Protein Analysis Using a Combination of an Online Monolithic Trypsin Immobilized Enzyme Reactor and Collisionally-Activated Dissociation/Electron Transfer Dissociation Dual Tandem Mass Spectrometry

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We demonstrated the combined applications of online protein digestion using trypsin immobilized enzyme reactor (IMER) and dual tandem mass spectrometry with collisionally activated dissociation (CAD) and electron transfer dissociation (ETD) for tryptic peptides eluted through the trypsin-IMER. For the trypsin-IMER, the organic and inorganic hybrid monolithic material was used. By employing the trypsin-IMER, the long digestion time could be saved with little or no sacrifice of the digestion efficiency, which was demonstrated for standard protein samples. For three model proteins (cytochrome *c*, carbonic anhydrase, and bovine serum albumin), the tryptic peptides digested by the IMER were analyzed using LC-MS/MS with the dual application of CAD and ETD. As previously shown by others, the dual application of CAD and ETD increased the sequence coverage in comparison with CAD application only. In particular, ETD was very useful for the analysis of highly-protontated peptide cations, *e.g.*, ≥ 3+. The combination approach provided the advantages of both trypsin-IMER and CAD/ETD dual tandem mass spectrometry applications, which are rapid digestion (*i.e.*, 10 min), good digestion efficiency, online coupling of trypsin-IMER and liquid chromatography, and high sequence coverage.

Key Words : Electron transfer Dissociation (ETD), Collisionally activated dissociation (CAD), Trypsin-immobilization, Immobilized enzyme reactor (IMER), Monolithic reactor

Introduction

In recent years, the technologies involved in mass spectrometry analysis of proteins has matured, which has enabled high throughput analysis of proteins at the proteome level.[1-3] The development of new mass spectrometric methods and the following implementation to the commercial mass spectrometers has been crucial in achieving the current state-of-the-art mass spectrometry. The particular examples of the new mass spectrometry techniques include the new ionization methods, such as electrospray ionization (ESI),[4] matrix assisted laser desorption/ionization (MALDI),[5-10] and desorption electrospray ionization (DESI),[11,12] ion-mobility techniques,[13-17] imaging mass spectrometry,[18,19] new types of ion trap devices (*e.g.* linear ion trap and orbitrap),[20-22] top-down mass spectrometry,[23,24] a variety of isotope-labeling methods,[25,26] and so on.

From the perspective of the tandem mass spectrometry techniques, electron capture dissociation (ECD) and electron transfer dissociation (ETD) have offered another dimension in protein/peptide identification and characterization.[27-36] When these odd electron species-based tandem mass spectrometry methods are used in combination of the traditional collision-based dissociation method (collision-induced dis-

sociation (CID)/collisionally-activated dissociation (CAD)), the protein/peptide sequence information becomes much richer by providing complementary information.[37] For example, Coon *et al*. demonstrated the so-called decision-tree algorithm approach in which for the low charge state (2+) peptides, only collision-based tandem mass spectrometry (CAD) is used, while both ETD and CAD is applied to the highly-charged peptides ions with certain *m/z* criteria.[38] The ECD and ETD have also been known to be important in the characterization of post-translational modifications (PTMs) of proteins.[28,33,34,39-45] Due to its excellent peptide sequencing ability and the unique advantage in the analysis of PTMs, the applications of these methods are now expanding.

On the other hand, there have been significant efforts to make the proteome analysis more automated and rapid. Among these, developing a trypsin-immobilized enzyme reactor (IMER) has been one of the active areas toward these goals.[46,47] The trypsin-IMER is known to have several advantages over the traditional in-solution digestion method; high enzyme-to-substrate ratio, high digestion efficiency, fast analysis speed, and repeated uses.[46-49] Furthermore, there has been a recent study that the digestion efficiency of the trypsin-IMER in a capillary format is higher even than that of trypsin-immobilized particles.[50] The main reason for these results can be found mainly in the fact that high enzyme/substrate ratio can be used in the trypsin-IMER

