Strategy for Analysis of Cardiac Troponins in Biological Samples with a Combination of Affinity Chromatography and Mass Spectrometry

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Background: Cardiac troponins are modified during ischemic injury and are found as a heterogeneous mixture in blood of patients with cardiovascular diseases. We present a strategy to isolate cardiac troponins from human biological material, by use of affinity chromatography, and to provide samples ready for direct analysis by mass spectrometry.

Methods: Cardiac troponins were isolated from human left ventricular tissue by affinity chromatography. Isolated troponins were either eluted and analyzed by Western blot or enzymatically digested while bound to affinity beads. The resulting peptide mixture was subjected to mass spectrometry for protein identification and characterization. The same method was used to analyze serum from patients with acute myocardial infarction (AMI).

Results: Affinity chromatography with antibodies specific for one cardiac troponin subunit facilitated the isolation of the entire cardiac troponin complex from myocardial tissue. The three different proteases used for enzymatic digestion increased the total protein amino acid sequence coverage by mass spectrometry for the three cardiac troponin subunits. Combined amino acid sequence coverages for cardiac troponin I, T, and C (cTnI, cTnT, cTnC) were 54%, 48%, and 40%, respectively. To simulate matrix effects on the affinity chromatography–mass spectrometry approach, we diluted tissue homogenate in cardiac troponin-free serum. Sequence coverages in this case were 44%, 41%, and 19%, respectively. Finally, affinity chromatography–mass spectrometry analysis of AMI serum revealed the presence of cardiac troponins in a wide variety of its free and/or complexed subunits, including the binary cTnI-cTnC and cTnI-cTnC-cTnT complexes.

Conclusions: Affinity chromatography–mass spectrometry allows the extraction and analysis of cardiac troponins from biological samples in their natural forms. We were, for the first time, able to directly confirm the presence of cardiac troponin complexes in human serum after AMI. This approach could assist in more personalized risk stratification as well as the search for reference materials for cardiac troponin diagnostics.

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In 1987, Cummins et al. (1) reported the first measurement of cardiac troponin I (cTnI)5 as a diagnostic marker for acute myocardial infarction (AMI). Since then, the development of increasingly sensitive and specific assays for cTnI and cardiac troponin T (cTnT) has led to the recommendation that these proteins be the new laboratory standard for AMI diagnosis (2). The ever-increasing number of different assays that are available documents the interest in cardiac troponin diagnostics (3).

A major drawback of cTnI assays is the lack of standardization (4–7). There are several reasons for this lack of standardization: The antibodies used in diagnostic assays are directed against different regions on the cTnI molecule (3). Proteolysis of cTnI has been shown to lead to detection difficulties when antibodies against proteolytically cleaved regions are used (8–10). Variations in cTnI assay results are also partly attributable to different calibrators that most likely differ from the actual analyte.
severity, and time after symptom onset of which may be directly linked to disease etiology, forms of the analyte present in a human body fluid, some release in the bloodstream, will determine the specific occurring either in the myocardium before release or after degradation, and N-terminal acetylation. cTnI from biochemical composition of the analyte (e.g., complex formation and oxidation) can have profound effects on assay response (12, 13). Troponins, as so-called type B analytes, are affected by pathologic changes and therefore appear as highly complex and heterogeneous mixtures in the sample in which they are measured (14). The precise nature of cardiac troponins once they are released from the myocardium and enter a patient’s circulation is still unknown; therefore, the actual target for the diagnostic assay is still unknown.

cTnI is susceptible to various modifications. In addition to complex formation with the other troponins [cTnT and cardiac troponin C (cTnC)] (12, 15–18), cTnI can undergo proteolysis (8–10, 16), phosphorylation (10, 19, 20), and oxidation (12). All of these cTnI modifications, occurring either in the myocardium before release or after release in the bloodstream, will determine the specific forms of the analyte present in a human body fluid, some of which may be directly linked to disease etiology, severity, and time after symptom onset (10, 13, 15, 20–22). Furthermore, if specific forms of cardiac troponins are the result of certain pathologies to the heart, these forms could be used for more precise diagnosis of cardiac disease. Ideally, calibrator materials and antigens used for raising antibodies should be chosen after the exact biochemical composition and structural configuration of an analyte are determined to make use of the additional information provided by disease-induced modifications to the analyte.

