RESEARCH ARTICLE

Effects of Electron-Transfer Coupled with Collision-Induced Dissociation (ET/CID) on Doubly Charged Peptides and Phosphopeptides

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Abstract

Electron-transfer dissociation (ETD) is a useful peptide fragmentation technique that can be applied to investigate post-translational modifications (PTMs), the sequencing of highly hydrophilic peptides, and the identification of large peptides and even intact proteins. In contrast to traditional fragmentation methods, such as collision-induced dissociation (CID), ETD produces c- and z'-type product ions by randomly cleaving the N–C α bonds. The disappointing fragmentation efficiency of ETD for doubly charged peptides and phosphopeptide ions has been improved by ETcaD (supplemental activation). However, the ETD data derived from most database search algorithms yield low confidence scores due to the presence of unreacted precursors and charge-reduced ions within MS/MS spectra. In this work, we demonstrate that eight out of ten standard doubly charged peptides and phosphopeptides can be effortlessly identified by electron-transfer coupled with collision-induced dissociation (ET/CID) using the SEQUEST algorithm without further spectral processing. ET/CID was performed with the further dissociation of the charge-reduced ions isolated from ETD ion/ion reactions. ET/CID had high fragmentation efficiency, which elevated the confidence scores of doubly charged peptide and phosphopeptide sequencing. ET/CID was found to be an effective fragmentation strategy in “bottom-up” proteomic analysis.

Key words: Collision-induced dissociation, Electron-transfer dissociation, Doubly charged, Phosphopeptides, Peptides

Introduction

In the past decade, tandem mass spectrometry (MS/MS) has played a major role in the bottom-up approach of peptide sequencing and protein identification [1–5]. The gas-

phase ion dissociation or fragmentation of isolated precursor ions generates consecutive sequence-informative product ions and reveals the identities of the corresponding amino acid sequences. The unique primary sequence of the digested peptide reveals an exact protein ID through in silico database search algorithms. Collision-induced dissociation (CID) is the most widely used peptide fragmentation technique in tandem mass spectrometry [6–9]. The precursor ions are kinetically excited and activated by collision with the buffer gas. During the activation process, the internal energy of precursor ions is accumulated and converted into

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vibrational energy, which is distributed throughout all of the covalent bonds. The fragmentation occurs when the distributed vibrational energy exceeds the required activation energy for bond cleavage. After ion fragmentation via CID, a consecutive series of b- and y-type product ions are produced due to the relatively low activation energy of amide bond cleavage. Unfortunately, the presence of certain amino acids (e.g., Asp, Glu, and Pro residues) [10] and post-translational modifications (PTMs) (e.g., Ser/Thr phosphorylations [11] and O-linked glycosylations) of the peptides can limit the sequence coverage provided by CID fragmentation.

Trypsin is the enzyme most commonly used in enzymatic protein digestion. Doubly charged protonated ions are dominantly produced following electrospray ionization (ESI) of tryptic peptides. Doubly charged protonated ions exhibit excellent fragmentation efficiency via CID [12]. However, the intense neutral-loss peaks and the relative absence of other product ions in CID spectra of phosphopeptides hamper the sequence identification of target phosphopeptides and the localization of phosphorylated sites (scheme 1, CID) [13, 14]. MS³ experiments are used to further activate the intense neutral-loss peaks, and to generate the sequence-informative fragment product ions. A multistage activation (MSA) strategy is also applied to reduce the duty cycle for phosphopeptide analysis [13].

Electron-based ion activation methods such as electron-capture dissociation (ECD) [15] and electron-transfer dissociation (ETD) [16] have been shown to be useful for investigating PTMs [16–20], sequencing highly hydrophilic peptides rich in Asp and Glu residues [20], and identifying large peptides and small proteins [20–22]. Electron-based ion activation methods are, therefore, complementary to CID in MS/MS sequencing analysis. When ECD is coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), odd-electron protonated ion species are generated when multicharged peptide cations capture the thermal electrons. When ETD is coupled with ion trap-type mass spectrometry (IT-MS), the ion/ion reactions between protonated ions and radical anions (generally fluoranthene) produce unstable odd-electron protonated ion species. Both of those methods favor cleavage of the N–C α bonds, thus forming c- and z'-type product ions.