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because trypsins are immobilized on the solid support; in-solution digest, 1:20-1:100 (w/w) enzyme/substrate ratio. The high enzyme/substrate ratio of the trypsin-IMER leads to much shorter incubation time; < a few min *versus* 5-24 h. The immobilization of trypsins also eliminates the possibility of the autodigestion of trypsin itself, thus avoiding the possible contamination. Furthermore, when made in the capillary format, the incorporation of the IMER into a variety of online LC-MS/MS configurations is possible and the details of the experimental configurations are well documented in the literature.[46,47]

The immobilization of trypsin proteases can be achieved on a solid support; the first example was reported by Wainer *et al.* in which trypsins were immobilized on silica material.[51] In their studies, the silica particles were grafted with hydrophilic polymers and treated with glutaraldehyde to generate aldehyde functionality at the polymer chain ends. Trypsin was then covalently attached to the surface by the Schiff base formation reactions, wherein the catalytic activities of immobilized trypsins were highly maintained. Alternatively, polymeric materials with a bimodal pore size distribution were used for trypsin immobilization.[52,53] Polymer particles were made to have large throughpores that allowed analyte molecules to perfuse through the particle and short diffusive pores wherein trypsins were immobilized. This flow-through type trypsin reactor possessed a high degree of protein digestion control, which is achieved by simply adjusting the flow rate of elution.

Monolithic materials were also introduced as a support for enzyme immobilization.[54,55] Fréchet and colleagues introduced macroporous monolithic polymeric materials based on poly-(glycidyl methacrylate-*co*-ethylene dimethacrylate) in which trypsins were immobilized.[56] This organic polymer-based bioreactor showed a variety of useful properties such as high flow-permeability, good biocompatibility, high enzyme stability, excellent pH stability, and fast mass transfer characteristics.[46,57] A silica-based inorganic monolithic material was also used for an enzyme immobilization.[58-61] The silica-based monolithic column material showed good chromatographic properties of dual pore size distribution; large macropores and thin, highly porous skeleton. In addition, this material offers high permeability, high mechanical strength, and good organic solvent tolerance.[62] However, these organic and inorganic monolithic materials also showed some weaknesses. The organic polymer materials suffer from swelling in organic solvent and the accompanying mechanical brittleness, while the silica-based inorganic materials require long synthesis time and showed poor reproducibility. To overcome these weaknesses, a hybrid organic-inorganic silica monolith was developed, and this material was used for trypsin immobilization.[62-65] Furthermore, in recent years, a microfluidic device including a trypsin-immobilized bioreactor channel is implemented in a commercial mass spectrometer, which improved the analysis speed and reproducibility of proteome analyses.[66]

Despite such obvious advantages of the application of the dual tandem mass spectrometry (*e.g.*, ETD + CAD) and the

trypsin-IMER, to our knowledge, there has been no experimental report on the combination of these two methodologies. Here, we present our recent studies in which the hybrid organic-inorganic silica monothic trypsin IMER in a capillary format was used for protein digestion and the eluted peptide fragments were analyzed using LC-MS/MS, particularly with dual application of both ETD and CID. This new type of combined experimental approach is demonstrated for three model proteins; cytochrome *c*, carbonic anhydrase, and bovine serum albumin. We hope that our demonstration would prompt a wide application of online trypsin-IMER in combination with ETD/CAD dual tandem mass spectrometry, which holds promising potential for the automated, on-spot identification and characterization of protein samples.

