**4th Joint EFLM-UEMS Congress "Laboratory medicine at the clinical interface"**, Warsaw, September 21-24th 2016, **Poster 0193**

# Human cardiac troponin T is cleaved by thrombin in serum samples

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## Introduction

CARDIAC TROPONIN T (cTnT) is one of the most important biomarkers of acute myocardial infarction (AMI) (1). After heart attack cTnT is released into the bloodstream from damaged myocardial cells and can be measured in blood samples by immunoassays. Analysis of cTnT in serum samples of AMI patients (2-4) or incubated *in vitro* (as a part of a native ternary complex (ITC complex)) in normal human serum (NHS) (4, 5) revealed a set of proteolytic fragments with apparent molecular masses of 29 (predominant form), 19, 18 and 16 kDa (4).

Experiments with cultivated cardiomyocytes showed that cTnT could be cleaved between R68/S69 amino acid residues (aar) by μ-calpain (calpain-1) (6). It was assumed that μ-calpain could cleave cTnT in serum as well (4). However, μ-calpain is a protease located intracellularly (6), so it is hard to expect its well-pronounced activity in blood. At the same time, no degradation of cTnT was observed during the incubation of ITC complex in normal heparin plasma (NHP) (5). Taking into consideration that serum differs from plasma by the activation of coagulation enzymes, the degradation of cTnT in serum could be explained by the action of these enzymes, rather than by the action of μ-calpain. So in the current study the influence of the most abundant coagulation protease, thrombin, on the cTnT degradation was analyzed.

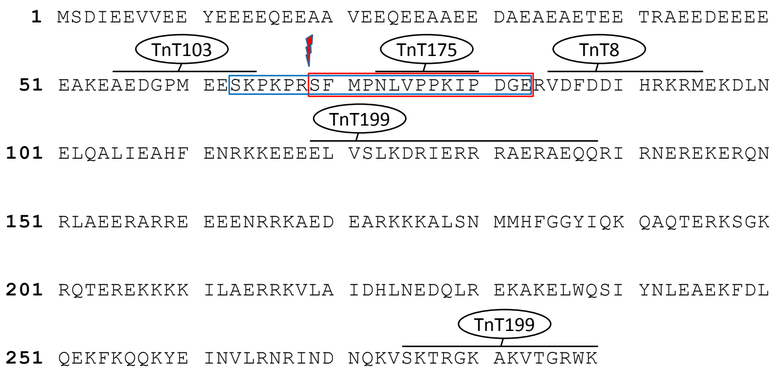


FIGURE 1. **The sequence of human cardiac TnT (isoform 6).** Epitopes of mAbs used in the study are indicated above the sequence.
Blue box - characteristic peptide identified by MS/MS after GluC proteolysis of the full-sized recTnT.
Red box - characteristic peptide identified by MS/MS after GluC proteolysis of the 29 kDa fragment of recTnT.

## Methods

*IN VITRO* CLEAVAGE OF cTNT BY THROMBIN was performed by spiking of human recombinant cardiac troponin T (recTnT) or ITC complex (HyTest) into NHS or NHP samples (both – at concentration 50 ng/ml of cTnT), or into buffer (20 mM Tris-HCl, pH 7.5; 150 mM KCl, 5 mM $CaCl_2$, 75 g/L bovine serum albumin, 0.15 g/L $NaN_3$) containing 5 NIH U/ml of human thrombin (Sigma). All solutions were incubated for 3h at 37°C. In control samples thrombin activity was inhibited by an addition of 50 USP U/ml of heparin or 50 IU/ml of recombinant hirudin before cTnT was added.

IMMUNOPRECIPITATION of cTnT was performed on affinity matrices containing mAbs, specific to the different epitopes of cTnT molecule (HyTest).

IMMUNODETECTION of cTnT and its fragments was performed by means of ECL with mAbs TnT103 (specific to 56-60 aar), TnT175 (72-82), TnT8 (82-96), TnT313 (119-138) and TnT199 (275-288), conjugated with HRP (Fig. 1).

MS OF THE INTACT recTNT AND ITS FRAGMENTS obtained by thrombin-mediated cleavage was performed in the linear mode of ultrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker). MS/MS analysis was performed in positive ions mode with reflectron. Prior to MS/MS proteins were separated via SDS-PAGE, bands corresponding to the full-sized recTnT and a 29-kDa fragment were excised from gel and digested by endoproteinase GluC (V8 Protease).

