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Top-down Targeted Proteomics for Deep Sequencing of Tropomyosin Isoforms

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SUMMARY

Tropomyosins (Tm) constitute a family of ubiquitous and highly conserved actin-binding proteins, playing essential roles in a variety of biological processes. Tm isoforms produced by multiple Tm encoding genes and alternatively expressed exons along with post-translational modifications (PTMs) regulate Tm function. Therefore, to gain a better understanding of the functional role of Tm, it is essential to fully characterize Tm isoforms. Herein, we developed a top-down high-resolution mass spectrometry (MS) based targeted proteomics method for comprehensive characterization of Tm isoforms. α -Tm was identified to be the predominant isoform in swine cardiac muscle. We further characterized its sequence and localized the PTMs such as acetylation and phosphorylation as well as amino acid polymorphisms. Interestingly, we discovered a “novel” Tm isoform that does not match with any of the currently available swine Tm sequences. A deep sequencing of this isoform by top-down MS revealed an exact match with mouse β -Tm sequence, suggesting that this “novel” isoform is swine β -Tm which is 100% conserved between swine and mouse. Taken together, we demonstrated that top-down targeted proteomics provides a powerful tool for deep sequencing of Tm isoforms from genetic variations together with complete mapping of the PTM sites.

Keywords

Tropomyosin; Muscle Contraction; Actin Filament; Mass Spectrometry; Electron Capture Dissociation

Introduction

Tropomyosins (Tm) are a family of ubiquitous and highly conserved proteins found in eukaryotic cells, playing an essential role in a variety of biological processes that include but not limited to muscle contraction, cell motility, cell transformation and cytokinesis.^{1–4} Tm are α -helical coiled coil dimers that form a head to tail polymer along the major groove of actin filaments in all muscle fibers and many nonmuscle cells. The most well-known biological function of Tm is its role in striated muscle. Tm was originally discovered as a

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structural component of the skeletal muscle contractile apparatus and was believed to be a precursor of myosin, hence the name “tropomyosin”.⁵ Muscle contraction depends on the interaction of actin thin filaments and myosin thick filaments of the sarcomere.^{6–8} As one of the primary components of the actin filament, Tm interacts with actin and, together with the troponin complex, regulates muscle contraction and relaxation in a calcium-dependent manner.^{3, 9}

Recently, Tm is becoming increasingly recognized for its function in the cytoskeleton upon the discovery of “nonmuscle contractile proteins” expressed in most tissues and cell types, regulating cell morphology, intracellular motility, cell transformation and migration among others.^{1, 3–4} It becomes clear that actin cytoskeleton is centrally involved in a diversity of functions in all eukaryotic cells. A plethora of Tm isoforms have been identified which significantly contribute to the diversity of actin filament function.^{1–4, 9–10}

Tm expressed in eukaryotic cells show an increased complexity in terms of protein isoforms from yeast to human.⁴¹¹ The isoform diversity results from multiple Tm encoding genes and alternatively expressed exons, along with post-translational modifications (PTMs).^{12–14} Tm are highly conserved proteins encoded by four different genes: *TPM1*, *TPM2*, *TPM3* and *TPM4*, while each gene utilizes alternative splicing, alternative promoters, and differential processing to encode multiple striated muscle, smooth muscle and cytoskeletal transcripts.^{3, 13–16} *TPM1* is the most versatile and complex gene that comprises 15 exons to encode a variety of tissue specific isoforms. There are two major striated and smooth muscle Tm isoforms, α -Tm and β -Tm encoded by *TPM1*, *TPM2* genes, respectively, whereas γ -Tm encoded by *TPM3* gene is present only in slow-twitch skeletal muscle.^{3, 17} All four Tm genes are expressed in non-muscle cells.^{3–4} The production of Tm isoforms has been demonstrated to be developmentally regulated and these isoforms exhibit distinct physiological properties which are characteristic of cell type and species.^{1, 10, 18–19}

The involvement of Tm in a variety of human diseases clearly underlines the high clinical importance of Tm.^{1, 3} Mutation of Tm genes are known to be associated with a number of myopathies, most notably dilated cardiomyopathy (DCM) and familial hypertrophic cardiomyopathy (FHC).^{3–4, 9, 20} Recently, a novel striated muscle specific isoform encoded by *TPM1* gene, TPM1 κ protein, was found to be significantly up-regulated in DCM and heart failure.¹⁷ Moreover, non-muscle Tm is shown to be differentially expressed in a variety of human tumors, suggesting its functional role in oncogenic signaling.⁴ Recent studies have demonstrated that specific isoforms of Tm may function as tumor suppressor with the ability to suppress the malignant growth of tumor cells.^{21–27} Hence, to better understand the functional role of Tm in both muscle and non-muscle cells, it is essential to fully characterize the sequences of Tm isoforms, which is still lacking.

Top-down mass spectrometry (MS) is emerging as a powerful tool to comprehensively characterize the protein isoforms resulting from alternative splicing and coding polymorphisms simultaneously with PTMs.^{28–31} Unlike the traditional bottom-up MS that requires proteolytic digestion prior to MS analysis, top-down MS measures the intact proteins without digestion, thus providing a “bird’s eye” view of all types of protein modifications including isoforms, mutations, PTMs of targeted proteins simultaneously in one MS spectrum without *a priori* knowledge,^{28–30, 32–41}. Individual protein isoforms can be isolated⁴² and then fragmented by tandem MS techniques such as collisionally activated dissociation (CAD)⁴³ and electron capture dissociation (ECD),⁴⁴ allowing deep sequencing to detect amino acid variations and reliably map the modification sites with full sequence coverage.

Herein, we have developed a top-down mass spectrometry (MS)-based targeted proteomics approach to purify Tm from tissues and comprehensively characterize its isoforms. We have rapidly and effectively purified Tm from swine cardiac tissues without the use of antibodies and performed a deep sequencing of all swine Tm isoforms using top-down MS/MS with ECD and CAD. Moreover, PTMs including acetylation and phosphorylation, as well as amino acid polymorphisms have been characterized for the Tm isoforms. To the best of our knowledge, this is the first comprehensive characterization of Tm isoforms directly purified from tissues with full sequence coverage and PTM mapping.

EXPERIMENTAL SECTION

Chemicals and reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. Complete protease inhibitor cocktail tablets were purchased from Roche Diagnostics Corp. (Indianapolis, IN). All solutions were prepared in Milli-Q water (Millipore, Corp., Billerica, MA). Bicinchoninic acid (BCA) protein assay reagents were from Thermo Scientific (Rockford, IL).