Experimental

Reagents and Chemicals. Tetraorthosilane (TEOS), 3-amino-propyltriethoxysilane (APTES), and cetyltrimethyl ammonium bromide (CTAB) were commercially available (Sigma, Seoul, Korea). Trypsin (bovine pancreas), ubiquitin (bovine, 76 amino-acids, 8.6 kDa), cytochrome *c* (horse heart, 105 amino-acids, 11.7 kDa), carbonic anhydrase (bovine erythrocytes, 260 amino-acids, 29.1 kDa), and bovine serum albumin (bovine serum, 607 amino-acids, 69.3 kDa) were also obtained from Sigma and used without further purification. Fused-sillica capillaries (100 μm i.d. × 375 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Organic solvents were all of HPLC grade. Water was obtained by purification by a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of Trypsin-Immobilized Bioreactor. A trypsin-immobilized monolithic enzyme reactor was made using the procedure of Ma *et al.*[62] A strategy of organic-inorganic hybrid silica monolith was utilized for the trypsin-IMER. This strategy was reported to be effective in ensuring high enzymatic activities. First, a silica-based polymeric network was formed in a fused-silica capillary (10 cm). A mixture solution of 112 μL of TEOS, 118 μL of APTES, 215 μL of ethanol, 8 mg of CTAB, and 32 μL water was prepared and vortexed at room temperature. This mixture solution was injected into a capillary using a syringe pump (KD Scientific, Holliston, MA, USA), and then the two ends of the capillary were sealed with parafilms. The sealed capillary was kept overnight in a water-bath maintained at 40 °C. The CTAB porogens were removed from the capillary by washing the capillary with ethanol and water for 30 min, respectively. The capillary silica monolith was activated by flowing 10% (v/v) glutaraldehyde in 100 mM potassium phosphate buffer (pH 8.0) through the capillary for 6 h. Trypsin enzymes were then immobilized within the capillary by pumping a trypsin solution through the capillary at 4 °C for 24 h. The trypsin solution was prepared using the following procedure. Trypsin was dissolved at the concentration of 2 mg/mL in 100 mM potassium phosphate buffer (pH 8.0), into which 50 mM benzamidine and 5 mg/mL sodium cyano-

borohydride (NaCNBH$_3$) were added. As a final procedure, unattached trypsin enzymes and the remaining aldehyde were removed by washing the capillary with 20% ACN in potassium phosphate buffer (4 h) and 1 M Tris-HCl (pH 8, 2 h). When the typsin bioreactor was not used, the reactor was kept at 4 °C in a 50 mM Tris-HCl buffer solution with 10 mM CaCl$_2$ and 0.02% NaN$_3$.

Protein Sample Preparation. Three proteins, ubiquitin (8.6 kDa), carbonic anhydrase (29.1 kDa), and cytochrome *c* (11.7 kDa), which do not contain a disulfide bond, were dissolved at 1 mM in 50 mM of ammonium bicarbonate solution, and 10% (v/v) acetonitrile was added to fully dissolve the proteins. Trypsin is known to retain its activity in 10% acetonitrile solution at pH between 7.6 and 9.0.[67] For bovine serum albumin (BSA, 69.3 kDa), which contains multiple disulfide bonds, a standard reduction and alkylation procedure was performed.

Online Digestion of Proteins. Online digestion of proteins into tryptic peptides and their separation by reversed-phase liquid chromatography were carried out in sequence using a six-port switching valve setup coupled with two pumping systems (see Scheme 1). Proteins loaded on a 2 µL injection loop were eluted through the trypsin-IMER (20 µm i.d. × 10 cm) for 10 min at a flow rate of 300 nL/min maintained at 37 °C with a thermostat. It was observed that the flow rate is very critical in determining the trypsin digestion efficiency, and 300 nL/min flow rate was found to be optimal. The length of the reactor has been reported to affect the digestion efficiency.[62] In the present study, a relatively long length of 10 cm was chosen to maximize the sequence coverage of proteins under examination. The eluted tryptic peptides were trapped on a C18 trapping column (180 µm i.d. × 20 mm, Symmetry, Waters, Seoul, Korea) using an isocratic pump (Acquity, Waters, Manchester, UK). The trapped peptides were separated using a home-made capillary column (75 µm i.d. × 15 cm) packed with C18 resin (100 Å, 3 µm, Magic C18, Bruker-Michrom, Auburn, CA, USA). For the separation of peptides, a nano-UPLC system (Acquity, Waters, Manchester, UK) was used with a linear gradient. Mobile phase A was composed of 0.1% formic acid-containing water, while mobile phase B was 0.1% formic acid in acetonitrile. The gradient began with 5% B for 15 min, ramped to 20% B over 3 min, increased to 95% B over 60

min, remained at 95% B over 12 min, and then decreased to 5% B for the next 2 min. The column was equilibrated with 5% B for 8 min before the next run.