## Results and discussion

COMPARISON OF cTNT FROM SERUM AND HEPARIN PLASMA SAMPLES OF AMI PATIENTS revealed that cTnT in heparin plasma samples was present mainly as the full-sized molecule, with minor presence of proteolytic fragments, whereas cTnT in serum samples collected from the same patients was present mainly as the 29-kDa fragment (Fig. 2).

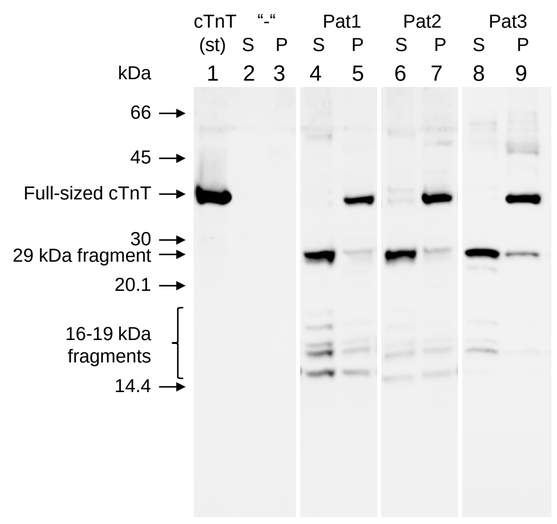


FIGURE 2. **Comparison of cTnT fragmentation in serum and heparin plasma samples of AMI patients.** cTnT and its fragments, immunoprecipitated from serum and heparin plasma samples of three representative AMI patients. Staining by mAb TnT313, specific to the 119-138 aar of cTnT.
1 – recTnT
2 – NHS
3 – NHP
4-9 – serum (S) and heparin plasma (P) samples of 3 AMI patients

THROMBIN-MEDIATED DEGRADATION OF cTNT. Incubation of cTnT in buffer containing thrombin or in NHS resulted in a formation of 29-kDa fragment similar to one seen in serum samples of AMI patients (Fig. 3). Incubation of cTnT in heparin NHP did not lead to any noticeable degradation of the protein, Addition of heparin or hirudin in NHS or buffer with thrombin resulted in an inhibition of cTnT degradation, meaning that it is thrombin that cleaves cTnT in serum.

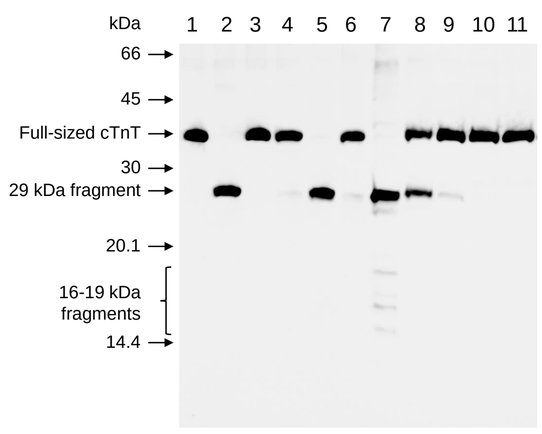


FIGURE 3. **Thrombin-mediated degradation of cTnT.** cTnT was incubated for three hours at 37°C in buffer with thrombin, NHS or NHP. cTnT and its fragments were extracted by means of immunoprecipitation and stained in WB by mAb TnT313.
1 - recTnT in buffer
2 - recTnT in buffer with thrombin
3 - recTnT in buffer with hirudin-pretreated thrombin
4 - ITC complex in buffer
5 - ITC complex in buffer with thrombin
6 - ITC complex in buffer with hirudin-pretreated thrombin
7 - cTnT extracted from AMI serum
8 - recTnT in NHS
9 - recTnT in heparin-pretreated NHS
10 - recTnT in hirudin-pretreated NHS
11 - recTnT in heparin NHP

LOCALIZATION OF THROMBIN CLEAVAGE SITE was performed by means of WB and MS. In WB TnT103 (55-64 aar) stained only the full-sized molecule and did not stain the 29-kDa fragment of cTnT, while TnT175 (73-80 aar) stained both bands, meaning that the site of cleavage is located between the epitopes of these two mAbs (Fig. 4). TnT8, TnT313 and TnT199 all stained the same fragment as the mAb 175, suggesting that there are no additional cleavage sites between the epitopes of mAbs TnT175 and TnT199 (275-288 aar).

