COVER SHEET

TITLE: Chamber-related Variation in Phosphorylation State of Cardiac Troponin Revealed by High Resolution Top-Down Mass Spectrometry

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ABSTRACT

Chamber-related Variation in Phosphorylation State of Cardiac Troponin Revealed by High Resolution Top-Down Mass Spectrometry

Cardiac troponin complex (cTn) is a key regulator of Ca^{2+} mediated cross-bridge cycling in cardiac muscle. Post-translational modifications, especially phosphorylation of cTn, are major mechanisms in modulation of contractile function. Two subunits of cTn, cTnI and cTnT, are considered as the "gold-standard" protein biomarkers for detecting acute myocardial infarction. Traditionally, the heart is considered homogeneous for analysis of cardiac proteins. However, both functional and morphological variations have been observed in four heart chambers. Herein we have employed high resolution top-down mass spectrometry to examine cTn purified from healthy swine myocardial tissues. Our data show varied phosphorylation levels in both cTnI and cTnT extracted from four cardiac chambers respectively, which is likely due to the functional and morphological differences.

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Introduction

Cardiac troponin (cTn) is a key regulator of heart contraction and relaxation. cTn has three subunits: inhibitory subunit (cTnI), tropomyosin-binding subunit (cTnT) and calcium-binding subunit (cTnC). When cytoplasmic calcium concentration is low, tropomyosin winds around actin filament and inhibits its binding by myosin. When an action potential is propagated to the muscle fiber via neuromuscular junction and raises intracellular calcium concentration, calcium binds to cTnC and causes allosteric change of the troponin complex. This in turn releases tropomyosin from actin, enabling cross bridge formation between the thick and thin filaments and muscle contraction occurs (Kobayashi and Solaro, 2005). cTnI and cTnT are unique to cardiac muscle and are released into circulation following damage of heart muscle tissue. Thus they have been used as the "gold-standard" serum biomarkers for acute cardiac injury (Babuin and Jaffe, 2005). Studies of cTn will contribute to our understanding of cardiovascular physiology and pathology, and hold the promise to developing reliable next generation biomarkers.

Post translational modifications (PTMs), especially phosphorylation of cTn causes conformational changes and provides a major mechanism to modulation of cardiac contractile function. *In vitro* studies have shown that phosphorylation of cTnI can be regulated by various protein kinases. For example, protein kinase A (PKA) phosphorylates cTnI at Ser 22/23 (Ser 23/24 when counting the N-terminal methionine) and reduces calcium-binding affinity of cTn complex under adrenergic stimulation. This generates the "fight and flight" response and increases heart contractility and heart rate. On the other hand, cTnI also has three protein kinase C (PKC) sites and phosphorylation by PKC has been shown to decrease the affinity to calcium.

Similarly, cTnT can potentially be phosphorylated by PKC on five amino acid residues. Changes of PTMs in cTn have been shown to be closely related to cardiac diseases (Layland, et. al, 2005). Hypophosphorylation of cTnI has been observed in patients with cardiac myopathy and is a possible mechanism for loss of cardiac functions.

Top-down mass spectrometry is a newly-evolved technology in which intact proteins are introduced directly into mass spectrometer and analyzed (Siuti and Kelleher, 2007). Compare to the bottom-up approach traditionally used in proteomics studies, in which proteins are first digested with proteolytic enzymes into peptides before being analyzes, top-down holds many significant advantages. First, it gives reliable mapping of all the PTMs without the risk of losing some of them during proteolytic digestion. It has also better chance to achieve full sequence coverage than the bottom-up approach as the digested peptides can sometimes be lost and not detected by the mass spectrometer. The mass spectrum (MS spectrum) gives a "bird's eye" view of all the protein species as well as modifications and isoforms present in the analyzed solution. Once the target peak is identified, it can be isolated in the mass spectrometer and cleaved via several different dissociation methods. This is called tandem mass spectrometry (MS/MS) and provides a map of the localization of PTM on the peptide. In this study, electron capture dissociation (ECD) was chosen as the best dissociation method (Ge, et. al, 2002; Kelleher, et. al, 1999). ECD is a mild cleavage method that breaks NH-CHR bonds and produces c and $z^{\bullet}$ ions. Its nonergodic nature enables the preservation of labile PTMs, especially phosphorylation.