Mass Spectrometry Analysis. For the identification of peptides, the eluted peptides were subjected to LC-MS/MS using a dual linear ion-trap mass spectrometer (Finnigan LTQ-Velos, Thermo Fisher Scientific, San Jose, CA, USA) equipped with ETD capability. The full mass survey scan and tandem mass spectrometry ETD or CAD MS/MS scans were operated in positive ion mode and also in data-dependent acquisition mode. The following mass spectromery parameters were used: spray voltage, +2.5 kV; capillary temperature, 220 °C; precursor isolation width, 1 *m/z*. The data dependent MS/MS analysis was performed for the three most abundant peaks observed in the full MS survey scan using both CAD and ETD; three CAD and ETD scans were performed alternatively (total cycle time for each precursor: 310 ms). CAD was performed with the normalized collision energy of 35% and with an automatic gain control (AGC) target value of 10,000 peptide cations. In ETD, an activation time of 80 ms was applied to the isolated precursors with an AGC target of 100,000, and fluoranthene was used as an ETD anion reagent. However, in our study, ETcaD, in which gentle collisional activation is applied to charge-reduced species with non-covalent interactions, was not used. The MS and MS/MS spectra were analyzed with Mascot search engine (v. x Matrix Science, London, UK); the search results were visually inspected in order to avoid false positive identifications. The product ion mass tolerance was set at ± 0.8 Da, and the precursor ion mass tolerance was ± 1.0 Da. Two missed trypsin cleavages were allowed, and the oxidation of Met and fixed modification of carbamidomethyl cysteine were also considered.

Results and Discussion

MALDI-TOF Evaluation of the Trypsin-IMER Activity. Before analyzing protein mixtures, the digestion efficiency of the home-made trypsin-IMER was evaluated for ubiquitin (0.01 mg, 76 amino acids, 8.6 kDa). Figure 1(a) shows the MALDI-TOF spectrum of ubiquitin tryptic peptides collected after elution through the trypsin-IMER (flow rate: 300 nL/min, 10 min). In this mass spectrum, ten tryptic peptides were observed, including LIFAGK (U1, *m/z* 648.2), QLEDGR (U3, *m/z* 717.1), MQIFVK (U4, *m/z* 765.2), EGIPPDQQR (U5, *m/z* 1,039.1), ESTLHLVLR (U6, *m/z* 1,067.4), TLSDYNIQK (U7, *m/z* 1,081.4), LIFAGKQLEDGR (U8, *m/z* 1,346.4), IQDKEGIPPDQQR (U9, *m/z* 1,523.4), AKIQDKEGIPPDQQR (U10, *m/z* 1,722.5), and TLSDYNIQKESTLHLVLR (U11, *m/z* 2,130.1). The four peptide fragments in the higher mass region of Figure 1(a), LIFAGKQLEDGR (U8, *m/z* 1,346.4), IQDKEGIPPDQQR (U9, *m/z* 1,523.4), AKIQDKEGIPPDQQR (U10, *m/z* 1,722.5), and TLSDYNQKESTLHLVLR (U11, *m/z* 2,130.1), resulted from missed cleavages by trypsins immobilized in the reactor. A slight difference in the digestion profiles between the trypsin-IMER and in-solution digestion for the same proteins has
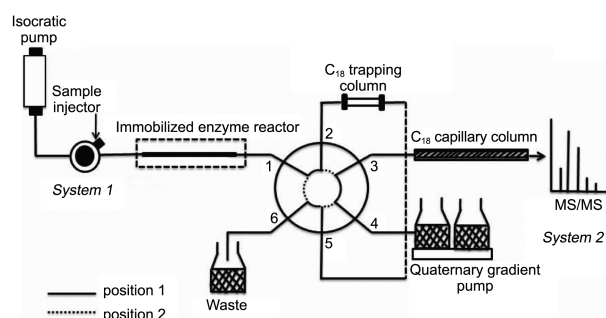
Scheme 1. The chromatography setup performing the tandem online digestion by trypsin-IMER and LC-MS/MS analysis of peptide mixtures eluted from trypsin-IMER.
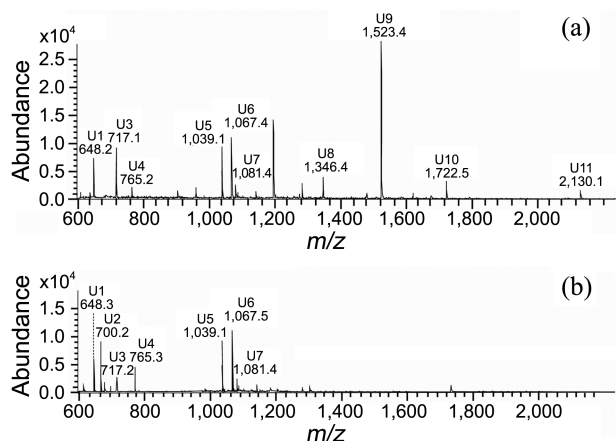
Figure 1. MALDI-TOF mass spectra of ubiquitin obtained from (a) trypsin-IMER and (b) in-solution digestion. DHB matrix (30:70 (v/v) acetonitrile: 0.1% triflouroacetic acid in water) was used for MALDI.