Purification of swine Tm

Swine cardiac tissue samples were excised from healthy juvenile Yorkshire domestic pigs, snap frozen in liquid N₂ and stored in -80 °C freezer. Hearts were harvested immediately after the animals were sacrificed. All procedures were approved by UW-Madison Animal Care and Use Committee. A rapid purification method with high reproducibility was established to purify Tm from tissues (Figure 1A). Briefly, ~0.1–0.4 g of heart tissue was homogenized with HEPES extraction buffer⁴⁵ (HEPES, 25 mM, pH 7.5, NaF, 50 mM, Na₃VO₄, 0.25 mM, PMSF (in isopropanol), 0.25 mM, EDTA, 2.5 mM, and half tablet of commercial protease inhibitor cocktail) twice using a Polytron electric homogenizer (Model PRO200, PRO Scientific Inc., Oxford, CT, USA) for 15 s. The homogenate was centrifuged at 16,100 g for 15 min at 4 °C (Centrifuge 5415R, Eppendorf, Hamburg, Germany) and supernatant was removed and saved as “HEPES extraction”. The pellet was then homogenized with TFA extraction buffer (TFA, 1%, TCEP, 1 mM) using the Polytron electric homogenizer. The homogenate was centrifuged (16,100 g, 4 °C, 15 min) to collect the supernatant. 8 VL of 10% NH₄OH solution was added to the 50 VL of collected supernatant drop-by-drop to neutralize the supernatant to a pH of 7.0. As the solution was neutralized, precipitation occurred. The mixture was then centrifuged at 16,100 g for 2 min at 4 °C to remove the precipitate. Finally the supernatant was desalted using the Amicon 100 K molecular weight cutoff (MWCO) filter with 0.1 % formic acid solution as the exchange buffer. Tm was retained on top of the 100 kDa MWCO filter likely due to its elongated α -helical coiled coil polymeric structure. Fractions were analyzed for protein content by SDS-PAGE on 12.5% polyacrylamide gels stained with Coomassie Blue (Figure 1B). BCA protein assay was employed to determine the total protein concentration with a Synergy™ 2 microplate reader (Bio-Tek Instruments, Winooski, VT). A total of 200 μ g Tm could be purified from 400 mg heart tissue.

Top-down MS

Approximately 2 μ g of purified swine Tm was analyzed each time using a 7 T linear ion trap/Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (LTQ/FT Ultra, Thermo Scientific Inc., Bremen, Germany) equipped with an automated chip-based nano ESI source (Triversa NanoMate; Advion Biosciences, Ithaca, NY, USA) as described previously (28, 32–36). The sample was introduced into the mass spectrometer with a spray voltage of 1.2–1.6 kV versus the inlet of the mass spectrometer, resulting in a flow of 50–200 nL/min. Ion transmission into the linear ion trap and subsequently into the FT-ICR cell

was automatically optimized for a maximal ion signal. The accumulated charge numbers for the full scan linear ion trap, FT-ICR cell (FT), MSⁿ FT-ICR cell and ECD were 3×10^4 , 8×10^6 , 8×10^6 , and 8×10^6 , respectively. The resolving power of the FT-ICR was typically set at 200,000, obtaining an acquisition rate of 1 scan/s. For CAD and ECD, the protein molecular ions of the individual charge states were first isolated and then fragmented by 12–25% normalized collision energy for CAD or 2.2–3% electron energy (corresponding to 1.0–1.8 eV) for ECD with a 70 ms duration and with no additional delay, respectively. Typically, 1,000–5,000 transients were averaged to ensure high-quality CAD and ECD spectra.

All MS and MS/MS data were processed by in-house developed MASH Suite software using THRASH⁴⁶ algorithm (version 1.0) with S/N threshold of 3 and a fit factor of 60% and manually validated. The fragments in MS/MS spectra were assigned based on the protein sequence of swine Tm obtained from Swiss-Prot protein knowledgebase (UniProtKB/Swiss-Prot). Allowance was made for possible PTMs such as acetylation of the N-terminus, phosphorylation and two amino acid polymorphisms using 1 Da tolerance for the precursor and fragment ions, respectively. The assigned ions were manually validated to ensure the accuracy of the assignments. For fragment ions containing possible phosphorylation site, the masses of fragment ions were manually validated for ~80 Da mass shifts to confirm or exclude the existence of phosphorylation. All the reported molecular weights (MWs) are the most abundant MWs.

Quantitative analysis of swine isoforms was performed as described previously.^{33, 38} All the spectra were acquired in full profile mode. Briefly, the integrated peak heights of the top five isotopomers were used to calculate the relative abundance of each detected protein form. The percentages of un- and mono-phosphorylated α -Tm, un- and mono-phosphorylated β -Tm were defined as the summed abundances of α -Tm, $p\alpha$ -Tm, β -Tm and $p\beta$ -Tm over the summed abundances of the entire Tm populations, respectively. Non-covalent phosphate adducts ($+H_3PO_4$) were also taken into account in the quantitation. For example, the abundance of Tm + H_3PO_4 peak is combined with that of the unphosphorylated Tm, whereas the abundance of $pTm + H_3PO_4$ is integrated with mono-phosphorylated Tm (pTm). Three swine hearts (three biological replicates) were investigated and each biological sample was run in triplicate (three technical replicates) to ensure the technical consistency. Data are represented as means \pm S. D.

RESULTS

SDS-PAGE and High-resolution MS of Swine Tm

The Tm purification process is comprised of four steps (Figure 1A): 1) HEPES extraction at neutral pH (~7.5) in the presence of a variety of protease and phosphatase inhibitors to preserve the endogenous modification stage.⁴⁵ This extraction contains mainly soluble cytoplasmic proteins. 2) TFA extraction of the insoluble portion from the HEPES extraction at acidic pH (~2.2) to solubilize myofilament proteins. 3) Neutralization of the TFA extraction to precipitate the majority of the myofilament proteins which are only soluble at very acidic pH. This step significantly enriched the Tm that remains soluble at the neutral pH (7.0). 4) Desalting with a membrane filter that effectively eliminates low MW species and purifies Tm that are then collected as “elution” for further MS analysis. The entire protein purification process takes less than 3 hours and was analyzed by SDS-PAGE (Figure 1B). The major band in the elution is a 32 kDa protein as shown in Figure 1B, indicating that Tm has been purified from swine heart tissue (Figure 1B). FT-ICR MS analysis with high-resolution and high mass accuracy provides an overview of all detectable protein forms present in swine Tm using the method employed here (Figure 2). The predominant peak with MW of 32735.92 likely corresponded to unphosphorylated swine Tm. The peak next to

the predominant peak showed a MW of 32815.90, with a mass increment of 79.98 Da, indicating it is a mono-phosphorylated form of the predominant swine Tm peak. High-resolution MS spectra of purified Tm from three different swine hearts exhibited highly comparable profiles (Figure 2, Supplemental Figure 1) and the predominant peak in these spectra demonstrated the same experimental MW value of 32735.92 (tentatively labeled as “Tm” in Figure 2).

Meanwhile, an interesting peak with MW of 32877.61 was observed with a mass increment of 141.69 compared to the predominant swine Tm peak of MW 32735.92. This mass increment indicates a possible isoform of swine Tm as it is rare to observe a PTM of 141 Da. Surprisingly, a thorough sequence homology analysis demonstrated that this isoform does not match any of the swine Tm sequences available in the Swiss-Prot and NCBI database (Supplemental Figure 2) including commonly occurring modifications such as N-terminal Met removal, acetylation and phosphorylation. Hence we tentatively labeled it as a “novel” isoform of Tm (nTm).

Minor peaks with MW of 32284.75 and 32535.83 were also detected in the spectra of swine Tm (Figure 2), likely representing trace degradation products. Other low abundance peaks with higher MW (+98 Da) than the major peak were also observed, which correspond to non-covalent phosphate adducts of swine Tm (Figure 2).

