A novel approach for quantitative peptides analysis by selected electron transfer reaction monitoring

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Abstract

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) with selective reaction monitoring (SRM) is a selective and sensitive method for quantitation of peptides. SRM is achieved via MS/MS utilizing collision-induced dissociation (CID) while monitoring unique precursor–product ion transitions. Low-energy CID tandem mass spectrometry has been, by far, the most common method used to dissociate peptide ions for sequence analysis. However, collisional scattering of product ions in CID results in decreased intensity of the primary product ion. The lower intensity of the targeted product ion can lead to a reduction in the sensitivity of a quantitative method that uses SRM. Electron transfer dissociation (ETD) is a fragmentation method that is complementary to CID. During the ETD reaction for doubly protonated peptides ([M+2H]2+), there is a significant shift toward nondissociative electron transfer (ET) product species ([M+2H]+). We utilized that particular defect in ETD to develop a new quantitative method for monitoring the transition of unique precursors ([M+2H]2+) to charge-reduced ions ([M+2H]+). We refer to this method as selective electron transfer reaction monitoring (SETRM). In ESI-MS, trypsin-digested peptides tend to generate doubly protonated peptide precursors. We found that SETRM was more suitable than SRM for these doubly charged tryptic peptides with nano-LC–MS/MS. The quantitative capabilities of SETRM provide a more sensitive way of performing quantitative experiments using the same instrument, thereby improving the application of electron transfer dissociation in proteomics.

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1. Introduction

The application of peptides as drugs in therapeutic treatment, or as biomarkers in clinical diagnosis is becoming more widespread. The establishment of a sensitive and accurate analytical methodology is indispensable for quantitative assessment of peptides. Mass spectrometric-based quantitative analysis has a high specificity for detecting peptide components in complex biological matrices such as plasma, urine, and tissue [1–5].

In the most common application of mass spectrometric-based quantitative analysis, specific peptides are measured using selected reaction monitoring (SRM) [4–6]. That technique monitors the product ion resulting from collision-induced dissociation (CID) of selected precursor ions when the sample is introduced into the MS/MS instrument. The sensitivity gains result from the greater signal-to-noise (S/N) ratio that is the characteristic of MS/MS. During CID processes, however, the fragmented product ions are dispersed, leading to a reduction in the number of ions reaching the detector and hence a reduction in the sensitivity of SRM-based measurement methods [7–9].

Electron transfer dissociation (ETD) is a recently introduced mass spectrometric technique that has been shown to complement CID. In ETD, multiple protonated peptide ion species react with negative ions, such as fluoranthene. The reaction induces fragmentation of the peptide backbone, causing cleavage of the N–Co bond. This creates complementary c-type and z-type fragment ions [10–12]. ETD is advantageous over CID because it is less dependent on cleavage site sequences and because it preserves labile post-translational modifications (PTMs), such as phosphorylation and glycosylation [13–17].

Although the dissociation efficiency of ETD is not dependent on peptide length, amino acid composition, or post-translational modifications, it is dependent on the charge state of the precursor ion [18]. During ETD, highly charged precursor ions (charge state ≥ 3) produce a series of c ions and complementary z ions, thereby allowing for almost complete sequence coverage. Fragmentation of doubly charged precursors is often limited to one or both ends of the peptide, and there is an apparent phenomenon toward electron
transfer as a function of reducing precursor ion charge [19–22]. The electron transfer reaction leads into connection between c ions and complementary z ions with noncovalent intramolecular interactions. The precursor ion charge is reduced by this electron transfer reaction [23]. Most proteomic approaches, however, rely on tryptic peptides that produce primary doubly charged ions. Improving the dissociation efficiency of ETD for doubly charged precursor ions might be possible by elevating the temperature or by collisional activation [18,20,23,24].

To our knowledge, this is the first report of using electron transfer reaction in the quantitation of doubly charged peptides. In this study we evaluated the effectiveness of selective electron transfer reaction monitoring (SETRM) of charge-reduced precursor ions, tryptic peptides especially (Fig. 1).