In ETD spectra, the unreacted precursor and charge-reduced ions produce major peaks in the product ion mass spectrum (Scheme 1, ETD). The charge-reduced ions are derived from precursor ions that receive at least one electron but do not dissociate further (ETnoD). This phenomenon is especially common in doubly charged precursor ions. Recently, Coon et al. demonstrated that supplemental activation (i.e., ETcaD) of the charge-reduced ions provides a way to enhance the efficiency of ETD fragmentation for the sequencing of doubly charged precursor ions (Scheme 1, ETcaD) [23]. ETcaD can supply “low-energy” CID to disrupt the noncovalent interactions between the undissociated c and z' ions [23, 24]. This

strategy generates ETD-specific product ions alone and improves sequence coverage. ETcaD has been employed to sequence doubly-charged peptide ions. However, in ETcaD, odd-electron c⁺ and even-electron z⁺ ions are generated from the hydrogen transfer between the c and z' ions [23]. The signals from the c⁺ and z⁺ ions usually dominate the ETcaD spectra. In SEQUEST algorithms, only the c (even-electron) and z' (odd-electron) ions in ETD can be considered real product ions.

SEQUEST is one of the most important database search algorithms for tandem MS spectra [25–27]. SEQUEST was originally designed to query CID-generated MS/MS data. However, in response to the increasing number of researchers using electron-based activation techniques in large-scale proteomic analyses, SEQUEST was modified to allow searches for c (even-electron) and z' (odd-electron) ions from ETD. The abundance of unreacted precursor and charge-reduced ions generated by ETD often suppress the peaks of product ions in ETD spectra. Even though ETcaD can eliminate the intense peaks from charge-reduced ions, this technique still results in a profusion of unreacted precursor ions. ETD data always yield low confidence scores, which results in the identification of fewer peptides using SEQUEST algorithms. Coon et al. showed that removing the unreacted precursor, the charge-reduced ions, and neutral losses from charge-reduced ions from the ETD spectra ultimately led to an increase in the relative abundance of ETD product ions [28]. The number of unique identifications was increased using the open mass spectrometry search algorithm (OMSSA) [29]. Nevertheless, this modified data format is not compatible with the SEQUEST algorithm in routine analysis.

Wu and Karger et al. [30] demonstrated MS³ experiments where “low-energy” CID was used to further dissociate the charge-reduced ions generated from ETD. Such low-energy CID only disrupted the noncovalent interactions of the undissociated c and z' ions and simplified the MS³ spectra, making it easy to locate PTM sites and determine the backbone. However, charge-reduced ions were still present in these MS³ spectra. Campbell et al. [31] performed ETD while simultaneously applying CID to doubly charged precursor ions in a single MS/MS experiment (ETD/CID). This ETD/CID approach increased sequence coverage, yielding more b and y ions through CID and c and z' ions from ETD. However, the charge-reduced ions in the ETD/CID spectra suppressed the relative abundances of the product ion peaks. Furthermore, Wu and Karger et al. [32] used “high-energy” CID for MS³ experiments to determine the disulfide linkages in recombinant therapeutic proteins through sequence-informative product ions (b, y, c, and z' ions, Scheme 2) from the highly charged precursor ions. The high-energy approach used in those MS³ experiments also played a key role in our study.

In this study, we first performed ETD reactions on doubly charged precursor ions (Scheme 1, ETD). The

isolated charge-reduced ions resulting from ETD were subsequently dissociated with “high-energy” CID (Scheme 1, ET/CID). Those ET/CID MS³ spectra contained b, y, c and z product ions with high relative abundances. We then compared the Xcorr values of the CID, ETD, ETcaD, and ET/CID spectra using the SEQUEST algorithm. We found that ET/CID was an effective fragmentation strategy for identifying doubly charged peptides and phosphopeptides ions without the need for further spectral processing.

Experimental

Chemicals and Reagents

A standard mixture of phosphopeptides (cat. no. P33357) and MassPREP phosphopeptide standard enolase (cat. no. 186003285) were purchased from Invitrogen (USA) and Waters (USA), respectively. Acetonitrile (ACN) and formic acid (FA) were obtained from Merck (Germany) and Fluka (Switzerland), respectively. DI water was derived from a Barnstead Nanopure system (>18 MΩ). The analytical standards were dissolved in 50% ACN with 0.1% formic acid to achieve final concentrations ranging from 0.5 μM to 1 μM. The peptides were then directly infused into the linear ion trap mass spectrometer via an electrospray source (LTQ XL with ETD, Thermo Fisher Scientific, USA).

