 = 0.994) with α-MyHC (726-741). **B.** β-MyHC (724-739) Standard Curve. β-MyHC (724-739) was mixed with 2 pmol IS peptide and the ion current ratio, β/IS, was measured. Each point is the average (+/- SD) of 10 measurements. The ion current ratio, β/IS, was linear (r^2 = 0.998) with β-MyHC (724-739).

Linearity of the assay with protein amount. An assay for a particular protein in a complex mixture should be linear with the amount of total protein analyzed. Myofibrillar

protein extract was electrophoresed on duplicate gels with loads of 0, 1, 2, 3, or 4 µg of total protein. The MyHC band was excised and processed as described. Tryptic digests were supplemented with 2 pmol of the IS peptide and subjected to MALDI-TOF MS. The ion current ratios of α/IS and β/IS were measured, and then converted to pmol of α-MyHC (726-741) and β-MyHC (724-739) by reference to the standard curves (Figure 6). The pmol of α-MyHC and β-MyHC are equivalent to the pmol of the peptides and were graphed against the µg of total protein (Figure 7). Regression analysis indicated that the amounts of α-MyHC and β-MyHC were linear with protein.

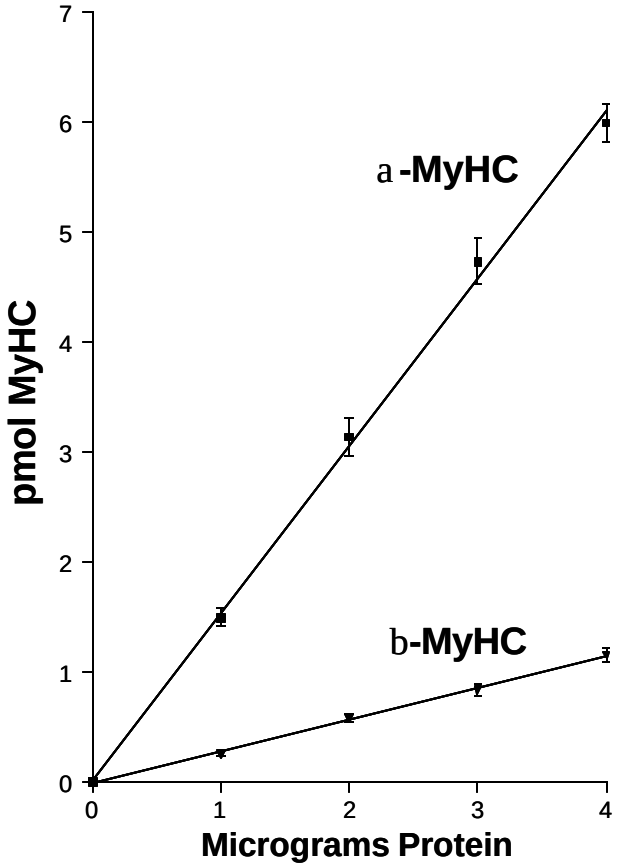

Figure 7. Assay Linearity with Protein Amount. Myofibrillar protein extract (patient 1) was analyzed as described to determine the amounts of α-MyHC and β-MyHC. Each

point is the average (+/- SD) of 10 measurements. α-MyHC (r^2 = 0.999) and β-MyHC (r^2 = 0.998) amounts were linear with respect to protein amount by regression analysis.

Quantification of a-MyHC and b-MyHC in Atrial Samples. The set of myofibrillar protein extracts was electrophoresed on duplicate gels using 3 μg total protein. The MyHC band was excised, processed as described, and the tryptic digests were supplemented with 2 pmol IS peptide. A representative MALDI-TOF MS spectrum is shown in Figure 3B. The ion current ratios of α/IS and β/IS peptide were measured and the pmol of α-MyHC (726-741) and β-MyHC (724-739) were determined by reference to the standard curves. These values are equivalent to the pmol of the parent α-MyHC and β-MyHC proteins (Table I). Both the pmol of α-MyHC and the pmol of β-MyHC were determined simultaneously in the atrial samples. From these amounts, the pmol α-MyHC/μg total protein and the pmol β-MyHC/μg total protein were calculated (Table 1). The absolute amounts of the isoforms were used to calculate the percentage of α-MyHC. These values are in good agreement with the relative amounts determined by the isoform ratio method described previously. From the combined amounts of α- and β-MyHC in each sample (i.e. 1.15-1.86 pmol/μg), and the MW of MyHC (223 kDa), we calculate that 26%-41% of the total protein in these myofibrillar protein extracts is MyHC. The approximate MyHC content of the extracts had previously been determined by densitometry of Coomassie stained gels. The amounts of MyHC measured by MALDI-TOF MS were consistent with the values determined by densitometry. The broad range of myosin content is due to the heterogeneity of the tissue pieces. These contain various

amounts of connective and other tissue types, mixed with myocardium (only myocardium expresses the cardiac isoforms).

