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Short communication

Temperature-dependent instability of the cTnI subunit in NIST SRM2921 characterized by tryptic peptide mapping

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ABSTRACT

In this study temperature-dependent instability of the cTnI subunit of the three-protein complex NIST SRM2921 was demonstrated using a mass spectrometric tryptic peptide mapping approach. The results were compared to the cTnI subunit obtained as a protein standard from Calbiochem with identical amino acid sequence. Both the three-protein complex from NIST as well as the cTnI subunit were incubated at elevated temperatures and then evaluated with respect to the primary sequence. The corresponding peptide maps were analyzed using LC–MS/MS. From a Mascot database search in combination with "semiTrypsin" tolerance it was found that two peptide backbone cleavages had occurred in subunit cTnI in NIST SRM2921 material upon incubation at 37 °C, namely between amino acids at 148/149 and 194/195. The Calbiochem standard did not show increased levels of "unexpected" peptides in tryptic peptide maps. One of the two peptide backbone cleavages could also be monitored using a "single-step" MALDI-MS approach, i.e. without the need for peptide separation. The amount of degradation appeared rather constant in replicate temperature-instability experiments. However, for accurate quantification internal labelled standards are needed.

1. Introduction

Cardiac troponin I (cTnI) was first reported as a marker for diagnosis of myocardial infarction (MI) in 1987 [1]. From the complex of three regulatory proteins (referred to as C, T and I) both the subunits cTnI and cTnT are now widely used as a "gold standard" cardiac biomarker for MI in a variety of immunoassays, the one for cTnT being patented [2–4]. In order to standardize clinical cTn-measurements the International Federation of Clinical Chemistry's Working Group for the Standardization of Troponin I (IFCC WG-TNI) has been established [5]. A human cardiac troponin complex from the National Institute of Standards & Technology (NIST) is widely used as a standard material (calibrant) in clinical labs. This three-protein complex is purified from human heart and referred to as standard reference material (SRM) 2921. From previous work and efforts made within this IFCC group a "stable region" has been determined in the cTnI peptide sequence [4]. Obviously, this part of the subunit is most attractive for the development of a capture monoclonal antibody (mAb). The terminology "stable region" implies the presence of a less stable part within the protein sequence. Indeed, data on time-dependent instability of cTnI have been reported, potentially influencing assay standardization [6–9].

In the current study we aim to obtain more detailed information on which parts of the polypeptide may suffer from degradation. A convenient approach to verify amino acid sequence is by creating a peptide map of the protein [10]. In order to obtain full sequence coverage often multiple experiments (with various specific enzymes) are needed. In combination with confident MS/MS-assignment of peptides many types of changes within a protein can be mapped. In general, proteins are relatively labile compounds with diverse properties. Factors that may promote degradation and instability are mechanical stress (shear), adsorption, heat, pH, ionic strength, denaturants and impurities. A notorious example of the latter one is the presence of proteases in biological samples. Chemical degradation pathways are hydrolysis, deamidation, oxidation, shuffling of disulfide bridges, deglycosylation or isomerization, whereas physical protein decomposition results from denaturation, aggregation, precipitation or adsorption. Obviously, awareness of possible instability of biological compounds is of great importance in analytical studies, and in the case of production of (bio)pharmaceuticals it is even *obligatory* to monitor (in)stability over time. Regulatory authorities require well-defined stability studies on biologically active compounds before (drug substance) and after (drug product) these are launched on the market. For this latter reason, peptide mapping based on MS has been extensively developed by industrial

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protein production groups [11–13]. Confirmation of the primary structure of therapeutic proteins is pivotal in quality control (QC) methodologies in (biotechnology) industry, as well as the detection of mutations or post-translational modifications (PTMs). Nevertheless, the term "peptide mapping" has become less popular due to the emergence of MS-based proteomics and metabolomics, which are now routine technologies in characterizing and quantifying a proteome or metabolome with still increasing numbers and types of applications [14–16].