Deep sequencing of the major swine Tm isoform

A thorough sequence homology analysis among all the available swine Tm sequences in Swiss-Prot and NCBI databases found the experimental MW of the predominant swine Tm form (32735.92) matches closest with the calculated MW (32705.75) of *Sus scrofa* α -Tm (UnitprotKB/Swiss-Prot P42639, TPM1_PIG) encoded by *TPM1* gene with a mass discrepancy of 30.17 Da (Supplemental Figure 2). Meanwhile the sequence of TPM1_PIG matches best with the MS/MS data among all available Tm sequences thus it was selected as a starting isoform for further comprehensive sequence mapping. Based on the mass discrepancy of 30.17 Da, it is reasonable to hypothesize that some modifications such as PTMs and/or amino acid polymorphisms have occurred in this predominant swine Tm isoform (defined as swine α -Tm hereafter). To confirm the possible modifications in the α -Tm chain, a single charge state of α -Tm precursor ions were isolated individually and fragmented by both ECD and CAD (Supplemental Figures 3, 4). Supplemental Figure 5 illustrated the effectiveness of a gas-phase isolation of a single charge state (M^{38+}) of un-phosphorylated Tm, mono-phosphorylated Tm, or a mixture of both.

In the representative ECD experiment of swine α -Tm, only 48 $z\bullet$ ions matched the C-terminal ions predicted from un-modified swine α -Tm sequence in the database (UnitprotKB/Swiss-Prot-P42639, TPM1_PIG) (Supplemental Figure 3A). The fact that no single ion matched N-terminal sequence indicates possible modifications at the N-terminus of the swine α -Tm. Given that the majority of eukaryotic proteins carry N-terminal acetylation, it is highly likely that this modification occurs to the swine α -Tm. As shown in Supplemental Figure 3B, after considering acetylation of the N-terminus of swine α -Tm, 30 c ions from the N-terminus were detected, which matched the predicted value of the fragment ions of the sequence before Arg38. The acetylation of swine α -Tm would give a mass increase of 42.01 Da. Meanwhile another 19 c ions after Arg38 were detected, with a mass decrease around 28.04 Da compared with the predicted values, which suggested that a potential amino acid polymorphism occurred at Arg38. The mass decrease of 28.04 Da possibly results from the amino acid polymorphism of Arg38→Gln38 (R→Q, $\Delta m = -28.04$, Supplemental Figure 3C). Another 31 c ions after Pro64 show a mass increase of 16.03 Da compared with the predicted values, indicating that another amino acid polymorphism occurred at Pro64 (Supplemental Figure 3D). The 16.03 Da mass increase

could be potentially generated from amino acid polymorphism from either Pro64→Leu64 (P/L) or Pro64→Ile64 (P/I) (P→I/L, $\Delta m = 16.03$). Indeed a homology sequence alignment indicates the presence of Gln38 and Leu64 in human, mouse, rabbit, rat, and bovine α -Tm sequences (Supplemental Figure 6). Moreover, comprehensive sequencing by ECD and CAD unambiguously identified these amino acid polymorphisms, Arg38→Gln38 and Pro64→Leu64 in swine Tm (Figure 3A, Supplemental Figure 3D).

One ECD experiment generated a total of 80 *c* ions and 48 *z*[•] ions according to the modified α -Tm sequence (Supplemental Figure 3D, Supplemental Table 1). After considering acetylation (+42.01 Da) and two amino acid polymorphisms (-28.04 Da, +16.03 Da, respectively), the calculated MW of swine α -Tm is 32735.75 (=32705.75+42.01-28.04+16.03), which matches well with the experimental MW of 32735.92 (5.5 ppm) (Figure 2). Figure 3 illustrated the representative product ions around Gln38 and Leu64 from the ECD and CAD spectra and resulting MS/MS ion map of swine α -Tm. 107 *b* ions and 15 *y* ions (118 cleavages) were generated in five CAD spectra, and 119 *c* ions and 78 *z*[•] ions (187 cleavages) were generated in four ECD spectra. A total of 228 out of 283 bond cleavages were obtained from the MS/MS data of swine α -Tm (Figure 3A). The top-down MS proteomics provided both the highly accurate MW measurement and effective sequencing, which unequivocally confirmed the modified sequence of swine α -Tm.

Mapping phosphorylation site in swine α -Tm

Swine α -Tm is present as both un- and mono-phosphorylated forms, at MWs of 32735.92 and 32815.90, respectively (Figure 2). The intensity of un-phosphorylated swine α -Tm is much higher than that of mono-phosphorylated α -Tm, indicating that swine α -Tm from healthy swine hearts is predominantly un-phosphorylated. No bis- or tris-phosphorylated swine α -Tm was detected, implying that higher-order phosphorylation was not present in the swine Tm samples or below the detection limit of the FTMS method applied in this study.

To localize the phosphorylation site of swine α -Tm, the precursor ion of monophosphorylated swine α -Tm was isolated and fragmented by ECD (Supplemental Figure 5B). 112 *c* ions and 79 *z*[•] ions were detected in combined four ECD spectra of mono-phosphorylated α -Tm; the representative fragment ions and the complete fragmentation map were demonstrated in Figure 4. As shown in the ECD spectra of isolated mono-phosphorylated swine α -Tm (*p* α -Tm), no phosphorylated product ions were detected for *c*₈ and *c*₃₃, nor other *c* ions detected in the ECD spectra (Supplemental Figure 7), which provided unambiguous evidence that the amino acid residues from the N-terminus of α -Tm do not contain a phosphorylation site (Figure 4A and 4B). In contrast, all 79 *z*[•] ions observed in these ECD spectra of mono-phosphorylated swine α -Tm were phosphorylated, suggesting the phosphorylation site is located near the C-terminus (Figure 4C and 4D). The smallest phosphorylated fragment ions detected was *z*[•]₉ in the ECD, which suggested the phosphorylation site was localized at one of the first 9 amino acids at the C-terminus (HALNDMTSI) (Figure 4E, Supplemental Table 2). Phosphorylation occurs most commonly on serine (~90%), then on threonine (~10%) and on tyrosine (~<0.05%).⁴⁷ Since there are only one serine (Ser283) and one threonine (Thr282) at this region, it is likely that Ser283 was phosphorylated as reported in the previous studies of Tm.⁴⁸⁻⁴⁹

Moreover, the representative ions from an individual ECD spectrum of un-, mono-phosphorylated swine α -Tm and their mixture were shown and compared (Supplemental Figure 7). Consistently, all the *c* ions in the spectra of un-, mono-phosphorylated α -Tm and their mixture were presented as un-phosphorylated form, indicating no phosphorylation site at the N-terminus. The *z*[•] ions observed were presented as un-phosphorylated, mono-phosphorylated, and a mixture of both un- and mono-phosphorylated forms in the ECD

spectrum of un-, mono-, and the mixture of un- and mono-phosphorylated swine α -Tm, respectively, suggesting the phosphorylation site at the C-terminus and presumably localized at Ser283.

Sequence determination of the “novel” swine isoform

To comprehensively characterize the “novel” isoform of swine Tm (nTm in Figure 2), we isolated this swine Tm isoform and then fragmented it by ECD and CAD. A single charge state of the swine Tm isoform was isolated and demonstrated a MW of 32877.61 with highly accurate mass measurement (Figure 5A). Both ECD and CAD spectra of the nTm generated sequence tags that match part of the N-terminal ions predicted from swine α -Tm with acetylation at N-terminus. The largest ions obtained from ECD and CAD spectra that match the sequence were c_{29} and b_{29} , respectively, suggesting a possible sequence difference after c_{29}/b_{29} . A thorough sequence alignment of both α - and β -Tm sequences among various species revealed that β -Tm among most mammalian species have one amino acid different at position 30 from N-terminus (Gln, Q) compared with swine α -Tm (Ala, A) (Figure 6, Supplemental Figures 6 and 8). Interestingly, analysis of MS/MS experiments of the swine Tm isoform discovered that this isoform matches exactly with the mouse β -Tm sequence (UnitprotKB/Swiss-Prot P58774, TPM2_MOUSE) encoded by *TPM2* gene (Figure 5B). 63 b ions and 15 y ions were detected from four CAD spectra, and 79 c ions and 69 z^* ions were detected from three ECD spectra, with the representative c , z^* , b , y ions shown in Figure 5C and the peak list of one ECD spectrum shown in Supplemental Table 3. Moreover, the calculated MW of the modified mouse β -Tm chain (acetylation at the N-terminus: 42.01) is 32877.90 (32835.79 + 42.01), which matches exactly with the experimental MW of this swine Tm isoform 32877.61 (5.8 ppm). As we have confidently excluded the possibility of potential contamination from mouse tissue samples, the only other explanation is that this “novel” swine Tm is swine β -Tm isoform that is 100% conserved with that of mouse β -Tm.