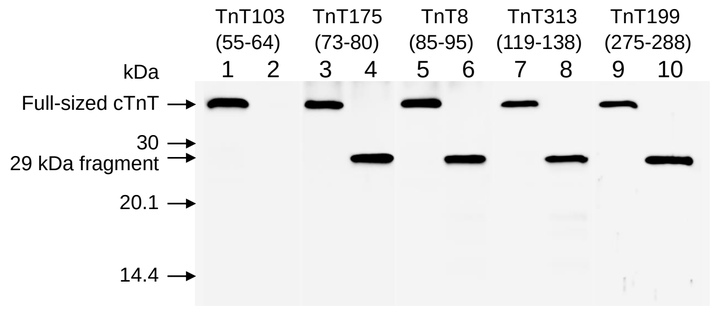


FIGURE 4. **Localization of thrombin cleavage site of cTnT in WB.** recTnT was incubated for three hours at 37°C in buffer without (tracks 1, 3, 5, 7, 9) or with (tracks 2, 4, 6, 8, 10) addition of thrombin. recTnT and it's fragments were stained in WB by mAb TnT103 (tracks 1, 2); TnT175 (tracks 3, 4); TnT8 (tracks 5,6); TnT313 (tracks 7,8) and TnT199 (tracks 9-10). Epitope of each mAb is indicated in brackets.

The MS of the proteins obtained after thrombin-mediated cleavage and immunopurified on the affinity matrix, specific to the N-terminal part of cTnT, showed two peaks of 7704 and 3852 Da (Fig. 5A). The mass of the first peak was in a good agreement with the mass of the 2-68 fragment of cTnT (estimated mass: 7703.7 Da), and the second peak corresponded to the doubly charged ion of the same peptide.

The MS of the proteins, immunopurified on affinity matrix, specific to the central/C-terminal part of cTnT, determined the presence of three peaks of 26797, 13384 and 8926 Da (Fig. 5B). The mass of the first peak went in a good agreement with the mass of 69-288 fragment of cTnT (estimated mass - 26773.3 Da), with two other peaks corresponding to the doubly and triply charged ions of the same peptide.

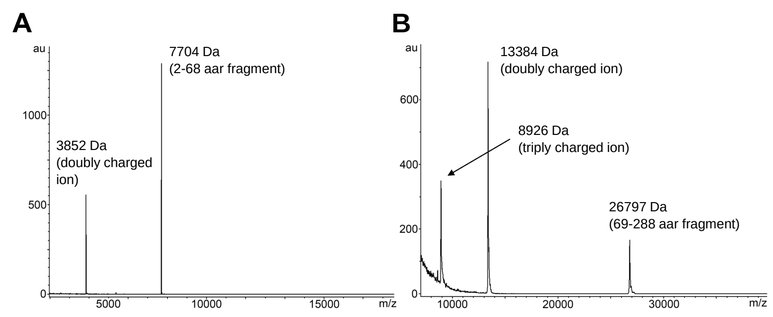


FIGURE 5. **MS of cTnT fragments obtained by thrombin cleavage in buffer solution.**
A – MS of the eluate from the matrix, specific to the N-terminus of cTnT
B – MS of the eluate from the matrix, specific to the C-terminal part of cTnT

The MS/MS of the peptides obtained after GluC-mediated cleavage of the full-sized recTnT resulted in a formation, among others, of a characteristic mass of 2334.1 Da that corresponded to the peptide SKPKPRSFMPNLVPPKIPDGE (62-83 aar, the expected molecular mass - 2333.3 Da) (Fig. 1, blue box). In the same time, the MS/MS of the peptides obtained after GluC-mediated cleavage of the 29-kDa fragment resulted in a formation of a mass of 1639.8 Da, corresponding to a peptide SFMPNLVPPKIPDGE (69-83 aar, the expected molecular mass - 1639.7 Da) (Fig. 1, red box). The results of MS and MS/MS confirm that the 29-kDa band corresponds to the 69-288 fragment of cTnT and locate the site of thrombin cleavage between amino acid residues R68/S69 of cTnT.

## Conclusions

We suggest that the degradation of cTnT that is observed in serum samples of AMI patients and results in a formation of the 29 kDa fragment occurs mainly due to the activation of thrombin during serum preparation and thrombin-mediated cleavage of cTnT molecule at the site R68/S69. This should be considered during the investigation of cTnT degradation and development of the new immunochemical methods for cTnT measurement.

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