Fragmentation

The peptides were fragmented with CID, ETD, ETcaD, and ET/CID. MS/MS conditions for each of the fragmentation methods were as follows. For CID, we used the MS² scan mode (~330 MS/scan), an activation time of 30 ms, an activation q_u of 0.25, and a normalized collision energy of 35%. For ETD, we used the MS² scan mode (~400 MS/scan). The reaction time with fluoranthene radical anions was 100 MS, and the anion automatic gain control (AGC) target value was 3×10^5 . For ETcaD, we used the MS² scan mode (~430 MS/scan), the default q_u value of 0.15, and a normalized collision energy of 12% for charge-reduced ions generated from precursor ions. For ET/CID MS³ experiments [~730 MS/scan (330 MS + 400 MS)], the isolated charge-reduced ions from ETD were subsequently dissociated with “high-energy” CID (an activation q_u of 0.25 and a normalized collision energy of 35% were used). A different activation q_u value and normalized collision energy were used to further activate the charge-reduced ions generated from the ETD reaction. All of the MS/MS spectra were acquired in three microscans.

SEQUEST Analysis

The raw files (MS/MS spectra) were searched against a homemade database containing only the peptides employed in this study using the SEQUEST algorithm (BioworksBrowsers 3.3.1 SP1, Thermo Fisher Scientific).

Xcorr was used to compare the tandem mass spectra and amino acid sequence information in the database. The value of Xcorr was found to be independent of database size, but it was dependent on peptide size and charge state. The search parameters were as follows. Enzyme restriction was set to “no enzyme.” The precursor mass tolerance was set at ± 2.0 Da, and the fragment ion mass tolerance was set at ± 0.5 Da. A low fragment ion mass tolerance was used to identify the unique c (even-electron) and z' (odd-electron) ions. The differential modification (phosphorylation) on STY residues was set at +97.96633 Da. In order to compare the Xcorr values (independent of database size) of the MS/MS spectra generated from the four MS/MS fragmentation methods, three groups of fragment ions (B + Y + C + Z, B + Y, and C + Z) were evaluated in each database search. C and Z corresponded to the c (even-electron) and z' (odd-electron) ions, respectively. To minimize the identification of false-positive product ions when evaluating product ion sequence coverage and peptide backbone cleavage efficiency, the product ions were counted and confirmed manually when the peak intensity was higher than 4% relative to that of the most intense matched product ion (excluding neutral-loss peaks). It has been reported that hydrogen atom transfer behavior between even-electron c ions and odd-electron z' ions produces odd-electron c⁺⁺ (1 Da lighter than c) and even-electron z⁺ ions (1 Da heavier than z') [23]. The SEQUEST algorithm interpreted even-electron c ions and odd-electron z' ions as real product ions rather than c⁺⁺ ions and z⁺ ions. In this study, we focused on even-electron c ions and odd-electron z' ions.

Results and Discussion

In this study, three unmodified peptides and seven phosphopeptides were examined. The sequences of these peptides are listed in Table 1. In order to simplify peptide annotations within this study, we gave a name to each peptide; for example, T18 indicates NVPL[pY]K, where [pY] indicates a phosphorylated residue. The MS/MS data were generated from peptides activated by CID, ETD, and ETcaD. The charge-reduced ions generated from the ETD were subsequently dissociated with CID (ET/CID) to produce the MS³ data. All spectra were searched using SEQUEST algorithm, and Xcorr values were acquired for the corresponding spectra. There were two major reasons for choosing the SEQUEST algorithm to evaluate the quality of the spectra: (1) SEQUEST is often used for ITMS coupled with ETD; (2) the Xcorr value for a specific spectrum was independent of the size of the database.

CID, ETD, and ETcaD of Doubly Charged Precursor Ions

In the CID analysis of doubly charged peptides, 6 of the 10 peptides had Xcorr values smaller than 2.2 (B + Y ion

Table 1. Information on the ten candidate peptides analyzed and the Xcorr values of the four fragmentation types obtained using the SEQUEST algorithm