The variety of diseases that can lead to increased cardiac troponin concentrations (23–28) suggests that different forms of modified cardiac troponins may be present with different diseases or disease states. Several attempts have been undertaken to identify the exact forms of cardiac troponins in the blood of patients with AMI (10, 12, 15–18) or unstable angina (18, 22) or who are undergoing coronary artery bypass graft surgery (20). Although these studies provided indirect evidence that cTnI is predominantly present as binary cTnI-cTnC or ternary cTnI-cTnC-cTnT complex and, in addition, extensively modified, as of yet, no precise information about the actual amino acid residues involved in such modifications has been published. Bunk et al. (29) used liquid chromatography–mass spectrometry to characterize cTnI standard materials (extracted from human hearts or expressed recombinantly) and found a variety of putative modifications (including phosphorylation, oxidation, degradation, and N-terminal acetylation). cTnI from biological samples, ideally serum, from patients should be analyzed next. To our knowledge, the potential of proteomic tools has not yet been fully used to analyze such samples. We present here an approach that uses affinity chromatography to extract cardiac troponins from human myocardial tissue and from serum of AMI patients and provides a sample ready to be used for subsequent mass spectrometric analysis.

Materials and Methods

SAMPLES

Human left ventricular tissue was obtained from a donor heart rejected by the recipient, immediately snap-frozen after explantation, and stored at −80 °C. A small piece of frozen tissue (~100 mg) was homogenized on ice under nondenaturing conditions in 10 volumes (mL/g of tissue) of buffer containing protease and phosphatase inhibitors (50 mmol/L Tris, 500 mmol/L NaCl, 1 µmol/L leupeptin, 1 µmol/L pepstatin A, 0.36 µmol/L aprotinin, 0.25 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L sodium fluoride, and 0.2 mmol/L sodium vanadate, pH 7.0). The homogenate was centrifuged for 10 min at 16,000g and 4 °C; the supernatant was diluted to a concentration of ~1 g/L (determined by Bradford assay) in homogenization buffer and subsequently subjected to affinity chromatography.

The same supernatant was also diluted 1:10, 1:20, 1:40, 1:80, and 1:160 in cardiac troponin-free serum (cTnT <0.01 µg/L by Elecsys 2010; Roche Diagnostics GmbH) from a healthy male volunteer, giving an approximate total myocardial protein concentrations of 1, 0.5, 0.25, 0.125, and 0.06 g/L, respectively. Before this, the troponin-free serum was mixed 1:1 with starting buffer containing 50 mmol/L Tris, 500 mmol/L NaCl, pH 7.0, and is subsequently referred to as “normal serum”.

Serum samples were taken from patients admitted to the Kingston General Hospital Emergency Department with chest pain and diagnosed for AMI, based on unequivocal symptoms and a significant increase in serum creatine kinase. Use of the samples described in this work was approved by the Human Research Ethics Board of Queen’s University. No further information about medical history or treatment of the patients could be obtained. Sample collection was performed according to routine care protocols and not by a defined study time course. Blood was collected in serum separator tubes, centrifuged, and assayed for cardiac markers. Samples were then frozen and stored at −80 °C until subsequent analysis.

AFFINITY CHROMATOGRAPHY

The following antibodies were conjugated to N-hydroxysuccinimide-activated Sepharose 4 Fast Flow (Pharmacia Biotech) according to the manufacturer’s protocol at a concentration of 100 µg of monoclonal antibody (mAb)/mL of beads: anti-cTnI mouse mAb 8I-7, which binds to amino acid residues 137–148 (Spectral Diagnostics Inc.); anti-cTnI goat polyclonal antibody (pAb) P1, which binds to amino acid residues 1–26 (BiosPacific); anti-cTnC mouse mAb 1A2 (BioDesign); anti-cTnT goat pAb P1, which binds to amino acid residues 3–15 (BiosPacific); and anti-cTnT mouse mAb JLT-12 (Sigma-Aldrich).