been previously reported.[47,62,68,69] Nevertheless, the above result clearly demonstrates that our home-made trypsin-IMER worked more or less efficiently for small proteins, such as ubiquitin. More importantly, with the trypsin-IMER, the current case, only 10 min was needed for protein digestion.

Protein Mixture Analysis by Dual Tandem Mass Spectrometry Applications. A mixture of the three proteins (cytochrome *c*, carbonic anhydrase, and bovine serum albumin) dissolved in 50 mM NH4HCO3 solution at a total concentration of 0.03 mg/mL (0.01 mg/mL for each) was analyzed. The injected volume of the protein mixture solu-
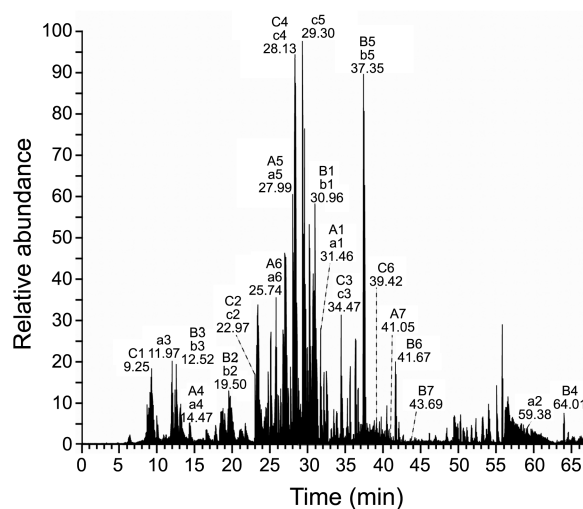

Figure 2. A base peak ion chromatogram obtained from the dual tandem ETD/CAD LC-MS/MS analysis of the online trypsin-IMER digestion mixture of the three proteins (cytochrome *c*, carbonic anhydrase, and bovine serum albumin).

tion was 2 μL, and the mixture was eluted through the trypsin-IMER at a flow rate of 300 nL/min. Peptides, digested with the trypsin-IMER, were separated by capillary C18 reversed-phase chromatography and then analyzed by application of both collisionally-activated dissociation (CAD) and electron transfer dissociation (ETD).