Peptide name	$[M + 2H]^{2+}$ (m/z)	$[M + 2H]^{++}$ (m/z)	Fragmentation type	Precursor ion (m/z)	Xcorr value ^b			Peptide sequences ^d
					B + Y + C + Z ions	B + Y ions	C + Z ions	
T18	407.20	814.40	CID	407.20	1.150	1.160	NM ^c	NVPL[pY]K
			ETD	407.20	1.387	0.647	0.735	
			ETcaD	407.20	1.271	NM	0.839	
			ET/CID ^a	814.40	1.771	1.002	0.790	
T19	432.21	864.42	CID	432.21	0.753	0.723	NM	HLADL[pS]K
			ETD	432.21	1.855	NM	1.690	
			ETcaD	432.21	2.044	NM	1.959	
			ET/CID	864.42	2.173	1.157	1.007	
A2	523.77	1047.54	CID	523.77	1.195	1.215	NM	DRVYIHPF
			ETD	523.77	0.947	NM	0.937	
			ETcaD	523.77	1.843	NM	1.783	
			ET/CID	1047.54	2.288	0.921	1.373	
A1	648.85	1297.70	CID	648.85	2.198	2.039	0.178	DRVYIHPFHL
			ETD	648.85	1.284	0.413	1.202	
			ETcaD	648.85	1.963	0.288	2.020	
			ET/CID	1297.70	3.399	1.366	2.240	
T431P	684.84	1369.68	CID	684.84	4.969	4.856	0.216	VNQIG[pT]LSESIK
			ETD	684.84	0.666	0.027	0.637	
			ETcaD	684.84	1.932	0.151	1.943	
			ET/CID	1369.68	3.766	1.140	2.816	
T432P	724.82	1449.64	CID	724.82	4.331	4.019	0.482	VNQIGTL[pS]E[pS]IK
			ETD	724.82	0.267	0.141	0.128	
			ETcaD	724.82	2.245	0.738	1.905	
			ET/CID	1449.64	3.500	1.553	2.384	
MBP104	789.93	1579.86	CID	789.93	2.006	1.740	NM	GKGRGLSLSRFSWGA
			ETD	789.93	0.703	0.015	0.685	
			ETcaD	789.93	1.349	0.209	1.179	
			ET/CID	1579.86	2.747	0.729	2.083	
MAPK177	835.34	1670.68	CID	835.34	5.230	5.283	NM	DHTGFL[pT]E[pY]VATR
			ETD	835.34	0.040	0.047	NM	
			ETcaD	835.34	2.018	NM	1.992	
			ET/CID	1670.68	5.728	3.125	2.994	
IR1142	851.88	1703.76	CID	851.88	2.864	2.829	0.210	TRDI[pY]ETDYYRK
			ETD	851.88	0.122	NM	0.146	
			ETcaD	851.88	0.968	0.180	0.784	
			ET/CID	1703.76	4.154	2.067	2.571	
pTp	860.95	1721.90	CID	860.95	0.270	0.289	NM	VPIGRFDRRV[pT]VE
			ETD	860.95	0.820	0.356	0.462	
			ETcaD	860.95	1.884	0.287	1.593	
			ET/CID	1721.90	3.315	0.981	2.385	

^aET/CID was MS³ fragmentation, in other words the charge-reduced ions generated from ETD were dissociated with CID (activation q_u of 0.25, normalized collision energy of 35%). The ion isolation width was m/z 50 for the MS³ step

^bXcorr value was calculated with three groups of product ions

^cNM no match using this product ion group

^dPhosphoserine, phosphothreonine, and phosphotyrosine are abbreviated to *pS*, *pT*, and *pY*, respectively

types, Table 1). In general, 2.2 was a relatively low criterion for identifying doubly charged peptides [33]. There were proline residues within the peptide sequences for peptides A2 and A1, and the peptide bonds at the N-termini of the proline residues were easy to fragment via CID. The other peptide bonds were not fragmented (T18 and A2). Boersema et al. reported a similar dissociation pattern for phosphoserine and phosphothreonine peptides [13]. Neutral losses of phosphate groups from phosphopeptides, $[M + 2H - 98]^{2+}$, were significantly stronger than b and y ions (T19 and pTp). The Xcorr values of those spectra did not always satisfy the criteria due to the relatively low abundance of product ions in the CID spectra. Using B + Y + C + Z ions to evaluate Xcorr values, we found that the values were

similar to the B + Y ion types due to fewer product ions of c and z' ions. Therefore, six of the ten peptides were not identified by CID fragmentation. In the ETD analysis of doubly charged peptides, no peptides or phosphopeptides could be identified because the Xcorr values were much smaller than 2.2 (C + Z ion types). ETD exhibited low efficiency at dissociating doubly charged peptides; the relative intensities of the c and z' ions were less than 5%. Unreacted precursor and charge-reduced ions were the dominant peaks in the ETD spectra of doubly charged peptides; therefore, ETD fragmentation does not produce adequate MS/MS spectra and sufficiently high Xcorr values to pass the criteria.