MS allows high-throughput and multiplexed molecular profiling of biological samples and has become a major player in the clinical research. In most of these approaches the protein is characterized through a bottom-up procedure, i.e. smaller peptides are obtained using an enzyme or a specific chemical cleavage. The resulting peptides are then separated by liquid chromatography (LC), and mass analyzed and sequenced using electrospray ionization (ESI) MS/MS. The chromatogram is referred to as a peptide map that functions as a basis to check multiple batches from similar protein productions. In these maps the previously identified peptides do not require further MS-analysis, only the newly appearing chromatographic peaks need full structure elucidation. Alternatively, the bottom-up peptides are mass analyzed in a matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) spectrum, yielding a peptide mass fingerprint. Although this strategy has become less popular because of early relatively low quality data, modern (high resolution) mass analyzers provide confident peptide identifications from a fingerprint. Moreover, often some peptides are not observed in an ESI peptide map and MALDI may provide complementary sequence coverage [17]. Finally, it should be noted that there is a growing interest in characterizing an intact protein. Such top-down strategies require the use of ultrahigh resolution analyzers (Fourier transform ion cyclotron resonance [FTICR], orbitrap or high-end TOF) that yield complex but extremely rich MS/MS-data [18,19]. The obvious advantage is that no protein cleavage is required, thus avoiding the introduction of a potential systematic error during sample workup. Recently, top-down MS has been used to fully characterize swine cTnI; however, this method is not suitable (yet) to measure the intact three-protein complex [20]. Moreover, top-down approaches have great potential, but have not yet been challenged with respect to quality guidelines. Therefore, in this work we have chosen for "classical" peptide mapping, which in addition is easier to implement in a clinical lab as a routine assay.

2. Materials and methods

2.1. Samples

Human cardiac troponin complex was obtained from NIST as SRM2921. The complex was produced by HyTest Ltd. (Turku, Finland) and purified from human heart. It consists of the three subunits troponin T, troponin I and troponin C. The SRM2921 is contained in 115 μl vials and was shipped on dry ice by NIST and stored at −80 °C after arrival until further use. The material used in this study was obtained from two independent shipments (received JUL2010 and OCT2011, respectively) with an identical NIST certificate of analysis (expiration date 30JUN2015). The troponin complex is supplied in 150 mM sodium chloride, 5 mM calcium chloride and 20 mM Tris buffer, pH 7.5. The material is used as the primary reference material for cardiac troponin I (refs) with a certified cTnI concentration of 31.2 ± 1.4 mg/l. The troponin T and C concentrations are 36.9 ± 3.8 mg/l and 24.2 ± 1.3 mg/l, respectively.

Cardiac human troponin subunit I (100 μg lyophilized powder purified from human heart, Calbiochem 648480; Merck, Darmstadt, Germany) was dissolved in MilliQ water to a concentration of 100 mg/l and kept on ice while aliquotting in 10 μl portions. The aliquots were stored at −80 °C in 0.5 ml polypropylene tubes (Sarstedt).

2.2. Temperature-dependent degradation studies

NIST SRM2921 was studied in a temperature-dependent degradation study by comparing an untreated sample ("t = 0") with one that was incubated at 37 °C for 48 h ("t = 48h@37C") and one that was placed on ice for 48 h ("t = 48h@4C"). For this purpose a 115 μl vial (see Sample description) was thawed on ice and aliquotted in eleven 10 μl vials. One aliquot was immediately snap frozen ("t = 0") and a second one was placed at 37 °C for 48 h in a Hettich incubator ("t = 48h@37C"). A third aliquot was placed on ice for 48 h ("t = 48h@4C"). The remaining aliquots were snap frozen and stored at −80 °C until further use. This temperature-degradation experiment was repeated a couple of weeks later using three of the remaining aliquots (same shipment, same 115 μl mother tube). During this second experiment a similar procedure was followed using aliquots prepared from the Calbiochem cTnI standard ("t = 0" + "t = 48h@4C" + "t = 48h@37C"). Again, a couple of weeks later a new 115 μl vial with NIST SRM2921 (different shipment) was aliquotted in eleven 10 μl vials, and a third temperature-degradation experiment was performed in a similar way. In addition, the Calbiochem cTnI standard was monitored in this third experiment.

2.3. Reduction, alkylation and tryptic digestion

To each 10 μl of troponin solution (approximately 923 ng protein, cTnI + C + T for NIST SRM2921 and 1000 ng cTnI Calbiochem) 2 μl 60 mM DTT in 25 mM ammonium bicarbonate (ABC) was added, followed by incubation for 45 min at 56 °C to reduce cysteins [21]. Then, 100 mM iodoacetamide (IAA) in ABC was added to a final IAA concentration 25 mM and the samples kept in dark for 1 h at room temperature to alkylate and protect the cysteins. The reduced and alkylated troponin subunits (cTnI, cTnT and cTnC) were overnight digested at 37 °C after adding 10 μl 100 ng/μl sequencing-grade trypsin (sequencing grade, Promega, Madison, WI, USA) in a freshly prepared 25 mM ABC solution. The digestion was quenched by adding 5 μl 10% TFA to lower the pH. The peptide digests were stored at −80 °C until analysis.