To simulate matrix effects of patient sera on the affinity
chromatography–mass spectrometry approach, tissue homogenate was diluted in normal serum. The high concentration of serum proteins, such as albumin and immunoglobulins, increased the amount of nonspecific binding to the affinity beads and, consequently, the number of proteins subjected to proteolytic digestion. This led to an increased number of contaminating masses submitted to peptide mass fingerprinting (PMF), making it more difficult to detect the cardiac troponins. Increasing the stringency in the washing step, by use of a buffer containing 1 mol/L urea and 1 g/L CHAPS, reduced nonspecific binding of serum proteins to an extent that allowed the identification of specifically bound cardiac troponins by mass spectrometry.

Antibody-conjugated beads (25–100 μL were used) were incubated batchwise with 1 medium volume (MV) of tissue homogenate, diluted in either homogenization buffer or normal serum, for 1 h at room temperature. Patient serum was diluted 1:1 in starting buffer, and five divided fractions (1 MV each) were incubated with antibody-conjugated beads for 45 min each at room temperature. The sample/bead mixture was then centrifuged, and the supernatant was removed. Before incubation with the next patient serum aliquot, beads were washed with 3 MV of starting buffer. After the last incubation, beads were washed with 15–20 MV of 50 mmol/L Tris, 500 mmol/L NaCl, pH 7.0 (for tissue homogenate), or 20 MV of 50 mmol/L Tris, 500 mmol/L NaCl, 1 mol/L urea, 1 g/L CHAPS, pH 7.0, followed by 15 MV of 50 mmol/L Tris, 500 mmol/L NaCl, pH 7.0 (for serum samples), and finally equilibrated with 6 MV of 25 mmol/L NH₄HCO₃.

Because eluting bound cardiac troponins from the beads with trifluoroacetic acid led to unsatisfying results (i.e., the substantial loss of cTnC after the lyophilization step), we developed an on-bead digestion procedure. On-bead digestion of the proteins was carried out while proteins were still bound to the affinity beads by incubating the beads for 24 h at 37°C with either 10 ng/L modified porcine trypsin (sequencing grade; Promega) in 25 mmol/L NH₄HCO₃ or with 5 mg/L Asp-N or Lys-C (sequencing grade; Sigma) in 25 mmol/L NH₄HCO₃. Typically, 25 μL of affinity beads was mixed with 10 μL of protease solution for on-bead digest. Supernatants (containing peptide fragments from digested proteins) were then separated from the beads and were ready for mass spectrometric analysis. Affinity chromatography and on-bead digestion were performed in 500-μL siliconized microtubes (Fisher Scientific) to minimize nonspecific protein loss.

**MASS SPECTROMETRY**

Peptide mixtures from on-bead digestion were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager DE Pro; Applied Biosystems) as described previously (30) with α-cyano-4-hydroxycinnamic acid used as an energy-absorbing matrix. External calibration was performed with peptide standard mixtures from Sequazyme Peptide Mass Standards Kit (PerSeptive Biosystems). Protein identification by PMF was carried out using the MS-Fit search engine (Protein Prospector; available online at http://prospector.ucsf.edu) with the following criteria: minimum matching peptides, 4; number of missed cleavages, 1; fixed modification, Met oxidation, Ser and Thr phosphorylation; variable peptide mass tolerance, SwissProt database. The total protein amino acid sequence coverage of an identified protein as a percentage is based on the amino acid sequence from the fragments observed by mass spectrometry. This amino acid sequence coverage does not reflect the absolute amount of the identified protein.

**WESTERN BLOT ANALYSIS**

Western blot analysis was carried out as described previously (10). Briefly, 2 μL of diluted serum, aliquots of beads used for affinity chromatography (with the captured proteins bound), or troponin-depleted samples after affinity chromatography was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (3.3 g/L sodium dodecyl sulfate, 3.3 g/L CHAPS, 3.3 mL/L Nonidet P-40, 0.1 mol/L dithiothreitol, 2 mol/L urea, and 50 mmol/L Tris, pH 6.8, in 500 mL/L glycerol) and boiled for 5 min. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5% stacking, 12% resolving gel) and transferred to Immobilon-P membranes (0.45 μm; Millipore) or nitrocellulose membranes (0.45 μm; Osmonics) in 10 mmol/L CAPS, pH 11.0, for 1 h at 100 V and 4°C. Membranes were blocked with 100 mL/L blocking reagent (Roche Diagnostics) and then probed with anti-cTnI mAb 81-7, anti-cTnC mAb 1A2, or anti-cTnT pAb P1 at a concentration of 0.5 mg/L (antibodies diluted in 10 mL/L blocking buffer). Secondary antibodies were conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories), and chemiluminescence was detected with use of an Immun-Star (BioRad).