To improve the power of ETD to dissociate doubly charged ions, supplemental activation was used to disrupt

noncovalent binding between c and z^{++} ions. Xcorr values of all ETcaD spectra were higher than ETD; however, the values did not satisfy the criteria. ETcaD gave lower charge-reduced ion intensities and more product ions than ETD. Although there was less interference with charge-reduced ions based on the results of the database search, there was still an overabundance of unreacted precursor ions. Xcorr values of ETcaD spectra were not sufficient to identify all 10 doubly charged peptides and phosphopeptides ($C + Z$ ion types, Table 1). Based on our results, doubly charged peptides and phosphopeptides could not

be identified directly in MS/MS spectra generated from ETD and ETcaD.

ET/CID of Doubly Charged Precursor Ions

Wu and Karger et al. [30] demonstrated charge-reduced-CID (MS^3) fragmentation for highly charged precursor ions. They applied “low-energy” CID (normalized collision energy of 10% and an activation q_u of 0.15) to further dissociate charge-reduced ions from ETD. In their report, highly charged precursor ions from Lys-C digestion were generated

Figure 1. CID (A), ETD (B), ETcaD (C), and ET/CID (D) spectra of product ions from the doubly charged peptide MBP104 (GKGRGLSLSRFSWGA). Note that the c and z labels shown in the spectra indicate the even-electron c ions and odd-electron z^+ ions, respectively

in electrospray ionization. Due to the “low-energy” CID, the charge-reduced ions in the MS³ spectra still produced interference with product ions. Coon et al. [23] utilized ETcaD MS² fragmentation, applying a normalized collision energy of 20% and an activation q_u value of 0.18 to disrupt charge-reduced ions and near-exclusively generate c and z' ions (including c⁺⁺ and z⁺⁺ ions). The unreacted precursor ions in the ETcaD spectra strongly suppressed the peaks of product ions. Campbell et al. [31] performed ETD while simultaneously applying CID to doubly charged precursor ions in a single MS/MS experiment (ETD/CID). The ETD/CID approach increased the sequence coverage of b and y

ions derived from precursor ions without affecting the number of c and z' product ions. ETD/CID improved the sequence coverage of doubly charged ions and eliminated the abundance of unreacted precursor ions that are normally generated by ETD. ETD/CID, however, could not remove the charge-reduced ions that inhibit the relative intensities of the product ions. Moreover, the number of ETD-derived product ions (c and z' ions) was not increased through the CID of even-electron precursor ions.

ET/CID experiments were conducted in the MS³ scan mode to increase the relative abundances of product ions in the MS spectra by eliminating the influence of unreacted

Figure 2. CID (A), ETD (B), ETcaD (C), and ET/CID (D) spectra of product ions from the doubly charged phosphopeptide pTp (VIPGRFDRRVP TVE). Note that the c and z labels shown in the spectra indicate the even-electron c ions and odd-electron z' ions, respectively. The neutral loss of phosphate is also shown

precursor and charge-reduced ions. These ET/CID spectra were used to directly identify doubly charged peptides and phosphopeptides without further spectral processing. We, therefore, applied a normalized collision energy of 35% and a q_u value of 0.25 to activate the isolated charge-reduced ions generated from ETD reactions, and found that b, y, c, and z' product ions were displayed in a single ET/CID spectrum. Using the SEQUEST search algorithm (Xcorr values >2.2 , C + Z ion types), we found that six of the ten standard peptides could be identified by ET/CID. By combining the b and y product ion results, eight peptides were recognized (B + Y + C + Z ion types). A larger number of peptides were identified via ET/CID than via CID, ETD and ETcaD (Table 1). A total of four peptides (A1, A2, MBP104 and pTp) were identified via ET/CID. The CID, ETD, ETcaD, and ET/CID spectra of MBP104 are shown in Figure 1. The enhanced relative intensities of product ions in the ET/CID spectra of MBP104 resulted in an Xcorr value of 2.747 (Figure 1d and Table 1). We could not directly identify MBP104 using CID, ETD, and ETcaD because of their low Xcorr values. CID, ETD, ETcaD, and ET/CID spectra of pTp are shown in Figure 2. pTp produced intense peaks from neutral losses of phosphate groups in the CID spectra, resulting in an Xcorr value of 0.270 (Figure 2a and Table 1). However, pTp was easily identified (Xcorr value = 3.315) by ET/CID without any spectral processing (Figure 2d and Table 1). Neutral losses of phosphate groups were indeed observed in the ET/CID spectra due to the fragmentation of the charge-reduced even-electron ions ($[M + H]^+$) derived from ETD side reactions (proton transfer

without dissociation) [11]. However, a number of product ions with high relative intensities were also generated, which provided a wealth of sequence information. It was proposed that both b and y ions were generated from the charge-reduced even-electron ions ($[M + H]^+$, Scheme 1 and 2) [32]. We were able to directly identify eight out of ten standard peptides and phosphopeptides due to the clarity of the ET/CID spectra without spectral processing (B + Y + C + Z ion types).