In routine proteomics studies of cardiac protein, the left ventricle (LV) is normally used as a representative or no specification is given for the region from which the tissue is used in the experiment. However, the four chambers of the heart, left ventricle (LV), left atrium (LA), right ventricle (RV) and right atrium (RA) differ in both function and morphology (Chinchoy, et. al,

2000). In this study, we employed high resolution top-down protein mass spectrometry to analyze the phosphorylation of cTn extracted from the four different chambers of domestic pig heart to reveal any chamber-related variations.

Methods

Immunoaffinity Chromatography

Healthy swine heart tissues were obtained from juvenile Yorkshire domestic pig as approved by the University of Wisconsin Animal Care and Use Committee and immediately frozen in liquid nitrogen and stored under -80°C. The tissues were then homogenized in wash buffer (5mM NaH_2PO_4, 5mM Na_2HPO_4, 5mM $MgCl_2$, 0.5mM EGTA, 0.1M NaCl, 1% Triton X-100, 5mM DTT, 0.75mg/mL protease inhibitor cocktail tablet, 1mM PMSF and 2ug/mL leupeptin, pH 7.4) using ceramic mortar. The mixture was centrifuged at 8000 rcf for 10min and the pellet was washed with wash buffer twice before being resuspended in extraction buffer (5mM EGTA, 0.1mM $CaCl_2$, 0.7M LiCl, 25mM tris, 5mM DTT, 0.75 mg/mL protease inhibitor cocktail tablet, 1mM PMSF and 2ug/mL leupeptin, pH 8.0) and incubated at 4 °C for one hour. After that the mixture was again centrifuged at 55,000 rpm using Beckman ultracentrifuge for 50 min and clean supernatant after centrifugation was incubated with 0.3mL of CNBr-activated Sepharose CL-4B conjugated with an anti-troponin I monoclonal antibody (MF4, Hytest, Finland) for another hour at 4°C. Flow-through was collected; the antibody column was then washed with 1.6mL extraction buffer. The protein was eluted with 100 mM glycine HCl (pH 2). 400mL of eluted sample were collected into 1.5mL microcentrifuge tubes containing 40mL MOPS (pH 9) for each elution. The flowthrough, wash and all elutions were analyzed with 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for an estimation of concentration before desalting.

Top-down Mass Spectrometry

Purified cTn complexes were desalted with offline reverse phase C18 protein microtrap and eluted with 1% acetic acid in 30:70, 50:50 and 75:25 methanol: water elution buffer. The sample is then introduced to the LTQ/FT mss spectrometer (Thermo Scientific, Germany) by an automated nano ESI source (Triversa NanoMate, Advion BioSciences). Fourier transform mass spectrometry (FTMS) spectrum is taken to show the contents of the protein mixture. The spray voltage was set at 1.35-1.55 kV. The resolution power of FTMS was set at 20000. 2% - 3% electron energy and 55ms duration time was used for ECD.

The average intensity of the top three isotopomers of each peak was used to calculate the relative abundance of observed protein species. The ECD spectrum was processed with Manual Xtract software (Thermo Scientific, Germany) and manually validated. Monoisotopic masses of observed ions were matched with the swine cTnI sequence obtained from Swiss Prot protein knowledgebase using ion assignment software.

Results

SDS-PAGE Analysis of Purified Swine cTn complex

As shown in Figure 1, SDS-PAGE shows three major bands corresponding to the three subunits of cTn at 18, 26 and 37 kDa for all four chambers. However the resolution of SDS-PAGE was too low to reveal any difference in PTMs. (Figure 1).