**RESULTS**

**HUMAN MYOCARDIAL TISSUE**

Affinity chromatography of the human cardiac tissue homogenate with anti-cTnI mAb 81-7 facilitated the specific enrichment of cTnI and its functional binding partners (i.e., the other subunits of the troponin complex) and separation from the rest of the myocardial proteome. Western blot analysis of the proteins bound to antibody-conjugated beads revealed, in addition to cTnI, the presence of cTnT and cTnC, which were co-extracted from the homogenate as troponin complex, and the specific antibodies for the respective subunits produced signals of comparable intensities (Fig. 1A). The entire isolated protein complex was digested enzymatically while still bound to the affinity beads, and the resulting peptide mixture was analyzed by MALDI-TOF mass spectrometry. PMF confirmed the presence of all three troponin subunits. Depending on the protease used for on-bead
digestion, different protein sequence coverages were obtained for the respective troponin subunits (Table 1). Not all proteases yielded peptide mixtures that would allow the identification of the various troponin subunits by mass spectrometry (Table 1). Combining amino acid sequence coverages from all digests, total protein sequence coverages for cTnI, cTnT, and cTnC were 54%, 48%, and 40%, respectively (Fig. 2).

The same experiments were also performed with the anti-cTnI pAb P1, anti-cTnT mAb JLT-12, anti-cTnT pAb P1, and anti-cTnC mAb 1A2. All of these antibodies facilitated the extraction of all three cardiac troponin subunits as confirmed by Western blotting and mass spectrometry; the protein sequence coverages, however, varied (data not shown). mAb 8I-7 showed the greatest protein sequence coverage for all subunits. It was therefore picked for the subsequent experiments on human serum.

**Matrix Effects on Cardiac Troponin Detection**

In the experiments evaluating matrix effects, total protein sequence coverages for cTnI, cTnT, and cTnC were 44%, 41%, and 19%, respectively (Fig. 3). Although cTnI and cTnT were detected in the first three dilutions (1:10, 1:20, 1:40), cTnC was detected only in the first two dilutions. At any dilution lower than 1:40, no cardiac troponins were detected (Table 2).

**AMI Serum**

To increase detection of the lower quantities of cTnI expected in AMI patient serum, 5 instead of 1 MV of diluted serum were incubated with affinity beads. As shown in Fig. 1C, the binding capacity of affinity beads exceeded the amount of cTnI present in the serum sample from patient 1. When affinity-bound proteins of this patient were analyzed by Western blotting, both cTnI (and some of its degraded products) and cTnC were readily detectable, whereas the cTnT signal was barely noticeable (compare panels A and B in Fig. 1). Furthermore, after on-bead digestion, mass spectrometric analysis revealed the presence of cTnI and cTnC with total protein sequence coverages of 42% and 19%, respectively. cTnT was not detected by mass spectrometry. This indicates that in this particular patient cTnI is present predominantly as either free cTnI or as a binary cTnI-cTnC complex. In the second patient, mass spectrometric analysis revealed the presence of cTnI, cTnT, and cTnC with total protein sequence coverages of 33%, 11%, and 28%, respectively. For this patient, we would also expect cTnI-cTnT or cTnI-cTnC-cTnT complexes to be present.

**Discussion**

The extreme pI values for cTnC (4.05) and cTnI (9.87), the strong binding affinity between the subunits, and the poor silver staining properties present a particular challenge to cardiac troponin analysis by a traditional proteomic approach, i.e., protein separation with two-dimensional gel electrophoresis, silver staining, and in-gel digestion for PMF by mass spectrometry (31). In comparison, the advantages of the affinity-chromatography/on-bead digestion approach, performed in a single microtube, are numerous. This approach reduces analysis time and the number of experimental steps, and therefore minimizes the possibility of introducing artificial modifications or the loss of proteins during sample handling and analysis.