Moreover, the effects of normalized collision energy (C) and activation q_u (Q) on the Xcorr values of the ET/CID spectra were examined (Table S1 of the Electronic Supplementary Material, ESM). The Xcorr values obtained using high-energy ET/CID (C35Q0.25) were significantly higher than those obtained using C12Q0.15 and C15Q0.15, due to the charge-reduced ions that remain when using low-energy ET/CID. In high-energy ET/CID spectra (C35Q0.25), the new b and y ions produced through the activation of the charge-reduced ions (even electron) enhanced the Xcorr values. Therefore, these data clearly show that ET/CID (C35Q0.25) is a powerful strategy that could be used in “bottom-up” proteomics to identify doubly charged peptides and phosphopeptides without further spectral processing. It had been reported that hydrogen atom transfer between even-electron c ions and odd-electron z' ions produced odd-electron c^{++} (1 Da lighter than c) and even-electron z^+ ions (1 Da heavier than z') [23]. The effect of c^{++} and z^+ ions upon the Xcorr values was also evaluated (Table S2 of the ESM). A higher mass tolerance of product ions (± 1.0 Da) was used to include the contribution from the H-transfer product ions (c^{++} and z^+ ions). However, this did not lead to any obvious

Scheme 1. Doubly charged phosphopeptides were activated by CID, ETD, ETcaD, and ET/CID. P indicates a phosphate group

Scheme 2. The procedure used in our ET/CID MS³ experiments. First, ETD was applied to the doubly charged precursor ions, and then the isolated charge-reduced ions were dissociated with “high-energy” CID. High relative intensities of product ions were observed in the resulting ET/CID spectra

enhancement of the Xcorr values. The use of a higher mass tolerance of product ions caused an increased risk of random matches during database searching.

Fragmentation Efficiency Calculations

Fragmentation efficiency was calculated in order to evaluate the confidence scores of matched peptide sequences. The fragmentation efficiency was derived from the product ion sequence coverage and the peptide backbone cleavage efficiency. Proline linkages were also calculated, although the N-terminus of proline was not cleaved by the ETD reaction.

Product ion sequence coverage (Table 2) was defined as

$$\frac{\text{no. of observed product ion}}{\text{no. of theoretical product ion}} \times 100$$

The peptide backbone cleavage efficiency (Table 3) was defined as

$$\frac{\text{no. of observed backbone cleavages}}{\text{no. of peptide backbone bonds}} \times 100$$

In ET/CID, the low-mass cutoff (LMCO) was increased because this isolates the larger m/z values of the charge-reduced ions for MS³ activation. The higher LMCO resulted in the loss of low m/z product ions. Even though ET/CID utilized

a higher LMCO for product ion detection in ion-trap mass spectrometry, ET/CID still led to high product ion sequence coverage (47.8%), while ETD and ETcaD yielded 25.4% and 44.5%, respectively (C + Z ion types, Table 2). ET/CID also led to higher product ion sequence coverage of B + Y + C + Z ions (41.2%) than observed for the other three fragmentation methods in a single spectrum (22.8%, 19.4%, and 29.0%, respectively). The LMCO problem could be solved by using other types of mass spectrometry equipped with ETD source (for example triple quadrupole ETD). Interestingly, for B + Y ions, we observed that b and y ions were also produced by ETD and ETcaD (13.3% and 13.4%, respectively). In particular, y ions were constantly generated in electron-based activation techniques due to the C-terminal residues of Arg and Lys derived from trypsin digestion. These data supported our reasoning behind increasing rather than decreasing the number of b and y product ions. The efficiency of ET/CID at cleaving B + Y + C + Z ions in the peptide backbone was 91.4% (Table 3). In addition, the efficiency of ET/CID at cleaving C + Z ions in backbone bonds was 84.4%. Therefore, ET/CID exhibited a high fragmentation efficiency that led to enhanced product ion sequence coverage for doubly charged peptides and phosphopeptides with the SEQUEST algorithm.