Table 1. Absolute Amounts of α- and β- MyHC Isoforms in Patient Samples.

Patient	pmol α-MyHC	pmol α-MyHC/ mg protein	pmol β-MyHC	pmol β-MyHC/ mg protein	% pmol α-MyHC
1	4.83 +/- 0.21	1.609 +/- 0.071	0.84 +/- 0.05	0.281 +/- 0.016	85.14 +/- 0.69
2	1.74 +/- 0.11	0.579 +/- 0.036	2.00 +/- 0.08	0.667 +/- 0.027	46.46 +/- 1.34
3	3.26 +/- 0.20	1.085 +/- 0.066	0.55 +/- 0.07	0.185 +/- 0.024	85.51 +/- 1.19
4	2.63 +/- 0.11	0.878 +/- 0.038	0.86 +/- 0.05	0.285 +/- 0.015	75.47 +/- 1.34
5	3.48 +/- 0.15	1.159 +/- 0.052	0.48 +/- 0.05	0.160 +/- 0.016	87.86 +/- 0.95
6	2.39 +/- 0.08	0.796 +/- 0.025	2.27 +/- 0.06	0.757 +/- 0.019	51.26 +/- 0.85
7	3.35 +/- 0.19	1.118 +/- 0.064	0.57 +/- 0.04	0.190 +/- 0.015	85.49 +/- 0.92

Myofibrillar protein extracts (3 μg) were electrophoresed on SDS gels and the MyHC band was excised for analysis. The amounts of the α- and β- MyHC isoforms are expressed as pmol and as pmol/μg protein. The values are used to calculate the % pmol α-MyHC (i.e. 100 x pmol α-MyHC/ (pmol α-MyHC + pmol β-MyHC). All values are averages +/- SD for ten measurements.

CONCLUSIONS

A novel MALDI-TOF MS method has been developed to quantify proteins by measuring the amounts of specific tryptic peptides. Both relative and absolute quantification have been achieved. The utility of the approach has been demonstrated by quantifying the relative and absolute amounts of the closely related human α- and β-MyHC isoforms. The silver stained gel approach[12, 26] that is currently the best available method to measure human α- and β-MyHC protein isoform ratios is cumbersome because it requires unusual gel conditions, long run times, and very low protein loads.[26]

The low protein loads necessitate silver staining which is difficult to quantify by densitometry and offers a very limited dynamic range.[28] This is probably responsible for the discrepancies between reports of the isoform ratios in normal human ventricle (i.e. 7.0% α-MyHC[11] compared to 2.5% α-MyHC[12]). The MALDI-TOF MS assay avoids these problems and should be more reliable. The peptide standards are relatively easy and inexpensive to produce using modern solid phase peptide synthesizers. This approach does not require expensive stable isotopes or necessitate difficult covalent labeling reactions such as the isotope-coded affinity tag (ICAT) approach.[29] Also, this method offers discrimination between extremely similar protein isoforms, such as skeletal and cardiac α-actin, which would be difficult to quantify with accuracy, except by MS based techniques.

The absolute amount of a given protein can also be quantified by using a specific tryptic peptide and an internal standard peptide that is similar in sequence but differs in mass. Quantification approaches such as stained gel densitometry and ICAT only measure relative ratios and do not measure absolute protein amounts. Although absolute protein amounts can be determined by employing stable isotope labeled peptides and HPLC/tandem MS[23, 24] this approach requires expensive isotopically labeled standards and the development of appropriate HPLC conditions. The method we describe is versatile, straightforward and utilizes MALDI-TOF MS technology. This approach can be applied to protein mixtures as long as the distinct ion currents arising from the peptides of interest are not subject to interference by other endogenous peptides. With the appropriate choice of protease, digestion conditions, and internal standard, it is possible

that relative and absolute determinations can be made for almost any protein by adopting these methods.

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