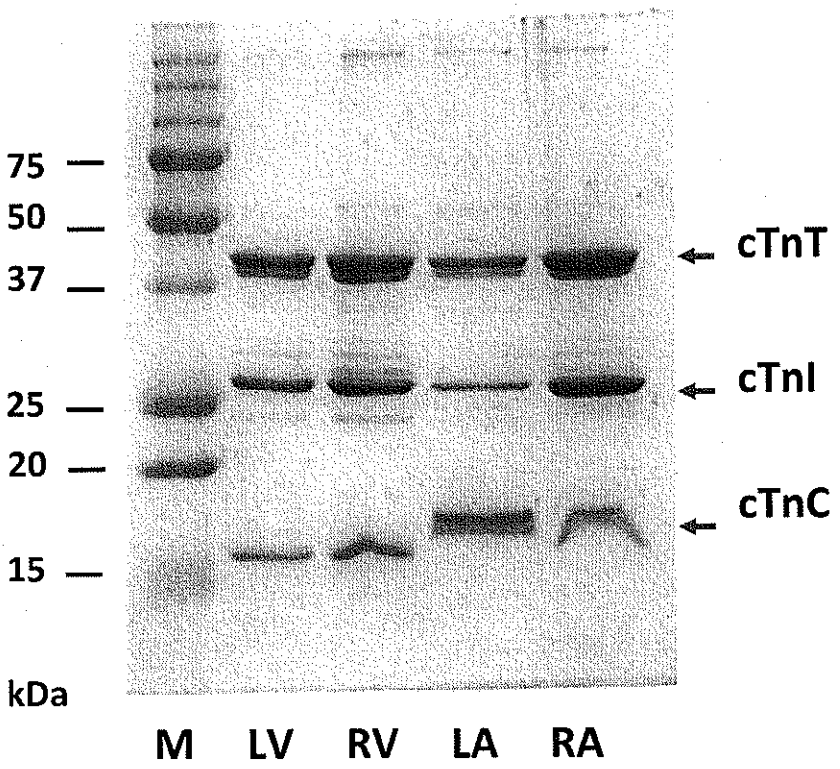

Figure 1. SDS-PAGE Analysis of Purified Swine cTn

Chamber-related Variation of Swine cTn Phosphorylation

After desalting, cTnT is mainly observed in the 30: 70 methanol: water elution, whereas cTnI elute mainly in the 75:25 methanol: water elution. The molecular weighted detected in the MS spectrum of both proteins matched the sequences with the removal of N-terminal methionine and addition of N-terminal acetylation. As shown in Figure 2 and 4, the experimental value of un-, mono- and bisphosphorylated cTnI are measured to be 23952.03 Da, 24032.00 Da and 24111.96 Da, while that of un- and monophosphorylated cTnT are 35130.05 Da and 35210.01 Da.

High resolution top-down mass spectra revealed that cTnI, the inhibitory subunit of cTn, have obvious chamber-related variation in phosphorylation level. The left atrium (LA) exhibits the highest phosphorylation level, with the relative percentages of un-, mono-, and bis-

phosphorylated cTnI forms of 8.4±0.8%, 30.0±3.9%, and 61.6±4.7%, respectively. This is followed by the left ventricle (LV), where the unphosphorylated form takes up 11.2±2.3% of total cTnI protein population, monophosphorylated 34.6±3.5%, bis-phosphorylated 54.2±5.0%, respectively. The relative percentages of un-, mono- and bis-phosphorylated forms in the right atrium (RA) are 19.5±2.5%, 37.7±3.5%, 42.8±5.9%; and those of the right ventricle (RV) are 25.0±3.9%, 41.2±2.0% and 33.8±4.4%, respectively. For cTnT, the tropomyosin-binding subunit of the cTn complex, only un- and monophosphorylated forms of swine cTnT are observed in the FTMS spectra. The chamber that contains the highest percentage of phosphorylated cTnT is LA, with 76.8±3.8% monophosphorylated and 23.2±2.5% unphosphorylated form of swine cTnT. The other three chambers, LV, RV and RA, respectively, have 64.5±3.7%, 69.9±3.5% and 70.0±4.1% of monophosphorylated swine cTnT. (Figure 2-5)