Evaluation by a Realistic Database

dCn (deltaCn) values—normalized scores obtained by calculating the difference between the first- and the second-ranked

Table 2. Product ion sequence coverages (%) of the four fragmentation types for the ten candidate peptides

Peptide name	B + Y + C + Z ions				B + Y ions				C + Z ions			
	CID	ETD	ETcaD	ET/CID	CID	ETD	ETcaD	ET/CID	CID	ETD	ETcaD	ET/CID
T18	10.0	45.0	35.0	35.0	20.0	40.0	30.0	40.0	0.0	50.0	40.0	30.0
T19	45.8	33.3	33.3	50.0	66.7	8.3	8.3	50.0	25.0	58.3	58.3	50.0
A2	10.7	14.3	28.6	64.3	21.4	0.0	7.1	64.3	0.0	28.6	50.0	64.3
A1	16.7	13.9	22.2	44.4	33.3	5.6	0.0	38.9	0.0	22.2	44.4	50.0
T431P	31.8	11.4	22.7	34.1	59.1	9.1	4.6	13.6	4.6	13.6	40.9	54.6
T432P	22.7	15.9	31.8	36.4	40.9	13.6	27.3	27.3	4.6	18.2	36.4	45.5
MBP104	28.6	12.5	28.6	32.1	50.0	7.1	7.1	14.3	7.1	17.9	50.0	50.0
MAPK177	39.6	25.0	35.4	56.3	75.0	33.3	16.7	54.2	4.2	16.7	54.2	58.3
IR1142	18.2	6.8	25.0	38.6	36.4	4.6	13.6	36.4	0.0	9.1	36.4	40.9
pTp	3.9	15.4	26.9	21.2	7.7	11.5	19.2	7.7	0.0	19.2	34.6	34.6
Average	22.8	19.4	29.0	41.2	41.1	13.3	13.4	34.7	4.5	25.4	44.5	47.8

Table 3. Peptide backbone cleavage efficiencies (%) of the four fragmentation types for the ten candidate peptides

Peptide name	B + Y + C + Z ions				B + Y ions				C + Z ions			
	CID	ETD	ETcaD	ET/CID	CID	ETD	ETcaD	ET/CID	CID	ETD	ETcaD	ET/CID
T18	20.0	100.0	100.0	80.0	20.0	80.0	60.0	80.0	0.0	80.0	80.0	60.0
T19	100.0	100.0	100.0	83.3	100.0	16.6	16.7	83.3	33.3	100.0	100.0	83.3
A2	28.6	57.1	85.7	100.0	28.6	0.0	14.3	100.0	0.0	57.1	85.7	85.7
A1	44.4	44.4	77.8	100.0	44.4	11.1	0.0	66.7	0.0	44.4	77.8	88.9
T431P	72.7	36.4	81.8	100.0	72.7	18.2	9.1	27.3	9.1	27.3	81.8	100.0
T432P	54.6	45.5	72.7	90.9	45.5	27.3	45.5	45.5	9.1	36.4	72.7	81.8
MBP104	64.3	35.7	92.9	100.0	57.1	14.3	14.3	28.6	14.3	35.7	92.9	92.9
MAPK177	83.3	75.0	91.7	100.0	83.3	66.7	33.3	91.7	8.3	33.3	91.7	100.0
IR1142	54.6	27.3	72.7	90.9	54.6	9.1	27.3	63.6	0.0	18.2	72.7	81.8
pTp	7.7	53.9	76.9	69.2	7.7	15.4	30.8	15.4	0.0	38.5	69.2	69.2
Average	53.0	57.5	85.2	91.4	51.4	25.9	25.1	60.2	7.4	47.1	82.5	84.4

sequences—were derived. The dCn values were dependent on database size, sequence homology, and search parameters (mass tolerance, enzyme restrictions). To search against a realistic protein database, an enzyme restriction (e.g., to trypsin) must be employed to avoid random matches in “bottom-up” proteomics. In this study, four out of six tryptic peptides (T431p, T432p, MAPK177, and IR1142) were successfully identified using ET/CID. The dCn values of those spectra were obtained by searching against a realistic human protein database (39,423 entries, Table 4), and the search parameters mimicked a general “bottom-up” proteomics strategy. The mass tolerances of the precursor and fragment ions were set to ± 2.0 Da and ± 0.5 Da, respectively. The enzyme restriction was set to trypsin, and two miscleavages were available. Carbamidomethyl cysteine was set as a fixed modification, while other modifications were set as variable modifications. More search result data are shown in Table S3 of the ESM. We observed that a higher Xcorr value did not always translate into a better match based on dCn values.