The sequence of swine β -Tm isoform identified here is significantly different from that of swine β -Tm chain reported in Swiss-Prot and NCBI database. The swine β -Tm sequence identified in our study has a total of 284 amino acids (Figure 3), in contrast to 287 amino acids in the hypothetical swine β -Tm sequence as reported in the database (Figure 6). Significantly, there are 39 amino acid differences between the observed (in this study) and the hypothetical swine β -Tm. Note that the hypothetical sequence for swine β -Tm encoded by *TPM2* gene (A1X899 available at Swiss-Prot databases) is marked as “un-reviewed”. The sequence alignment analysis indicates that a significant sequence difference exists between un-reviewed swine β -Tm (A1X899_PIG) and reviewed mouse and human β -Tm (P58774-TPM2_MOUSE, P07951-TPM2_Human) in the Swiss-Prot database (Supplemental Figure 8a), which challenges the un-reviewed swine β -Tm sequence and supports that the sequence characterized in this study is the correct swine β -Tm isoform. More interestingly, a further investigation of β -Tm across different species found all the reviewed β -Tm sequences are highly conserved among mammals (Supplemental Figure 8b). In fact, β -Tm chains are 100% identical among mouse, rat, rabbit, pig and bovine, whereas there is only one amino acid difference in β -Tm between human and other mammals at amino acid position 66. This further supported our hypothesis that swine β -Tm sequence is 100% conserved with mouse β -Tm.

In summary, both the MS/MS data and highly accurate MW measurement unambiguously define the correct sequence for swine β -Tm isoform. The three major peaks in the spectra with MW of 32735.92, 32815.90 and 32877.61 have been defined as un-phosphorylated swine α -Tm, phosphorylated α -Tm and un-phosphorylated β -Tm, respectively. Meanwhile, low MW peaks at 32284.75 and 32535.83 were assigned as a C-terminal degradation product Tm [1-280], Tm [1-282] and their isoforms d Tm [1-280], d Tm [1-282],

respectively (Figure 2). Due to the low intensity of these peaks, these degradation product isoforms have not been investigated further in this study.

Relative quantification of Tm isoforms and their phosphorylated form

To investigate the composition of Tm isoforms in swine hearts, the percentages of swine α -Tm, β -Tm and their corresponding mono-phosphorylated forms were calculated based on the molecular ion abundances (M^{35+} and M^{36+}) of Tm purified from three swine hearts. The reported results are from the three swine hearts, each with three technical replicates. Percentages of un- and mono-phosphorylated swine α -Tm and un- and mono-phosphorylated β -Tm were shown in Figure 7. The percentages of swine α -Tm are comparable among three distinct swine hearts, $66.2 \pm 0.3\%$, $68.4 \pm 3.2\%$, $70.7 \pm 1.7\%$ and the percentages of the mono-phosphorylated form ($p\alpha$ -Tm) are $18.8 \pm 0.2\%$, $16.4 \pm 3.1\%$, and $16.5 \pm 1.5\%$, respectively (Figure 7A-C). The percentages of the swine β -Tm and its mono-phosphorylated form ($p\beta$ -Tm) exhibited in the three different swine hearts are $12.3 \pm 0.3\%$, $12.6 \pm 0.6\%$, $10.3 \pm 1.7\%$ and $2.7 \pm 0.2\%$, $2.6 \pm 0.6\%$, $2.6 \pm 0.4\%$, respectively (Figure 7A-C). The summed percentages of the Tm isoforms and their corresponding mono-phosphorylation forms over three biological replicates with triplicate measurements are $68.5 \pm 2.8\%$, $17.2 \pm 2.2\%$, $11.7 \pm 1.4\%$, and $2.6 \pm 0.4\%$ for un- and mono-phosphorylated swine α -Tm and un- and mono-phosphorylated β -Tm, respectively (Figure 7D). The ratio of swine β -Tm of the total Tm population (including all Tm species regardless of isoforms and PTMs) is $14.3 \pm 1.2\%$. The phosphorylation occupancy of α -Tm and β -Tm is $20.1 \pm 1.8\%$ and $18.3 \pm 1.5\%$, respectively.

DISCUSSION

The highly conserved Tm sequences

Tm are highly conserved proteins found in eukaryotic cells and encoded by a family of four alternative spliced genes: *TPM1*, *TPM2*, *TPM3* and *TPM4*. Sequence homology alignment of α -Tm encoded by *TPM1* among various mammalian species demonstrated that α -Tm is highly conserved and there are only four amino acid substitutions at positions 38, 52, 64 and 220 from the N-terminus (Supplemental Figure 6). Moreover, after considering two amino acid polymorphisms of swine α -Tm at R38→Q and P64→L, there are only two amino acid differences (positions 52 and 220) across these species. Not only α -Tm encoded by *TPM1* but β -Tm chains encoded by *TPM2* exhibit highly conserved sequences among different species (Supplemental Figure 8). β -Tm chains of mouse, rat, rabbit and bovine have exactly the same protein sequence and these β -Tm chains have only one amino acid discrepancy from human β -Tm at amino acid position 66 from the N-terminus (Supplemental Figure 8b). Interestingly, as discovered in this study, the corrected sequence of swine β -Tm is 100% conserved as that of mouse β -Tm, suggesting the high sequence similarity among mammals β -Tm. Meanwhile, top-down targeted proteomics analysis of swine Tm isoforms characterized here revealed that 85.6% sequence similarity was exhibited between swine α -Tm and β -Tm from the swine heart tissue with 41 amino acid differences distributed throughout the entire sequence of 284 amino acids.