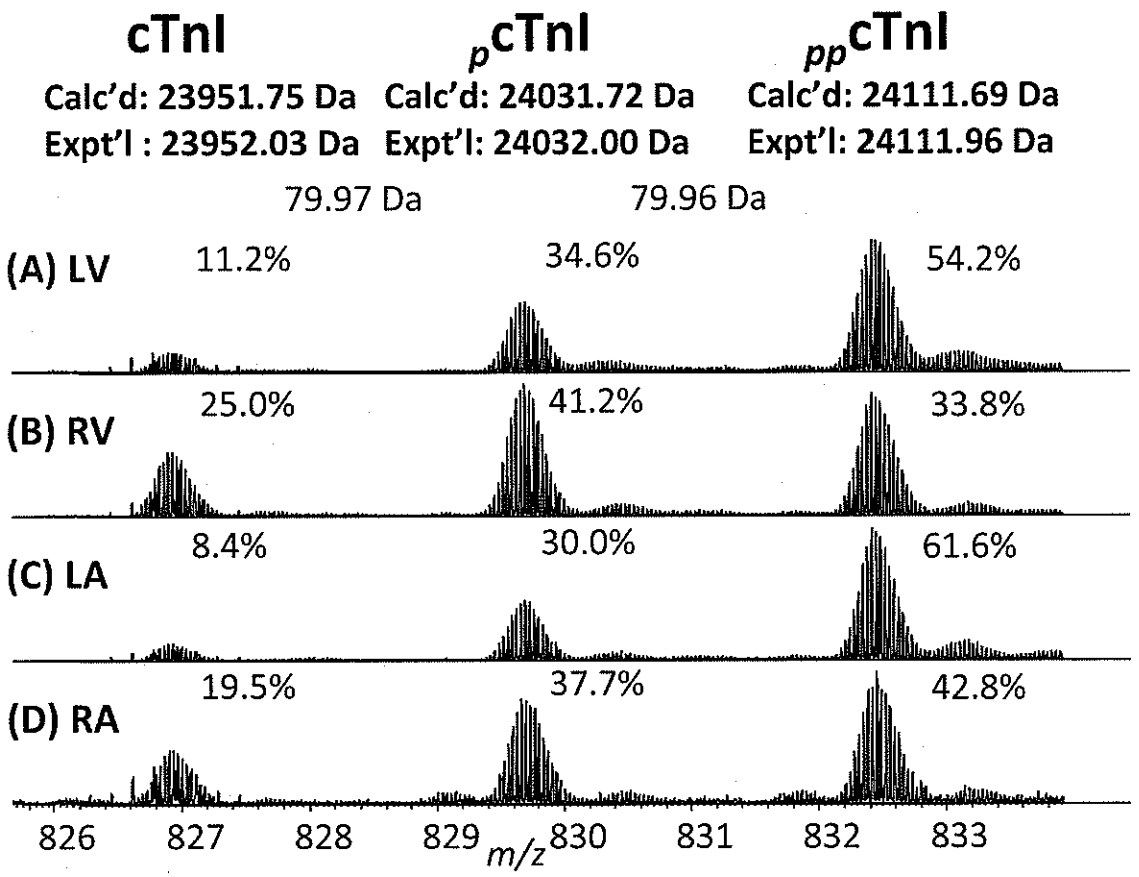

Figure 2. Chamber-related variation of swine cTnI phosphorylation

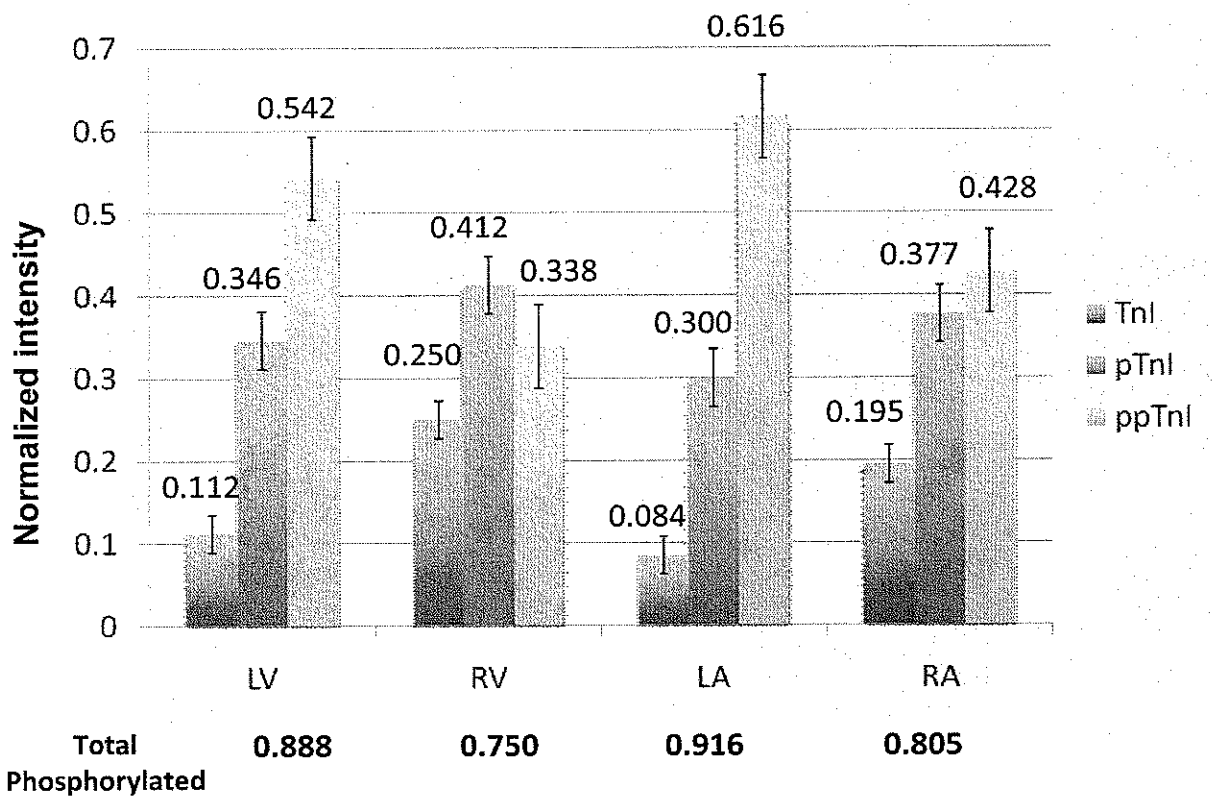

Figure 3. Quantified Result of Chamber-related variation of swine cTnI phosphorylation