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**Table 1. Total protein amino acid sequence coverage for cardiac troponins from human myocardial tissue.**

<table>
<thead>
<tr>
<th></th>
<th>Trypsin</th>
<th>Asp-N</th>
<th>Lys-C</th>
<th>Combined</th>
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<tr>
<td>cTnC</td>
<td>Not found</td>
<td>40 (aa&lt;sup&gt;a&lt;/sup&gt; 25–161)</td>
<td>Not found</td>
<td>40 (aa 25–161)</td>
</tr>
<tr>
<td>cTnI</td>
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<td>36 (aa 104–209)</td>
<td>34 (aa 40–205)</td>
<td>54 (aa 27–209)</td>
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<tr>
<td>cTnT</td>
<td>18 (aa 126–285)</td>
<td>Not found</td>
<td>29 (aa 78–287)</td>
<td>48 (aa 78–287)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total protein amino acid sequence coverage as assessed by MALDI-TOF mass spectrometry for digestion products produced by three different enzymes (with first and last covered amino acid).

<sup>b</sup> aa, amino acid.
processing. It does so while at the same time producing highly concentrated samples, ready for mass spectrometric analysis. This last feature is of particular importance for the investigation of serum, in which the concentrations of proteins such as albumin and immunoglobulins exceed those of cardiac troponins by several orders of magnitude. Because of the high sensitivity of MALDI-TOF mass spectrometry, the addition of a huge number of peptides (from contaminating serum proteins) to a sample can dramatically decrease the ability to detect low-abundance proteins to such an extent that PMF is impossible. This explains the need for a more stringent washing step (with a buffer containing 1 mol/L urea and 1 g/L CHAPS) for the analysis of serum to reduce the amount of protein.

**Fig. 2.** Total protein amino acid sequence coverage for cardiac troponins from human myocardial tissue as assessed by MALDI-TOF mass spectrometry.

Gray boxes represent peptides found by mass spectrometry. Sequences used were obtained from the SwissProt database (http://ca.expasy.org/sprot/). a, cTnT isoform 6 was used for PMF.

**Fig. 3.** Combined total protein amino acid sequence coverage for cardiac troponins from human myocardial tissue diluted in normal serum as assessed by MALDI-TOF mass spectrometry.

Gray boxes represent peptides found by mass spectrometry. Sequences used were obtained from the SwissProt database (http://ca.expasy.org/sprot/). a, cTnT isoform 6 was used for PMF.
nonspecifically bound to the surface of the affinity beads. At the same time, such stringent washes increase the risk of altering the antibody-binding affinity of cTnI as well as the affinity between the troponin subunits, reducing the yield of analyte. Although disruption of a cardiac troponin complex by such washes cannot be completely ruled out, we showed in our previous work (31) that far more stringent buffers (containing 6 mol/L urea, 2.5 mol/L thiourea, 2.5 mol/L NaCl, and 40 g/L CHAPS) were required to separate the cardiac troponin subunits from each other. Reduction in the nonspecific binding of serum proteins to the bead surface, by either altering surface characteristics or pretreatment of the sample before affinity chromatography, could allow a decrease in the stringency of the washing buffer, minimizing the risk of protein loss during washing steps.