2. Experimental

2.1. Chemicals and reagents

[Glu^1]-Fibrinopeptide B peptide, somatostatin, angiotensin I, ACTH 1–17, and ACTH 7–38 and MiniTip™ C18 were purchased from Sigma–Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Pierce (Rockford, IL). BSA digested standard was purchased from Waters (Milford, MA). Acetonitrile (ACN) was purchased from Waters (Milford, MA). Acetonitrile (ACN) was purchased from Pierce (Rockford, IL). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Fluka (Buchs, Switzerland). All samples were used without further purification.

2.2. In-solution digestion

BSA was diluted in 50 mM NH₄HCO₃ to reach a final concentration of 100 μg/mL. Disulfide bonds were reduced with 10 mM dithiothreitol for 1 h at 56 °C, and then alkylated with 50 mM iodoacetamide for 30 min at room temperature in the dark. Subsequently, alkylated proteins were digested with trypsin at a ratio of 1:20 (w/w, trypsin/protein), and incubated at 37 °C overnight. After digestion, the peptides were dried in a vacuum centrifuge, and then resuspended in 10 μL of 0.1% TFA for MiniTip™ C₁₈ desalting.

2.3. Liquid chromatography and mass spectrometry

Synthetic peptides were dissolved in 50% acetonitrile with 0.1% formic acid and infused into the mass spectrometer via an electrospray source. Analytes for LC–MS/MS analyses were introduced into the mass spectrometer via high-performance liquid chromatography using an Agilent (Palo Alto, CA) 1200 series binary HPLC pump and an LC packings FAMOST™ well-plate microautosampler. For each analysis, sample was loaded into a 2 cm × 75 μm i.d. trap column. The trap column was connected to a 11 cm × 75 μm i.d. analytical column and the columns were rigidly packed in-house with 5 μm C₁₈ reversed-phase packing material. Mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of 0.1% formic acid in 100% acetonitrile. The flow rate was 250 nL/min with linear gradients of: (1) 5–40% B for 27 min; (2) 40–85% B for 3 min; (3) isocratic at 85% B for 10 min.

All mass spectrometric analyses were performed on a Thermofisher Scientific LTQ XL (San Jose, CA) linear ion trap mass spectrometer equipped with a chemical ionization source for the generation of radical anions (fluoranthene) for ETD reactions. For both CID and ETD analyses, precursor ion width was set at 2 Da and the automatic gain control (AGC) of the precursor cations for MSⁿ scan was set at 1 × 10⁵. For the CID experiment, the q-value was equal to 0.25 and the normalized collision energy ranged from 0% to 35%. For the ETD experiment, the reaction time ranged from 50 ms to 300 ms and the AGC target for fluoranthene anions ranged from 1 × 10⁵ to 3 × 10⁶.

2.4. Quantitative analysis

For [Glu^1]-Fibrinopeptide B human peptide, the SRM transition was m/z 786.2 (doubly charged precursor ion) → 480.3 (y₄ ion) and the SETRM transition was m/z 786.2 (doubly charged precursor ion) → 1571.3 (charge-reduced ion). A standard curve was established using different amounts of [Glu^1]-Fibrinopeptide B human peptide (500 amol–1 pmol) and the area under the curve was calculated for all transitions using the vendor-supplied Xcalibur data system. Method validation was carried out using a set of standards (0.5–50 fmole) analyzed in triplicate. For the matrix effect test, a 25 μL aliquot of [Glu^1]-Fibrinopeptide B human peptide (1 fmol/μL) prepared in 50 mM NH₄HCO₃ was evaporated using a speed vacuum concentrator and then dissolved in 10 μL of 0.1% TFA for MiniTip™ C₁₈ desalting.

2.5. Desalting peptides with MiniTip™ C₁₈

The 10 μL MiniTip™ pipette tips contain a C₁₈ spherical silica (50–60 μm, 200 Å pore size) sorbent bed bonded at the working end of the tip, were used for the desalting of the peptides. The tips were first wetted with 10 μL of 0.1% TFA in 70% ACN, then were equilibrated with 10 μL of 0.1% TFA. The peptides dissolved in 10 μL of 0.1% TFA, then were bound to MiniTip™ by fully depressing the pipette plunger to a dead stop. Samples were aspirated and dispensed for 10 cycles. Then the tips were washed with 10 μL of 0.1% TFA twice. The peptides on the tips were eluted out using 10 μL of 0.1% TFA in 70% ACN. After desalting, the peptides were dried in a vacuum centrifuge, and then resuspended in 0.1% FA (25 μL for [Glu^1]-Fib B) before MS analysis.