The functional significance of Tm isoforms

Tm are known to have a large number of isoforms which play important roles in regulating physiological function in various developmental stages.^{3-4, 9} Isoform switching accompanies morphogenesis and alterations in Tm isoforms have been detected in cancer cells.⁴ In striated muscle, there are three primary Tm isoforms, α -Tm, β -Tm, and γ -Tm. α -Tm is present in various tissue types and is the predominant form expressed in cardiac tissue, which is consistent with our findings here. β -Tm is expressed in both cardiac and skeletal muscles whereas γ -Tm is primarily expressed in slow twitch skeletal muscle.^{3, 9}

The ratio of α -Tm to β -Tm present in the muscle cells is characteristic of the muscle type and species and also dependent on the developmental stages.³ Although both α - and β -Tm isoforms are expressed in fetal heart tissue, α -Tm appears to be expressed nearly exclusively in the adult myocardium of small mammals.⁹ Indeed, Wieczorek and co-workers showed that the endogenous β -Tm protein expression is absent (<2%) in the healthy adult mouse heart.⁵⁰ In contrast, β -Tm is expressed in large animal and human myocardium in a much higher ratio (approximately 1:5 of β -Tm: α -Tm), which agrees well with our current data. The difference of α - and β -Tm ratio in cardiac muscle may be dependent on the size and contraction speed of the heart,⁵¹ with the ratio of α -Tm growing with increasing muscle speed.³ In large and slowly beating hearts such as pig and human, the ratio of β -Tm to α -Tm is much higher than that of small and faster beating hearts such as in rabbit, guinea pig and rat, where the β -Tm component appear to be nearly absent.⁴⁹

Meanwhile, compelling evidence shows that different Tm isoform expressions in muscles are functionally distinct.^{1-4, 9, 10} The Wieczorek lab has utilized transgenic mouse models to define the functional differences among these isoforms in regulating cardiac physiology.^{9, 52-53} Partial replacement of α -Tm with β -Tm in the adult mouse heart significantly increased Ca^{2+} sensitivity of the force and decreased the rates of contraction and relaxation.⁵²⁻⁵³ Increased expression level of β -Tm (>75%) leads to significantly altered diastolic function of the myocardium and these transgenic mice die between 10-14 days postnatally.⁵² In contrast, γ -Tm overexpression to ~50% of total Tm population leads to decreased myofilament Ca^{2+} sensitivity and increased rates of contraction and relaxation.^{9, 15} Thus, these studies suggest Tm isoforms are not functionally equivalent and alteration of the Tm composition may directly modulate the muscle physiology.

Regulation of Tm function by PTMs

Our top-down MS/MS data has located the site of N-terminal acetylation and suggests phosphorylation at Ser283. N-terminal acetylation is a common PTM for eukaryotic proteins. The functional role of N-terminal acetylation remains enigmatic although it is typically perceived to increase the stability and half-life of proteins in the cell.⁵⁴ Interestingly, Tm is among one of the few proteins in which the N-terminal acetylation has been recognized to be functionally significant. It has been demonstrated that Tm require NatB-mediated N-terminal acetylation for proper function actin-tropomyosin interaction and actomyosin regulation.⁵⁵ Since Tm are coiled coil dimers which form head-to-tail polymers along the major grooves in the actin thin filament, it is reasonable to believe that the modifications on the N- and C-termini are especially important for Tm polymerization. Indeed, only N-terminally acetylated Tm has been shown to have normal actin affinity and polymerization ability, underscoring the importance of Tm N-terminal acetylation.⁵⁴

Here our data also suggested a single phosphorylation site at Ser283 with partial phosphorylation occupancy. This is consistent with previous studies showing the presence of phosphorylated Tm in both skeletal and cardiac muscle from various species including frog, rabbit, rat, mouse, and chicken.^{3, 49} Moreover, with ³²P labeling and amino acid analysis, Ser283 was identified as a single phosphorylation site in α -Tm, but not β -Tm, isolated from skeletal muscle of adult frog despite of the similarity of the C-terminal sequence between β -Tm and α -Tm.⁴⁹ Nonetheless, the phosphorylated β -Tm has been detected in developing striated muscles of the rat and mouse although the percentage of phosphorylation is relatively lower in β -Tm than α -Tm,³ which is in agreement with our findings. The fact that a higher extent of phosphorylation was detected in both α -Tm and β -Tm indicates a more prominent role of Tm in early development.⁴ Importantly, phosphorylation of Tm has been shown to regulate the function of actin filament and remodeling of the actin skeleton.^{3, 56} Phosphorylation of Ser283 on each of Tm chains can form salt bridges with e-NH₂ groups of Lys6 and Lys12 respectively, which may enhance the stability of the head to tail

interaction involved in Tm polymerization^{3, 49}. In addition, phosphorylation of α -Tm may also promote the actomyosin interaction and activate myosin Mg^{2+} -ATPase.⁵⁶ A recent report shows that dephosphorylation of α -Tm mediated by p38-MAPK in murine hearts leads to depressed contractility.⁵⁷

Top-down targeted proteomics for deep sequencing of protein isoforms

Top-down MS is an attractive and unique method for analysis of protein isoforms because of its inherent sensitivity, resolution and speed, providing an overview of all possible isoforms as a result of multiple gene coding, alternative splicing and polymorphisms together with PTMs of the targeted protein in one mass spectrum.^{28–30, 39} Moreover, top-down MS with ECD and CAD is able to provide deep sequencing to define the specific sequence variation and PTM sites to a single amino acid. In this study, we have identified two swine Tm isoforms: swine α -Tm encoded by *TPM1* gene and another “novel” isoform with a mass increase of 141.67 Da from swine α -Tm in the same spectra. Interestingly, this “novel” isoform does not match any of the swine Tm sequences at NCBI or Swiss-Prot database with possible modifications. Our MS/MS with ECD and CAD allowed a deep sequencing of this “novel” isoform which suggested it was swine β -Tm and verified that it matched exactly with the mouse β -Tm sequence encoded by *TPM2* gene. Apparently, the original swine β -Tm sequences deposited in the NCBI or Swiss-Prot database are incorrect although we cannot exclude the possibility that it represents a new isoform that is yet to be confirmed. Furthermore, a thorough homology sequence alignment revealed that β -Tm is highly conserved among mammals and β -Tm chains are 100% conserved among pig, mouse, rat, rabbit and bovine (Supplemental Figure 8). Moreover, the top-down MS/MS deep sequencing also mapped two amino acid polymorphisms in the sequence of swine α -Tm and localized its mono-phosphorylation site. Admittedly, most of the top-down MS studies to date have focused on the analysis of protein PTMs.^{29, 32–41, 58–60} Despite fewer applications, top-down MS has shown to be equally powerful for sequencing protein isoforms resulting from alternative splicing and coding polymorphism.^{28, 30–31, 61–63} Although protein isoforms from genetic variation can also be identified by DNA sequencing, the unique advantages of top-down proteomics are the ultimate accuracy for sequencing protein isoforms purified directly from tissue and cells as well as the concurrent characterization of protein sequence variants (e.g. alternative splicing, polymorphisms, mutations) together with PTM mapping. With the further development of advanced instrumentation,^{64–65} we expect that the top-down MS-based targeted proteomics will be utilized routinely for sequencing protein isoforms with high throughput and high sensitivity.

CONCLUSIONS

In this study, we have developed a top-down targeted proteomics method which effectively and highly reproducibly purified Tm from muscle tissues without the use of antibodies and deeply characterized the sequence of all Tm isoforms as well as PTMs. We can define the sequence variants and PTM sites down to a single amino acid. The ubiquitous expression of Tm in eukaryotic cells from yeast to human underscores the high importance of Tm. Indeed, the role of Tm in the regulation of actin filament is increasingly appreciated and compelling evidence exists that Tm isoforms are functionally distinct in different cell types and species. The top-down targeted proteomics method we have established here provides comprehensive characterization of Tm isoforms from cardiac tissue, which can be applied to all striated muscle tissues and other tissue/cell types (with minor modifications) to facilitate a better understanding of the functional role of Tm isoforms in muscle and nonmuscle cells.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

| | |
|-----------------|---|
| Tm | Tropomyosin |
| MS | Mass spectrometry |
| DCM | Dilated cardiomyopathy |
| FHC | Familial hypertrophic cardiomyopathy |
| CAD | Collisionally activated dissociation |
| ECD | Electron capture dissociation |
| PTM | Post-translational modification |
| MWCO | Molecular weight cutoff |
| FT-ICR | Fourier transform ion cyclotron resonance |
| SDS-PAGE | Sodium dodecyl sulfate – polyacrylamide gel electrophoresis |
| ESI | Electrospray ionization |