2.4. Tryptic peptide mapping using LC–MS/MS

For LC–MS/MS experiments, an Amazon ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) was coupled on-line to a parallel, splitless NanoLC-Ultra 2D plus system (Eksigent, Dublin, CA, USA) with additional loading pumps for fast sample loading and washing, which resulted in high chromatographic peak capacity [21]. The LC-system was configured with a 15-cm 300 μm-i.d. ChromXP C18 column purchased from Eksigent and a linear 90 min gradient from 4 to 44% acetonitrile in 0.05% formic acid was applied at a flowrate of 4 μl/min. The LC system was controlled by HyStar 3.2–3.4 with a plugin from the LC manufacturer, and the ion trap by Bruker esquireControl 6.2. The acquired data was automatically transferred to a dedicated server and processed as follows. Each individual MS/MS dataset provided by the ion trap was converted to MGF files using DataAnalysis (Bruker Daltonics). The datasets were separately searched using Mascot 2.1. All identified peptides with a best Mascot ion score of at least 25 were used for further analysis.

2.5. Tryptic peptide mapping using MALDI-FTICR-MS

MALDI-FTICR experiments were performed on a Bruker 15 tesla solariX^{TM} FTICR mass spectrometer equipped with a novel CombiSource (Bruker Daltonics) [22]. The MALDI-FTICR system was controlled by Compass solariXcontrol software and equipped with a Bruker Smartbeam-II^{TM} Laser System that operated at a frequency of 500 Hz. The "medium" predefined shot pattern was used for the irradiation. Each mass spectrum was obtained from a single scan of 600 laser shots using 512 K data points. Typically, the target plate offset was 100 V with the deflector plate set at 180 V. The ion funnels operated at 100 V and 6.0 V, respectively, with the skimmers at 15 V and 5 V. The trapping potentials were set at 0.60 V and 0.55 V, the analyzer entrance was maintained at −7 V, and side kick technology was used to further optimize peak shape and signal intensity. The required excitation power was 28% with a pulse time of 20.0 μs.

3. Results and discussion

Both intact troponin (the three-protein complex NIST SRM2921) as well as the purified subunit cTnI (Calbiochem) were used for the described experiments. An aliquot of the thawed material was snap frozen and stored at −80 °C until further analysis before starting a temperature-dependent degradation study. Such aliquots are referred to as "t = 0" samples. Possible degradation of the polypeptide backbone was monitored by incubating freshly thawed material for 48 h at either 4 °C or at 37 °C, yielding "t = 48h@4C" and "t = 48h@37C" samples. After incubation these samples were snap frozen and stored at −80 °C until further analysis. The thus obtained samples (SRM2921 and Calbiochem cTnI; t = 0, t = 48h@4C and t = 48h@37C) were all processed at the same time according to a standard reduction/alkylation/digestion protein sample preparation protocol [21]. In short, disulfide bonds in the polypeptides were reduced and alkylated, and then the polypeptides were digested with trypsin. The tryptic peptides were analyzed in an LC–MS experiment, using reversed-phase liquid chromatography (RP-LC) coupled with an ion trap (IT). During each LC–MS run tryptic peptides were identified by automatic selection of precursor ions for MS/MS according to a standard workflow [21]. A typical example of such an experiment for sample SRM2921/t = 0 is depicted in Fig. 1a. In the upper panel the total ion current (TIC) of the full run is shown. In the middle panel of Fig. 1a the extracted ion chromatogram (EIC) is shown for tryptic peptide ISADAMMQALLGAR (observed as a doubly charged ion at m/z 724.4), and in the lower panel for tryptic peptide NIDALSGMEGR (observed as a doubly charged ion at m/z 581.7). Both peptides were also observed as triply charged ions and were sequenced at least two times per LC-run yielding high Mascot ion scores (typically between 40 and 70). Note that this latter peptide has been reported previously in a study to quantify cTnI via an MRM-assay [4]. Moreover, in the same LC–MS run multiple tryptic peptides from cTnT as well as cTnC were identified with high ion scores (>40, data not shown). Interestingly, additional (human) proteins were identified in sample SRM2921/t = 0, namely mitochondrial ATP synthase beta-subunit (Uniprot P06576), cardiac actin (P62736), myosin-binding protein C (Q14896), creatine kinase S-type (P17540), tropomyosin 1 (P09493), myomesin 1 (P52179), and myomesin 2 (P54296). Previously, some of these proteins were identified [23]. Their presence follows from the source of the isolated troponin. It is possible that these proteins play an important role in the here reported sample instability of NIST SRM2921 (as shown further). This hypothesis is currently being investigated, in combination with the effect of changing the buffer system or cross-linking the protein backbone.