Fig. 1. SRM vs. SETRM in doubly-charged peptide quantitation.
3. Results and discussion

3.1. Comparison of CID to ETD for doubly charged precursor ions

To compare the dissociation efficiency between CID and ETD for doubly charged precursor ions, we employed [Glu1]-Fibrinopeptide B human peptide ([Glu1]-Fib) as the model analytic species. [Glu1]-Fib via ESI primarily generated doubly charged precursor ions (data not shown). Using the CID approach, doubly charged precursor ions of [Glu1]-Fib generated a large number of b- and y-type product ions (Fig. 2A) and collisional scattering of the product ions decreased in intensity for the targeted product ion (y4 ion). Using the ETD approach, doubly charged precursor ions of [Glu1]-Fib reacted with fluoranthene anions generating only a few fragment ions (Fig. 2B); however, there was a general shift toward nondissociative electron transfer as the charge of the precursor species ([M+2H]+) decreased. Herein, we refer to nondissociative electron transfer precursor species as charged-reduced ions. The intensity of the charge-reduced ions (3.2 × 10^5) in ETD was higher than that of the major product ions (1.9 × 10^5) in CID. For this reason, we developed a new approach based on the detection of specific transitions between the precursor and charge-reduced species of peptides. This approach is referred to as selective electron transfer reaction monitoring (SETRM).

3.2. The charge state of precursor ions impacts on CID and ETD fragmentation

We found that the charge state of precursor ions was the critical factor affecting the efficiency of ETD reaction; therefore, we selected five synthetic peptides with differently charged states to observe the correlation between the charge-reduced trend and the charge state of precursor ions. The major doubly charged precursor ions were generated both in [Glu1]-Fib and somatostatin under ESI. The most abundant charge state distributions were triply charged ions of angiotensin I, quadruply charged ions of ACTH 1–17, and quintuply charged ions of ACTH 7–38 (data not shown).

Similar to [Glu1]-Fib (Fig. 3A), the intensity of charge-reduced ions ([M+2H]+) with ETD was higher than that of the major product ions for somatostatin using CID (Fig. 3B). An analogical reaction was observed in antiotensin I (Fig. 3C). For ACTH 1–17 and ACTH 7–38, however, the intensity of charge-reduced ions with ETD was lower than that of the target product ions with CID (Fig. 3D and E). During ESI, the ACTH 1–17 and ACTH 7–38 synthetic peptides produced quadruply and quintuply charged precursor ions, respectively. Because highly charged precursors (>3) are partial to fragmentation into c- and z-type ions, the intensity of the charge-reduced ions was weak. This trend suggested that SETRM is better than SRM at detecting doubly and triply charged precursor ions.

3.3. The charge state distribution of tryptic peptides

In proteomics, the most common enzyme used for digestion is trypsin, an agent that cleaves protein sequences with positive residues lysine or arginine (K or R). We used BSA tryptic peptides to observe the distribution of charged precursor ions (Table S1). Note that the doubly charged precursor ions were most abundant in tryptic peptides. This suggests that SETRM is appropriate for quantifying tryptic peptides.
3.4. Optimization of MS/MS process for CID and ETD

Collision energy is the most important factor affecting dissociation in CID reactions. We altered the collision energy to acquire the optimal condition for CID reaction. As shown in Fig. 4, when the collision voltage of precursor ions increased by more than 23%, the y₄ ion of [Glu₁]-Fib produced stable ion intensity. This means that [Glu₁]-Fib had the best dissociation efficiency when the collision voltage was greater than 23%. We, therefore, selected 30% collision voltage in later SRM experiments.

In ETD, the amount of time it takes for precursor ions to react with negative ions (fluoranthene) and the amount of automatic gain control (AGC) are the most important factors affecting the efficiency of dissociation. Fig. 5 shows that the intensity of [Glu₁]-Fib charge-reduced ions (m/z 1571) was enhanced regardless of the reaction time or the amount of AGC. In order to get the maximum ion intensity of charge-reduced species, we used a reaction time of 250 ms and an AGC of 3 × 10⁵ in later SETRM experiments.