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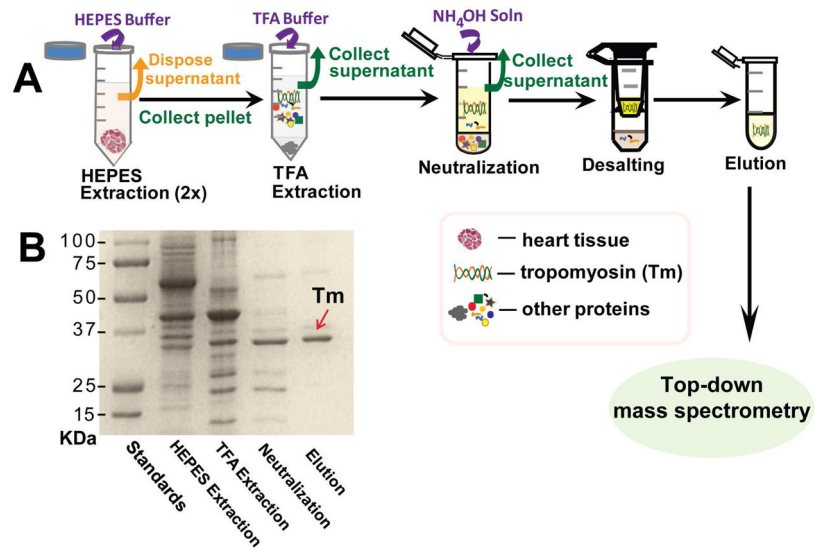


Figure 1. Flow diagram (A) and SDS-PAGE (B) analysis of swine Tm purification and characterization procedures

Tm was purified from swine heart tissue. Each lane of the SDS-gel represents different stages of the purification procedure.

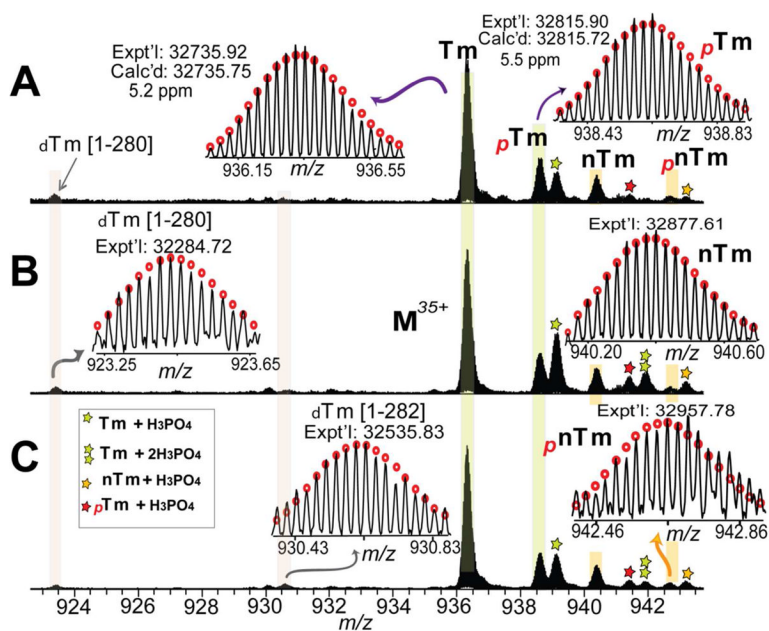


Figure 2. High-resolution MS analysis of intact Tm purified from three different swine hearts (A), (B) and (C)

Tm: swine α -Tm. nTm: a “novel” isoform of swine Tm. $_d$ Tm[1-282] and $_d$ Tm[1-280], C-terminal degradation products of swine α -Tm. $_p$ Tm and $_p$ nTm represent mono-phosphorylated Tm and nTm. The peaks marked with stars represented non-covalent phosphoric acid adducts. Red circles: the theoretical isotopic abundance distribution of the isotopomer peaks corresponding to the assigned mass. Calc’d, calculated most abundant molecular weight; Expt’l, experimental most abundant molecular weight. Molecular weights were calculated based on DNA-predicted sequence of *Sus scrofa* Tm (UnitprotKB/Swiss-Prot P42639, TPM1_PIG) with acetylation of the first amino acid at the N-terminus (+42.01) and two amino acid polymorphisms, P38→Q (−28.04) and P64→L (16.03).

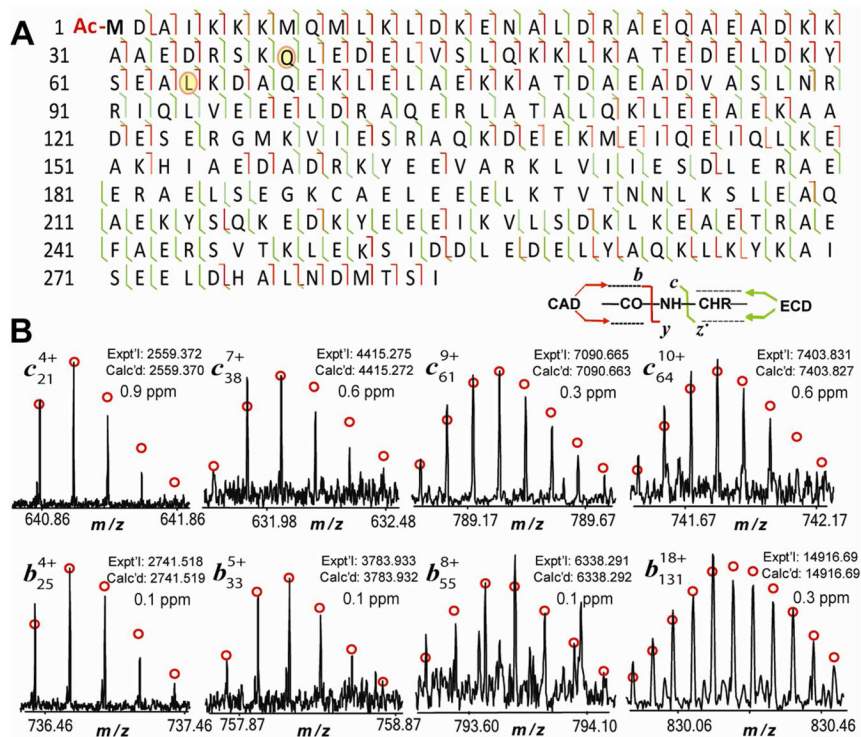


Figure 3. MS/MS product ion map (A) and representative product ions (B) from both ECD and CAD spectra of unphosphorylated swine α -Tm

Fragment assignments were made to the DNA-predicted sequence of *Sus scrofa* Tm (UnitprotKB/Swiss-Prot P42639, TPM1_PIG) with acetylation of the first amino acid at the N-terminus(42.01) and two amino acid polymorphisms, P38→Q (-28.04) and P64→L (16.03). A total of 228 out of 283 bond cleavages from five CAD and four ECD spectra are shown. Two amino acid polymorphisms were highlighted in orange circles. Circles: the theoretical isotopic distribution of isotopomer peaks. Calc'd, calculated most abundant molecular weight (MW). Expt'l, experimental most abundant MW.