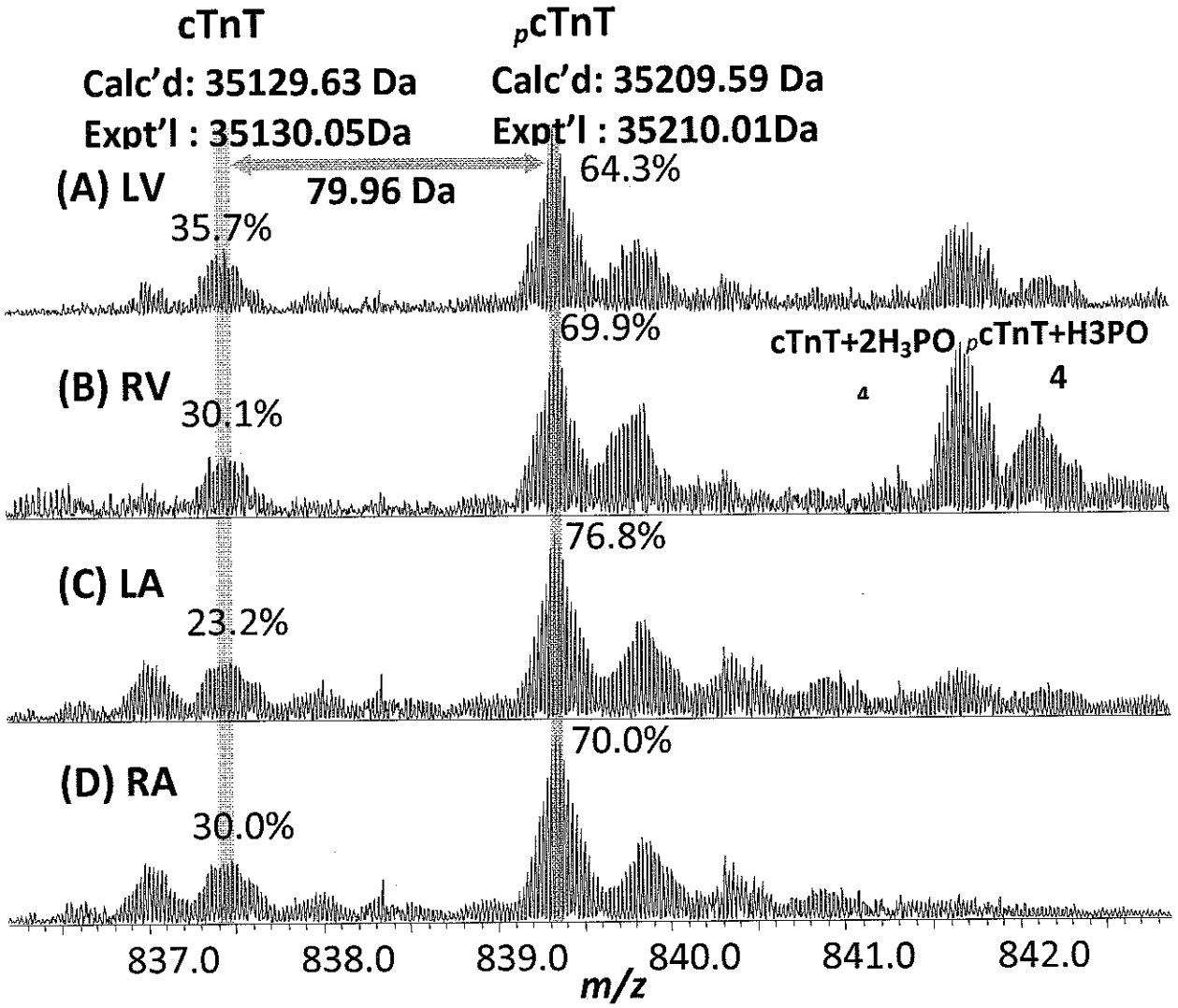

Figure 4. Chamber-related variation of swine cTnT phosphorylation

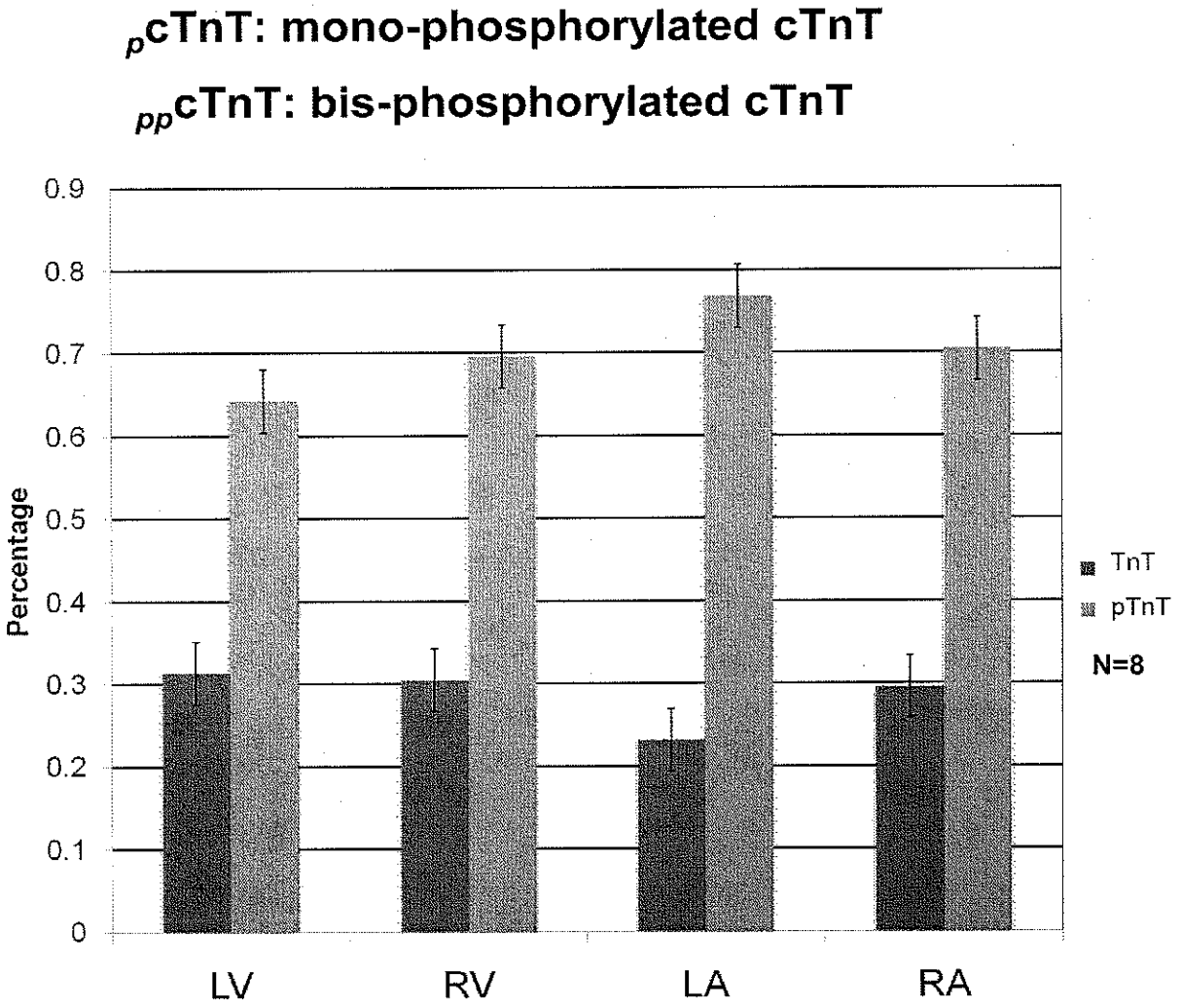

Figure 5. Quantified Result of Chamber-related variation of swine cTnT phosphorylation

Localization of Phosphorylation Sites in Swine cTnI

The ECD spectrum of isolated bisphosphorylated cTnI is shown in Figure 6. 45 *c* ions and 53 $z^{\bullet}$ ions were generated, achieving 100% sequence coverage. *c* ions at the N-terminal, including c_6, c_7, c_{11}, c_{15}, c_{18}, c_{19}, c_{20} and c_{21} do not contain any phosphorylation. However, a monophosphorylated c_{22} was observed and all *c* ions after c_{23} are bisphosphorylated with no un- or mono- phosphorylated peak detected. This result is sufficient to demonstrate that Ser 22 and Ser 23 are the only sites and are completely phosphorylated. The cleavage map is shown in Figure 7.