3.5. Quantitative analysis by SRM and SETRM

Two sets of quantification experiments were performed – (i) SRM transition from precursor ion m/z 786 ([M+2H]+²) to product ion m/z 480 (y₄ ion) and (ii) SETRM transition from precursor ion m/z 786 ([M+2H]+²) to charge-reduced ion m/z 1571 ([M+2H]+•). At least seven concentrations were analyzed and the linearity of peak area with respect to concentration was evaluated under optimal conditions and described as the regression equation. Limits of detection (LOD) and quantification (LOQ) under the chromatography conditions used were separately determined at signal-to-noise ratios (S/N) of 3 and 20, respectively. As shown in Fig. 6, both of SRM and SETRM were given in good linearity (r² > 0.99) in a relatively wide concentration range. Moreover, the LODs were 0.1–0.5 (fmol) of SRM and 0.02–0.05 (fmol) of SETRM. Their corresponding LOQs were 0.5–2.5 (fmol) and 0.1–0.5 (fmol), respectively (Table S2).

The accuracy of the method was measured by determining the mean concentration at various concentrations of analyte. Both the intra- and inter-day accuracy and precision of the method were determined by triplicate analysis of [Glu₁]-Fib at five concentrations. Precision was estimated as the relative standard deviation (RSD) of the analyses and the accuracy was calculated as percentage error of theoretical versus measured concentrations. The estimated amounts of [Glu₁]-Fib were in good agreement with the expected amounts, the intra- and inter-assay precision were less than 13% for SRM and SETRM (Table S3). The values for intra-day and inter-day accuracy were 90.65–107.94% and 94.63–104.91% of SRM, 96.70–113.78% and 99.02–111.89% of SETRM, respectively. The intra-day and inter-day precision calculated as RSD were within the range of 0.82–5.13% and 0.38–4.61% of SRM, 1.16–12.00% and 1.00–13.02% of SETRM, respectively (Table S3). Furthermore, the peak areas of [Glu₁]-Fib (5 fmol) were similar for solvent and matrix prepared [Glu₁]-Fib. [Glu₁]-Fib in matrix was prepared in 50 mM NH₄HCO₃ and then desalted with MiniTip™ C₁₈ prior to LC–MS/MS analysis (Table S4).

3.6. Quantification of BSA tryptic peptides by SRM and SETRM

As shown in Table S1, doubly charged precursor ions were the major charge state in ESI, suggesting that SETRM is an appropriate method for quantifying tryptic peptides. Here, four doubly charged tryptic precursor peptides were selected to compare the two methods. Because the efficiency of ionization from these four peptides was not equal, the precursor ion intensity of these four peptides was different. However, during MS/MS, the intensity of charge-reduced ions by ETD was greater than that of the target product.
Fig. 7. Graph comparing the peak area intensity of SRM and SETRM from four doubly charged BSA digestion peptides.

ions by CID (Fig. 7). The amino acid sequence of each peptide in Fig. 7 was described in the supporting information (Table S5). This result demonstrates that SETRM transition is suitable for peptide quantification.

3.7. Applicability of the SETRM to proteomic studies

In order to test the efficiency of the SETRM method, the peptide quantitation was executed with more complicated protein digests. SRM and SETRM mass chromatograms of [Glu\(^+\)]-Fibrinopeptide B standards (500 fmol) which mixed with BSA digestion peptides (500 fmol) were shown in Fig. 8. The peak area of charge-reduced ions by SETRM was greater than that of the target product ions by SRM. This result demonstrates that SETRM transition is also suitable for peptide quantification in complicated digestion peptides.

4. Conclusions

We demonstrated that the charge state of precursor ions has a significant impact on the efficiency of electron transfer dissociation. The intensity of charge-reduced ions in ETD is greater than that of the target product ions in CID for doubly charged peptides. In this study, we used this phenomenon to develop a new strategy of peptide quantification, namely selective electron transfer reaction monitoring (SETRM). The linearity, dynamic range, precision and accuracy of quantification obtained for doubly charged peptides in SRM were similar to those in SETRM. However, SETRM has a low LOD because the intensity of charge-reduced ions in ETD is higher than the intensity of target product ions in CID. Furthermore, we found that SETRM is suitable for quantifying tryptic peptides.