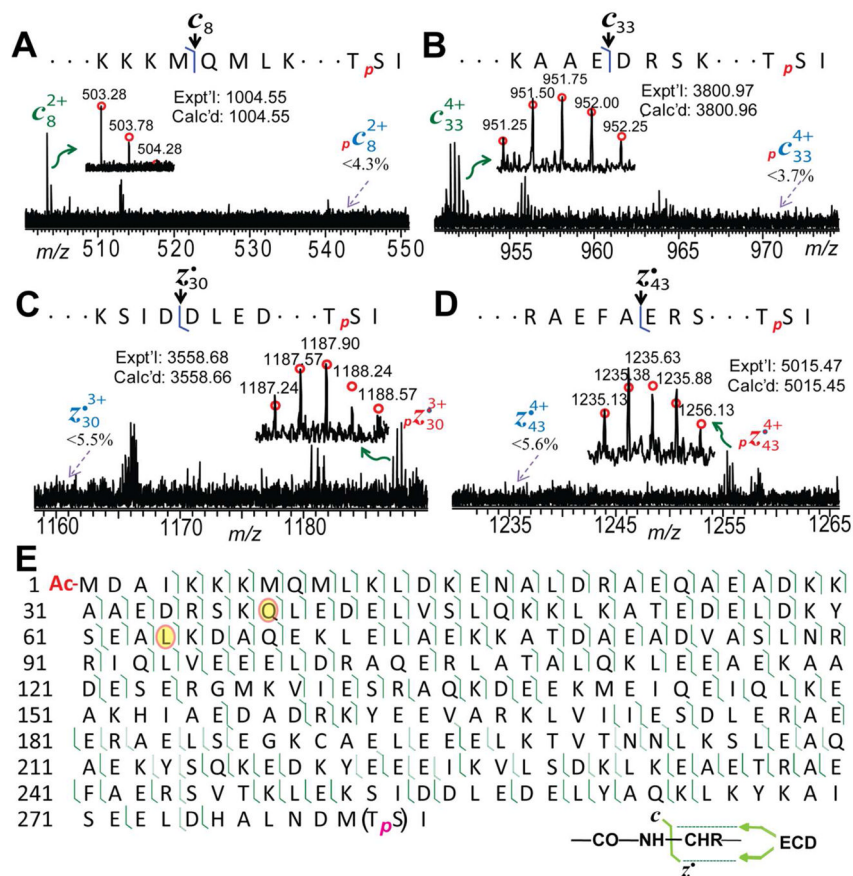


Figure 4. ECD for mapping the phosphorylation site of swine α -Tm

(A)–(D), Representative MS/MS spectra of c ions and z^* ions from ECD spectra of mono-phosphorylated swine α -Tm. (E) MS/MS ion map from ECD spectra of mono-phosphorylated swine α -Tm. The two amino acid polymorphisms were highlighted in orange circles and p presents the mono-phosphorylation site at Ser283. A total of 173 out of 283 bond cleavages from four ECD spectra are shown.

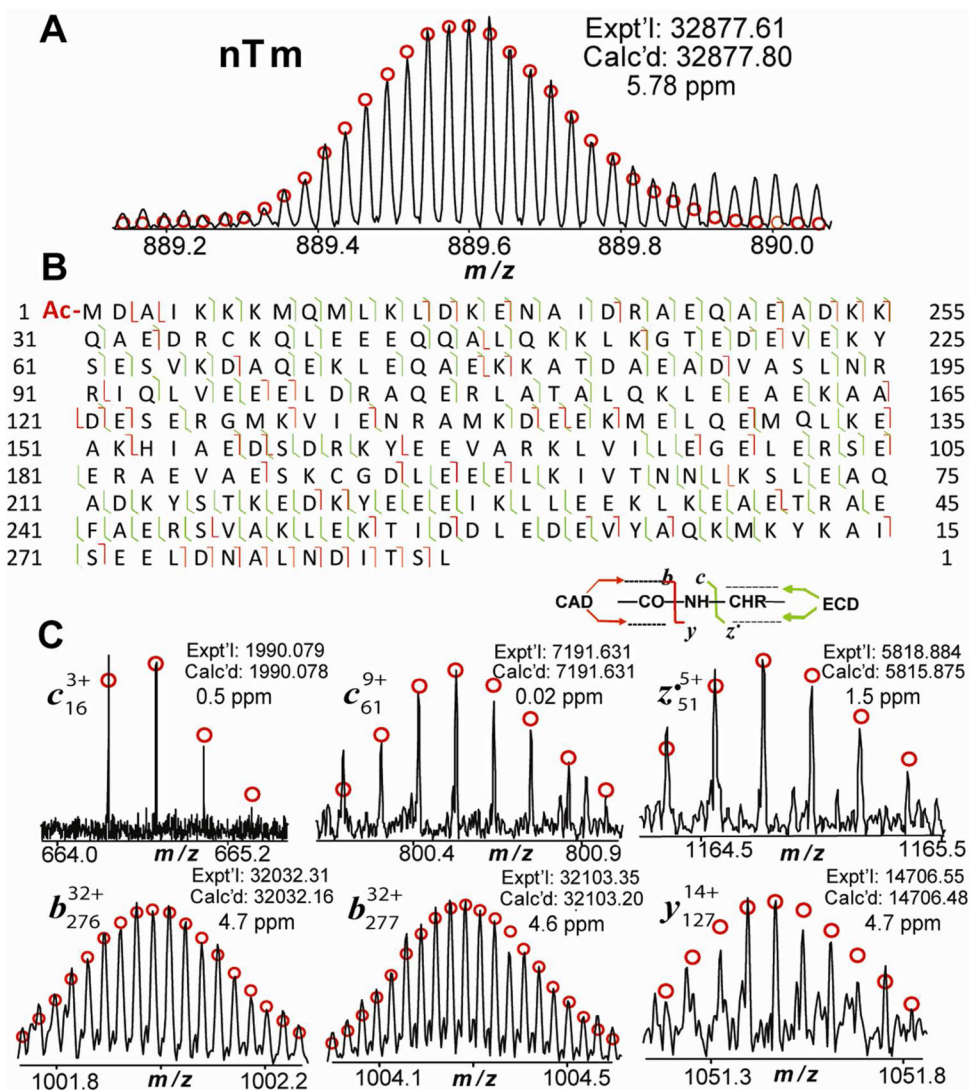


Figure 5. MS/MS characterization of a “novel” isoform of swine Tm (nTm)

(A) High-accuracy mass measurement of nTm (M^{37+}). (B) Fragmentation ion map of nTm from both ECD and CAD spectra. Fragment assignments were made to the DNA-predicted sequence of mouse β -Tm (UnitprotKB/Swiss-Prot P58774, TPM2_MOUSE) with acetylation of the first amino acid at N-terminus. A total of 137 out of 283 bond cleavages from 6 CAD and 2 ECD spectra are shown. (C) Representative ECD and CAD fragment ions from nTm.