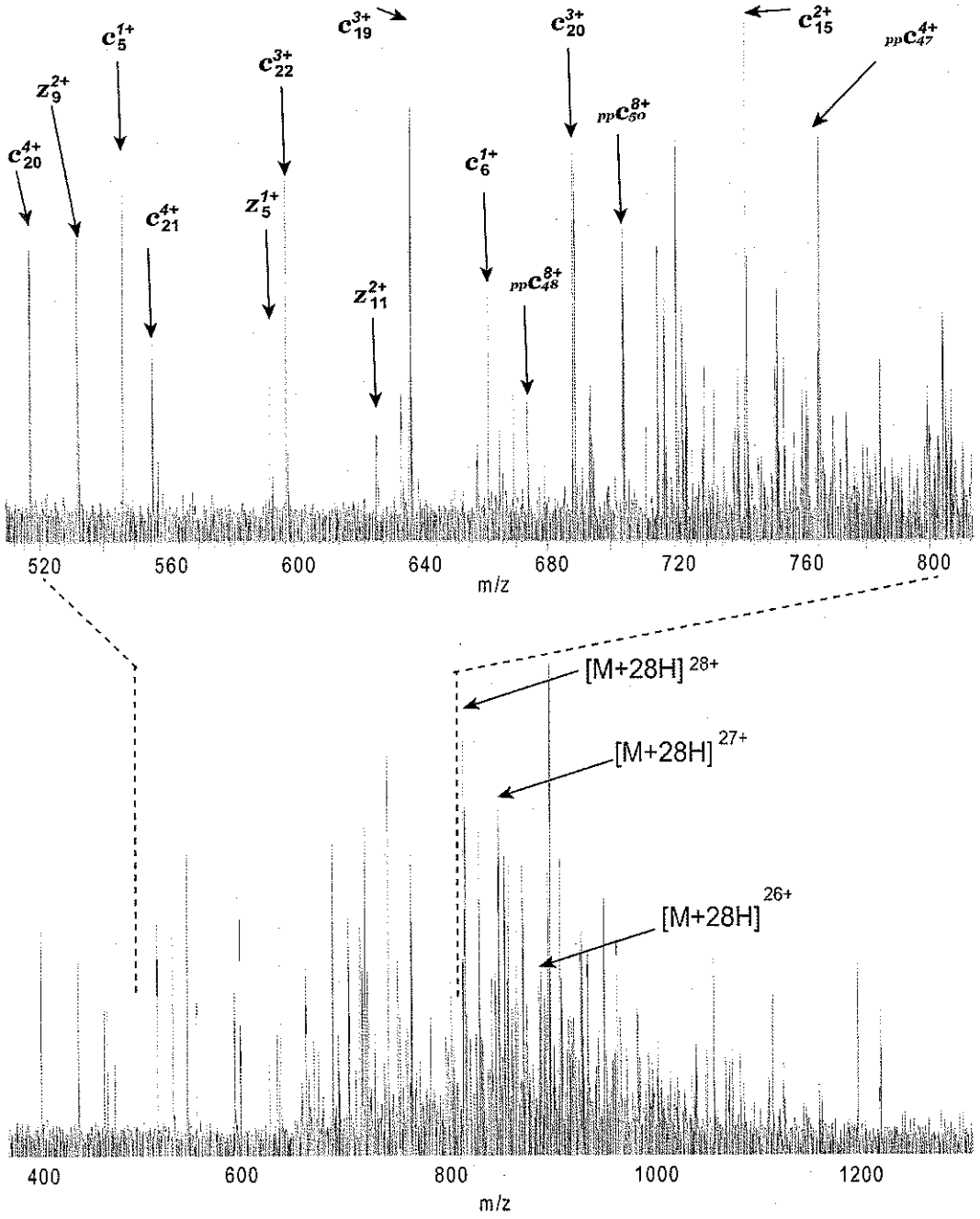

Figure 6. ECD Spectrum of isolated bisphosphorylated cTnI

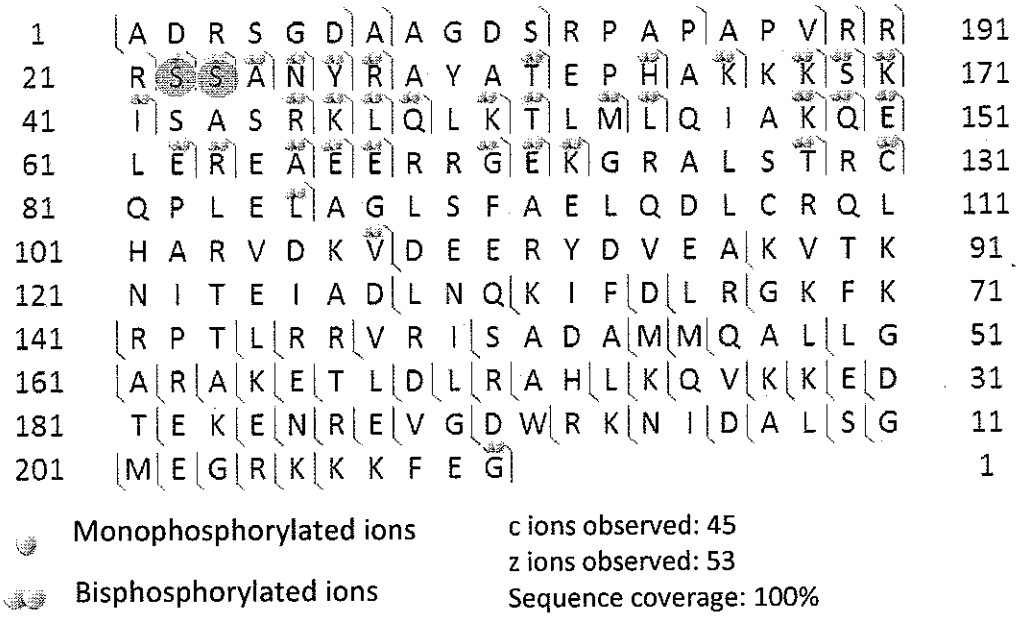

Figure 7. ECD fragementation product ion map for bisphosphorylated swine cTnI.

Discussion

This study demonstrated the great power of top down protein mass spectrometry in revealing, quantifying and localizing all protein PTMs. Top down MS provides us with comprehensive, unbiased and accurate characterization of intact proteins, while ECD analysis can reliably localize the amino acids on which the PTMs are placed. The experimentally detected molecular weight matched accurately with theoretical value. Compare to the bottom-up approach which heavily depends upon existing protein sequence, the top-down method allows *in vivo* analysis without the need of any *priori* knowledge. Moreover, with full sequence coverage, top-down eliminate the possibility of overlooking any PTMs as long as they are present within detection limit. This is also partly because the ionization efficiency of top-down does not change with the presence of PTMs.

The result showed that phosphorylation levels are different in both cTnI and cTnT in tissue from four chambers of swine heart. This evidence suggested that chamber-related variations in phosphorylation state needs to be considered in further cTn studies as LV does not give a comprehensive representation of the entire heart. In the heart used in this study, chambers on the left side are collectively more phosphorylated than those on the right side. However, it requires more biological replicates to ensure if this phenomenon is universal within the species or it is individualized. More studies are also being conducted on different animal models in order to confirm evolutionary coherence.

The phosphorylation sites of swine cTnI were determined to be Ser22/23, which is consistent with our previous results on mouse, rat and human cTnI. Phosphorylation on these sites had been shown to be regulated by PKA, PKC and other protein kinases in previous *in vitro* studies (Layland, et. al, 2005). This study reveals that these two sites are basally phosphorylated *in vivo* in healthy pig and is very likely important for proper functionality of cardiac muscle.

More studies are needed to understand the physiological significance underlying this observed chamber-related variation in cTn phosphorylation as phosphorylation of these proteins is shown to be closely related to cardiac functionality and contractility. Functional and morphological assays can be coupled to the proteomics methods used in this study in order to build the connection between the difference in PTMs and the difference in chamber morphology and functionality.

Conclusion

Combined with immunoaffinity choromatography, top-down high resolution mass spectrometry gives comprehensive characterization of PTMs without a *priori* knowledge. In this study, difference in phosphorylation state of muscle tissue from four different chambers of swine

heart was revealed using this method. The phosphorylation sites of cTnI were identified to be Ser22/23, which is consistent with previous results in other species. The result provides useful indications to future proteomics studies of cardiac protein that chamber-related variation should be considered as a factor that might influence the level of PTMs.

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