The quantitative capabilities of SETRM provide a more sensitive way of performing quantitative experiments using the same instrument, thereby improving the application of electron transfer dissociation.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.08.068.

References

Table S1. Charge distribution of total ions which were selected for MS/MS under data dependant mode in BSA tryptic digests.

<table>
<thead>
<tr>
<th>Charge distribution</th>
<th>Number of peptide ions a</th>
<th>% of total a)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1</td>
<td>26</td>
<td>10</td>
<td>0.15</td>
</tr>
<tr>
<td>+2</td>
<td>227</td>
<td>84</td>
<td>0.03</td>
</tr>
<tr>
<td>+3</td>
<td>17</td>
<td>6</td>
<td>0.28</td>
</tr>
</tbody>
</table>

a) Average of three time experiments by using LC-MS/MS data dependant mode
Table S2. LODs and LOQs for [Glu\(^1\)]-Fibrinopeptide B standards obtained on linear ion trap with SRM and SETRM.

<table>
<thead>
<tr>
<th>Quantitative mode</th>
<th>LOD (femtomole)</th>
<th>LOQ (femtomole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRM</td>
<td>0.1-0.5</td>
<td>0.5-2.5</td>
</tr>
<tr>
<td>SETRM</td>
<td>0.02-0.05</td>
<td>0.1-0.5</td>
</tr>
</tbody>
</table>
Table S3. Intra- and inter-day precision and accuracy for [Glu<sup>1</sup>]-Fibrinopeptide B standards obtained on linear ion trap with SRM and SETRM.

<table>
<thead>
<tr>
<th>Quantitative mode</th>
<th>Nominal (femtomole)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>R.S.D. (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td>SRM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.71</td>
<td>4.44</td>
<td>108.21</td>
</tr>
<tr>
<td>5</td>
<td>5.06</td>
<td>0.82</td>
<td>101.11</td>
</tr>
<tr>
<td>10</td>
<td>9.07</td>
<td>3.37</td>
<td>90.65</td>
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<tr>
<td>25</td>
<td>26.99</td>
<td>5.13</td>
<td>107.94</td>
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<tr>
<td>50</td>
<td>49.14</td>
<td>1.09</td>
<td>98.28</td>
</tr>
<tr>
<td>0.5</td>
<td>0.57</td>
<td>12.00</td>
<td>113.78</td>
</tr>
<tr>
<td>2.5</td>
<td>2.58</td>
<td>9.51</td>
<td>103.16</td>
</tr>
<tr>
<td>SETRM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.15</td>
<td>1.30</td>
<td>103.06</td>
</tr>
<tr>
<td>10</td>
<td>9.67</td>
<td>1.16</td>
<td>96.70</td>
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<tr>
<td>25</td>
<td>26.60</td>
<td>3.55</td>
<td>106.39</td>
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</tbody>
</table>
Table S4. Peak area of [Glu$^1$]-Fibrinopeptide B standards obtained on linear ion trap with SRM and SETRM.

<table>
<thead>
<tr>
<th></th>
<th>prepared in solvent&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>prepared in matrix&lt;sup&gt;b)&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Peak area</td>
<td>R.S.D.%</td>
</tr>
<tr>
<td>SRM</td>
<td>1.97E+04</td>
<td>3.67</td>
</tr>
<tr>
<td>SETRM</td>
<td>3.15E+04</td>
<td>4.28</td>
</tr>
</tbody>
</table>

<sup>a</sup>) Sample dissolved in 0.1% FA
<sup>b</sup>) Sample dissolved in 50 mM NH$_4$HCO$_3$ and then desalted with MiniTip<sup>TM</sup> C$_{18}$. 


Table S5. The amino acid sequence of BSA tryptic peptides for SRM and SETRM quantitation.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Amino acid sequence (^a)</th>
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<tbody>
<tr>
<td>722</td>
<td>YICDNQDTISSK</td>
</tr>
<tr>
<td>789</td>
<td>LKPDPNTLCDEFK</td>
</tr>
<tr>
<td>572</td>
<td>KQTALVELLK</td>
</tr>
<tr>
<td>710</td>
<td>SLHTLFGDELCK</td>
</tr>
</tbody>
</table>

\(^a\) The underlined “C” denotes carbamidomethylated cysteine.