CLUSTAL O(1.0.0) multiple sequence alignment

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SP|P42639|TPM1_PIG   MDAIKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLEDELVSLQKLLKATEDELDKY 60
SP|P58771|TPM1_MOUSE MDAIKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLEDELVSLQKLLKATEDELDKY 60
SP|P09493|TPM1_HUMAN MDAIKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLEDELVSLQKLLKATEDELDKY 60
TR|A1X899|A1X899_PIG MDAIKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLEDELVSLQKLLKATEDELDKY 60
SP|P58774|TPM2_MOUSE MDAIKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLEDELVSLQKLLKATEDELDKY 60
SP|P07951|TPM2_HUMAN MDAIKKMQMLKLDKENALDRAEQAEADKKAEDRSKQLEDELVSLQKLLKATEDELDKY 60
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

SP|P42639|TPM1_PIG   SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVVEEELDRAQERLATALQKLEEAEKAA 120
SP|P58771|TPM1_MOUSE SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVVEEELDRAQERLATALQKLEEAEKAA 120
SP|P09493|TPM1_HUMAN SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVVEEELDRAQERLATALQKLEEAEKAA 120
TR|A1X899|A1X899_PIG SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVVEEELDRAQERLATALQKLEEAEKAA 120
SP|P58774|TPM2_MOUSE SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVVEEELDRAQERLATALQKLEEAEKAA 120
SP|P07951|TPM2_HUMAN SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVVEEELDRAQERLATALQKLEEAEKAA 120
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

SP|P42639|TPM1_PIG   DESERGMKVIESRAQKDEEKMEIQ--EIQLKEAKHIAEDADRYEEVARKLVIESDLE 177
SP|P58771|TPM1_MOUSE DESERGMKVIESRAQKDEEKMEIQ--EIQLKEAKHIAEDADRYEEVARKLVIESDLE 177
SP|P09493|TPM1_HUMAN DESERGMKVIESRAQKDEEKMEIQ--EIQLKEAKHIAEDADRYEEVARKLVIESDLE 177
TR|A1X899|A1X899_PIG DESERGLKVIENRAMKDEEKMEIQEMQEMQLKEAKHIAEDSDRYEEVARKLVILEGELE 180
SP|P58774|TPM2_MOUSE DESERGMKVIESRAQKDEEKMEIQ--EMQLKEAKHIAEDSDRYEEVARKLVILEGELE 177
SP|P07951|TPM2_HUMAN DESERGMKVIESRAQKDEEKMEIQ--EMQLKEAKHIAEDSDRYEEVARKLVILEGELE 177
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

SP|P42639|TPM1_PIG   RAERAELSEGKCAELEELKVTNNLKSLEAQAEKYSQKEDRYEEEEKVLSDKLKEAET 237
SP|P58771|TPM1_MOUSE RAERAELSEGKCAELEELKVTNNLKSLEAQAEKYSQKEDRYEEEEKVLSDKLKEAET 237
SP|P09493|TPM1_HUMAN RAERAELSEGKCAELEELKVTNNLKSLEAQAEKYSQKEDRYEEEEKVLSDKLKEAET 237
TR|A1X899|A1X899_PIG RSEERAQVSRARQLEEEELRTMDQALKSIMASEEYESTKEDRYEEEEKVLSDKLKEAET 240
SP|P58774|TPM2_MOUSE RSEERAQVSRARQLEEEELKVTNNLKSLEAQADKYSTKEDRYEEEEKVLSDKLKEAET 237
SP|P07951|TPM2_HUMAN RSEERAQVSRARQLEEEELKVTNNLKSLEAQADKYSTKEDRYEEEEKVLSDKLKEAET 237
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

SP|P42639|TPM1_PIG   RAEFAERSVTKLEKSIDDELELYAQKLYKAISEELDHALNDMTSI 284
SP|P58771|TPM1_MOUSE RAEFAERSVTKLEKSIDDELELYAQKLYKAISEELDHALNDMTSI 284
SP|P09493|TPM1_HUMAN RAEFAERSVTKLEKSIDDELELYAQKLYKAISEELDHALNDMTSI 284
TR|A1X899|A1X899_PIG RAEFAERSVAKLEKTIDDELELYAQKLYKAISEELDHALNDMTSI 287
SP|P58774|TPM2_MOUSE RAEFAERSVAKLEKTIDDELELYAQKLYKAISEELDHALNDMTSI 284
SP|P07951|TPM2_HUMAN RAEFAERSVAKLEKTIDDELELYAQKLYKAISEELDHALNDMTSI 284
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

TR|A1X899|A1X899_PIG: TPM2
    
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Figure 6. Protein sequence homology alignment of Tm isoforms among various species
 The α - and β -Tm isoforms presented here are detected in pig, mouse and human, encoded by *TPM1* and *TPM2* genes. Highlighted areas marked in purple and green indicate amino acid substitutions between α -Tm and β -Tm across different species, respectively. Asterisks indicate the conserved sequence regions. A1X899_PIG is encoded by *TPM2* gene but the sequence is marked as “unreviewed” in the Swiss-Prot database.

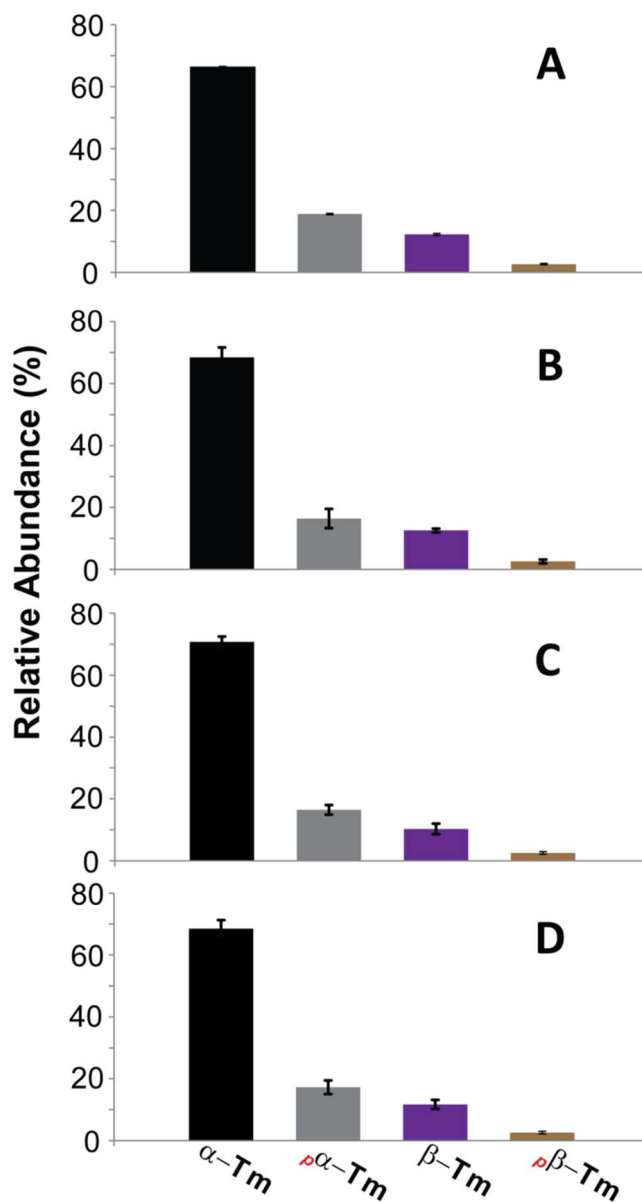


Figure 7. Relative quantification of two swine Tm isoforms (α -Tm and β -Tm) and their phosphorylation forms ($p\alpha$ -Tm and $p\beta$ -Tm) from three different swine hearts (A), (B) and (C), respectively. (D) Summary plot of Tm isoforms and their phosphorylated forms from a total of three biological replicates

The percentage of α -Tm, $p\alpha$ -Tm, β -Tm and $p\beta$ -Tm was defined as summed abundances of α -Tm, $p\alpha$ -Tm, β -Tm and $p\beta$ -Tm over the summed abundances of the entire Tm populations, respectively. The bar graphic data were expressed with means \pm S.D. Samples are run in triplicate.