The antibody we chose for our experiments binds (because of its binding site in the center of the molecule) the majority of possible degradation products of cTnI (10). It has been shown that the NH₂ and COOH termini of cTnI are susceptible to proteolytic cleavage (8, 10, 16). Because these regions contain binding sites for both cTnT and cTnC (32), the binding characteristics between the three cardiac troponin subunits will very likely change as a result of such proteolysis. Hence, use of anti-cTnI antibodies with epitopes near the NH₂ and COOH termini of the molecule bears the risk of selective extraction of intact and complexed cTnI. Although affinity chromatography on tissue and tissue added to normal serum yielded trimeric cTnI-cTnC-cTnT complex, we found that the predominant forms of cTnI in serum from AMI patient 1 were free cTnI and a binary cTnI-cTnC complex (Fig. 1, A and B). Patient 2, however, appeared to also have cTnT complexes present. These serum results are in accordance with data in the literature (12, 15–18). The methods used to date have allowed only indirect identification of the cTnI binding partners. One of these studies used gel-filtration chromatography with subsequent analysis of the fractions with commercial immunoassays (12). Others used two-site immunoassays with antibody pairs binding to different cardiac troponin subunits or whose binding affinities to cTnI were affected by troponin complex formation (15, 17, 18). Morjana (16) performed native polyacrylamide gel electrophoresis with Western blotting and showed cross-reactivity of antibodies against all three cardiac troponin subunits to a high-molecular-weight protein complex affinity-purified from serum from a AMI patient, whereas no free cardiac troponin subunits were found. Using our affinity chromatography–mass spectrometry approach, we were able to directly confirm the presence of cardiac troponin complexes in sera from AMI patients.

The identification of (disease-induced) modifications to cardiac troponins is of particular importance for the selection of antibodies and standard materials for diagnostic assays. Although we were able to enrich the cardiac troponins and separate them from myocardial as well as serum proteins, the analysis of digested peptides by MALDI-TOF mass spectrometry did not generate information about the entire sequence of each cardiac troponin subunit (Table 1). Possible reasons for this are numerous. MALDI-TOF mass spectrometry in the setting used for this work accurately detects peptides between 500 and 4000 Da. Peptides outside this range would not be detected. Certain regions within the cTnT sequence show clusters of negatively charged amino acids (i.e., aspartic acid). Such clusters can lead to problems with peptide ionization and subsequent detection by MALDI-TOF mass spectrometry. These peptides will therefore not be represented in the mass spectrum. The same problem can occur if phosphorylated amino acid residues are present in a peptide. The additional negative charge from the phosphorylated residue is known to suppress ionization and, consequently, the detection of such peptides. The absence of matching fragments for the NH₂ termini of all three troponin subunits might be caused by an N-terminal blocking group, which again may interfere in peptide detection. Finally, binding of the cardiac troponin subunits to each other may limit the access of peptides to their respective cleavage sites, producing large peptides. Such protection against proteolysis most likely explains the absence of peptides covering amino acids 138–147 on cTnI because this region forms the main part of the binding site for the capture mAb 8I-7 (amino acids 137–148). It also has to be stated that some therapeutic interventions (i.e., lytic therapy) can have substantial impact on the number and forms of modified troponins (33). We have no information about the therapies applied to our patients and can therefore draw no conclusion about how such therapies could have influenced our results.

Although this method is capable of detecting small amounts of cardiac troponins in the serum of patients with AMI, MALDI-TOF mass spectrometry is not quantitative and the current setup is not applicable for routine clinical analysis. A fully automated system for high-throughput separation, using ProteinChips with surface-enhanced laser desorption, ionization mass spectrometry, is under development and could be a next step in the analysis of cardiac troponins from biological samples.

### Table 2. Total protein amino acid sequence coverage for cardiac troponins from human myocardial tissue diluted in normal serum.

<table>
<thead>
<tr>
<th>Total protein amino acid sequence coverage, %</th>
<th>1:10</th>
<th>1:20</th>
<th>1:40</th>
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<tr>
<td>cTnC</td>
<td>15 (aa⁴ 25–161)</td>
<td>19 (aa 25–161)</td>
<td>Not found</td>
</tr>
<tr>
<td>cTnI</td>
<td>40 (aa 50–209)</td>
<td>40 (aa 50–209)</td>
<td>27 (aa 40–204)</td>
</tr>
<tr>
<td>cTnT</td>
<td>30 (aa 78–264)</td>
<td>41 (aa 78–287)</td>
<td>23 (aa 78–287)</td>
</tr>
</tbody>
</table>

*Total protein amino acid sequence coverage as assessed by MALDI-TOF mass spectrometry for three dilutions after digestion with three different enzymes, trypsin, Asp-N, and Lys-C (with first and last covered amino acid).

† aa, amino acid.
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References