Based on the Xcorr and dCn values, all of the peptides were easy to identify in ET/CID spectra (Table 4). Moreover, the phosphorylated site was clearly apparent in the ET/CID spectra of IR1142 (Table S3 of the ESM). Those data strongly indicate that ET/CID spectra yield specific results for trypsin-based “bottom-up” proteomics.

Comparison of ETcaD (With Spectral Processing) and ET/CID (Without Spectral Processing)

ETcaD data were processed manually using spectral processing methods [28] in order to remove the peaks from unreacted precursor ions, charge-reduced ions, and neutral losses from charge-reduced ions. The resulting .dta files were then searched with the SEQUEST algorithm, and higher Xcorr values were obtained for these new ETcaD spectra (see Table S4 of the ESM). The Xcorr values of all ion types (B + Y + C + Z, B + Y, and C + Z ions) were

Table 4. Trypsin-dependent dCn evaluations of four tryptic peptides using a realistic human protein database (with 39423 entries) and the SEQUEST algorithm

Peptide name	Fragmentation type	Precursor ion (m/z)	Xcorr value ^b			dCn ^d		
			B + Y + C + Z ions	B + Y ions	C + Z ions	B + Y + C + Z ions	B + Y ions	C + Z ions
T431P	CID	684.84	4.969	4.856	NM ^c	0.140	0.165	—
	ETD	684.84	0.666	NM	NM	0.030	—	—
	ETcaD	684.84	1.932	NM	1.943	0.135	—	0.137
	ET/CID ^a	1369.68	3.766	NM	2.816	0.213	—	0.139
T432P	CID	724.82	4.331	4.019	NM	0.108	0.077	—
	ETD	724.82	NM	NM	NM	—	—	—
	ETcaD	724.82	2.245	NM	1.905	0.170	—	0.355
	ET/CID	1449.64	3.500	NM	2.384	0.144	—	0.106
MAPK177	CID	835.34	5.230	5.283	NM	0.246	0.250	—
	ETD	835.34	NM	NM	NM	—	—	—
	ETcaD	835.34	2.018	NM	1.992	0.052	—	0.065
	ET/CID	1670.68	5.728	3.125	2.994	0.117	0.005	0.100
IR1142	CID	851.88	2.864	2.829	NM	0.157	0.140	—
	ETD	851.88	NM	NM	NM	—	—	—
	ETcaD	851.88	NM	NM	NM	—	—	—
	ET/CID	1703.76	4.154	NM	2.571	0.092	—	0.196

^aET/CID was MS³ fragmentation, in other words, the charge-reduced ions generated from ETD were dissociated with CID (activation q_u of 0.25, normalized collision energy of 35%). The ion isolation width was m/z 50 for the MS³ step

^bXcorr value was calculated with three groups of product ions

^cNM no match using this product ion group

^ddCn (deltaCn) was calculated with three groups of product ions

enhanced due to the elevated relative abundances of the product ions. However, the Xcorr values obtained using the ET/CID data were still higher than those obtained with the processed ETcaD data, due to the abundance of B + Y ions.

Conclusions

ETcaD is widely used to sequence doubly charged peptide ions, and it improves the utility of ETD in "bottom-up" proteomics. Although ETD complements CID in MS/MS peptide sequencing analysis, the abundance of unreacted precursor and charge-reduced ions generated by ETD often suppress the peaks of product ions from doubly charged peptides in ETD spectra. Therefore, without further spectral processing, it is difficult to identify doubly charged peptide sequences by ETD and ETcaD. Most of the peptides chosen for this study were doubly charged phosphopeptides, which are notorious for producing poor ETD spectra. Eight out of the ten standard doubly charged peptides and phosphopeptides were identified by ET/CID directly using the SEQUEST algorithm without spectral processing. ET/CID conducted in the MS³ scan mode increased the relative abundances of product ions by minimizing the influence of unreacted precursor and charge-reduced ions. The ET/CID data were conveniently analyzed using the SEQUEST algorithm. ET/CID exhibited a high fragmentation efficiency that assisted in peptide sequencing and PTM site location for doubly charged peptides and phosphopeptides.

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