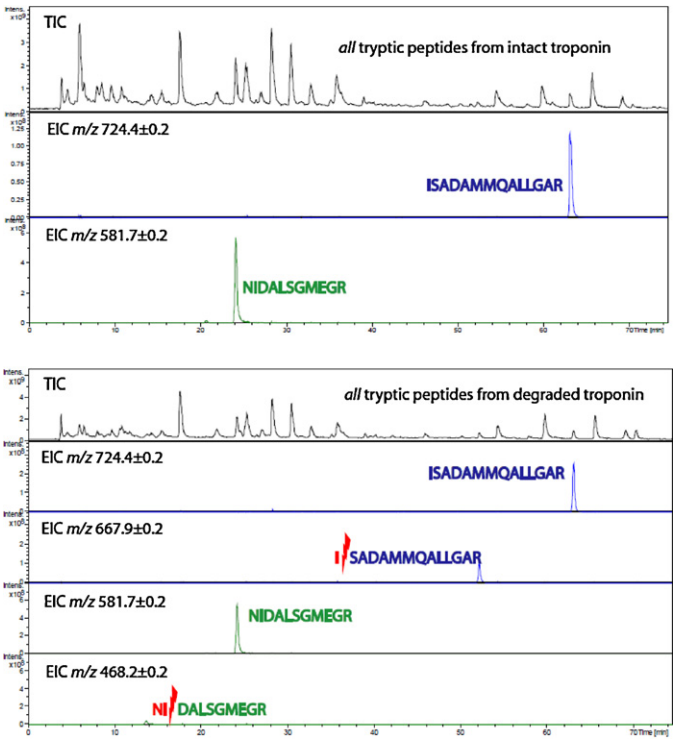

Fig. 1. LC–MS/MS analysis of tryptic peptides obtained from SRM2921/t = 0 (a) and SRM2921/t = 48h@37C (b).

From the LC-run depicted in Fig. 1a, in SRM2921/t = 0 ten different tryptic peptides from cTnI were identified with high ion scores when performing a standard Mascot search. Taking into account one or two missed cleavages in the tryptic digest yielded another five (partially overlapping) peptides. These 15 peptides add to a total sequence coverage of 58% as overviewed in Scheme 1a. The LC–MS run of the tryptic peptides obtained from the same material after temperature incubation (SRM2921/t = 48h@37C) is depicted in Fig. 1b. In this sample two additional tryptic peptides were observed both containing one missed cleavage, namely GRALSTR and FKRPTLR (Scheme 1b). These peptides result in an increased sequence coverage (65%), but are not directly informative about

a

ADGSSDAARE PRPAPAPIRR RSSNYRAYAT EPHAKKKSKI SASRKLQLKT LLLQIAKQEL

EREAEERRGE KGRALSTRCQ PLELAGLGFA ELQDLCRQLH ARVDKVDEER YDIEAKVTKN

ITEIADLTQK IFDLRGKFKR PTLRRVRISA DAMMQALLGA RAKESLDLRA HLKQVKKEDT

EKENREVGDW RKNIDALSGM EGRKKKFES

b

ADGSSDAARE PRPAPAPIRR RSSNYRAYAT EPHAKKKSKI SASRKLQLKT LLLQIAKQEL

EREAEERRGE KGRALSTRCQ PLELAGLGFA ELQDLCRQLH ARVDKVDEER YDIEAKVTKN

ITEIADLTQK IFDLRGKFKR PTLRRVRISA DAMMQALLGA RAKESLDLRA HLKQVKKEDT

EKENREVGDW RKNIDALSGM EGRKKKFES

Scheme 1. (a) Tryptic peptide sequence coverage of subunit cTnI from intact troponin (NIST SRM2921). Tryptic peptides observed in the LC–MS analysis are singly underlined. Doubly underlined amino acids were parts of peptides containing one or two missed cleavages. (b) Tryptic peptide sequence coverage of subunit cTnI from degraded troponin (NIST SRM2921). Tryptic peptides observed in the LC–MS analysis are singly underlined. Doubly underlined amino acids were parts of peptides containing one or two missed cleavages. The four peptides indicated in grey were additional ones compared to the tryptic peptide map from intact troponin. Note that the peptides SADAMMQALLGAR and DALSGMEGR result from temperature-dependent cleavages between amino acids 148 and 149, and 194 and 195, respectively.

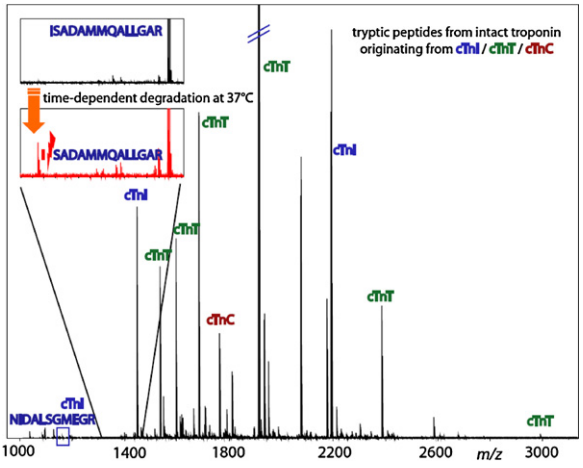

Fig. 2. MALDI-FTICR spectrum of tryptic peptides obtained from SRM2921/t = 0. In the inset the m/z-range from 1300 to 1460 is enlarged, of which the second panel displays a new tryptic peptide obtained from SRM2921/t = 48h@37C.

possible degradation pathways. In contrast, two other identified peptides in this sample *are* indicative for polypeptide backbone degradation, namely SADAMMQALLGAR and DALSGMEGR. The first peptide results from temperature-dependent cleavage between amino acids 148 and 149, the second one from cleavage between amino acids 194 and 195 (Scheme 1b). Both of these peptides are outside the previously defined "stable region" of the cTnI sequence [4]. It is important to note that these two peptides can only be found in a database search when allowing for one specific cleavage, i.e. in Mascot referred to as "semiTrypsin". The inclusion of this tool in Mascot was stimulated by the MS-based proteomics community to allow for the identification of endogenous peptides or perform other searches on non-matched MS/MS-spectra. As pointed out, the presence of these two "semiTryptic" peptides in a tryptic digest hints towards cleavage at the intact protein level, i.e. the cTnI subunit, during temperature-degradation. Thus, the use of this tool can be helpful in finding instability-indicating peptides. Nevertheless, when used as such it is important to carefully map *all* a-specific peptides since it is known that low amounts of "semiTryptic" peptides may be present in a tryptic digest. Thus, in order to further verify the observed backbone cleavages in cTnI the LC–MS runs were inspected with regard to *all* "semiTryptic" peptides in the digests. To this end, the EIC's of m/z 667.9 (doubly charged SADAMMQALLGAR) and m/z 468.2 (doubly charged DALSGMEGR) were compared for t = 0, for t = 48h@4C, and t = 48h@37C material. It was found that the two "degradation-indicating" peptides were neither present in trypsin-digested starting materials SRM2921/t = 0 and Calbiochem/t = 0, nor in SRM2921/t = 48h@4C and Calbiochem/t = 48h@4C. In SRM2921/t = 48h@37C both peptides were present, namely at chromatographic intensities of 30% and 7%, respectively (compared to its "expected" counterpart in the same LC–MS run). Remarkably, in Calbiochem/t = 48h@37C the peptides SADAMMQALLGAR and DALSGMEGR were fully absent. For both types of samples, i.e. NIST and Calbiochem, no significant increase or decrease in the intensity of any other "semiTryptic" peptide was observed upon temperature-degradation, albeit that these peptides were still identified with high ion scores.

Finally, the tryptic digests of all samples were analyzed using an ultrahigh resolution MALDI-FTICR platform [22]. Such a platform provides a fast and single-step experiment to map tryptic peptides at sub-ppm mass precision and thus monitor possible degradation of proteins. A typical example of a MALDI-FTICR spectrum of SRM2921/t = 0 is shown in Fig. 2, with tryptic peptides observed for all three subunits. The expected tryptic peptides from the cTnI subunit ISADAMMQALLGAR and NIDALSGMEGR were observed as singly protonated species at m/z-values 1447.740 and 1162.552, respectively. In the degraded material SRM2921/t = 48h@37C these peptides were still present, and an additional signal appeared at m/z 1334.656, which corresponds to singly protonated SADAMMQALLGAR (see inset Fig. 2). The ratio between the intensities of and m/z 1334.656 and m/z 1447.740 was approximately 1–9. Note that this "degradation-indicating" peptide was found in the LC–MS peptide map at an intensity ratio of 3 to 7, compared to ISADAMMQALLGAR. From this difference between MALDI- and ESI-quantification it follows that for an accurate determination of the amount of degradation synthetic labelled analogues are required. Unfortunately, the peptide DALSGMEGR (singly protonated at m/z 935.426) was not observed in any of the MALDI-FTICR spectra of degraded material, implying the intensity was below the limit of detection (since this peptide was observed in the corresponding LC–MS runs). In conclusion, the MALDI-FTICR experiments can be used for mapping possible degradation of troponin, but the more time-consuming LC–MS runs provide more elaborate and in-depth data of all the peptides that result from a tryptic digestion.

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