Figure 2 shows the base-peak ion chromatogram obtained for the three protein mixture. On the base-peak chromatogram, only representative peaks with large peak areas are

Table 1. Representative tryptic peptides shown in Figure 2. The peptide peaks annotated as a, b, and c denote peptides that are digested from cytochrome *c*, carbonic anhydrase, and bovine serum albumin, respectively, and are identified by CAD, whereas peptides A, B, and C are identified by ETD

| Site	Sequence	# of missed cleavage	m/z	Charge	CAD /ETD
a1, A1: (10-14)	IFVQK	0	633.4	2+	CAD/ETD
a2: (1-8)	MGDVEKGK	1	862.4	2+	CAD
a3: (1-9)	MGDVEKGKK	2	990.5	3+	CAD
a4, A4: (29-39)	TGPNLHGLFGR	0	1167.6	2+	CAD/ETD
a5, A5: (29-40)	TGPNLHGLFGRK	1	1295.7	2+	CAD/ETD
a6, A6: (41-54)	TGQAPGFSYTDANK	0	1456.1	2+	CAD/ETD
A7: (74-87)	KYIPGTKMIFAGIK	2	1566.4	2+	ETD
b1, B1: (159-167)	VLDALDSIK	0	973.1	2+	CAD/ETD
b2, B2: (28-36)	QSPVDIDTK	0	1002.1	2+	CAD/ETD
b3, B3: (149-158)	VGDANPALQK	0	1011.5	2+	CAD/ETD
B4: (213-224)	EPISVSSQQMLK	0	1347.1	2+	ETD
b5, B5: (37-57)	AVVQDPALKPLALVYGEATSR	0	2197.2	2+, 3+	CAD/ETD
B6: (227-251)	TLNFNAEGEPELLMLANWRPAQPLK	0	2852.3	3+	ETD
B7: (28-58)	QSPVDIDTKAVVQDPALKPLALVYGEATSRR	2	3338.3	2+	ETD
C1: (29-34)	SEIAHR	0	711.4	2+	ETD
c2, C2: (257-263)	LVTDLTK	0	788.5	2+	CAD/ETD
c3, C3: (549-557)	QTALVELLK	0	1014.0	2+	CAD/ETD
c4, C4: (402-412)	HLVDEPQNLIK	0	1305.7	1+, 3+	CAD/ETD
c5: (437-451)	KVPQVSTPTLVEVSR	1	1639.2	2+	CAD
C6: (249-263)	AEFVEVTKLVTDLTK	1	1691.9	3+	ETD
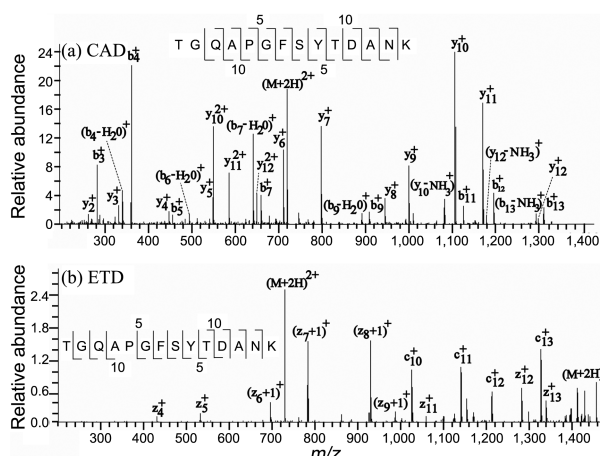
Figure 3. The tandem mass spectra of doubly-protonated TGQAPGFSYTDANK (retention time (rt) = 25.7 min) from cytochrome c obtained using (a) CAD and (b) ETD tandem mass spectrometry methods, respectively. The peptide backbone fragmentations are summarized on the linear sequences.

annotated, where a, b, and c denote peptides from cytochrome c, carbonic anhydrase, and bovine serum albumin, respectively, identified by CAD, whereas A, B, and C indicate identification by ETD. These representative peaks are summarized in Table 1. In total, 30, 19, and 72 peptides were identified for cytochrome c, carbonic anhydrase, and bovine serum albumin, respectively, from chromatogram analysis using the Mascot search engine with the option of two missed trypsin cleavages. The detailed analysis results are described in the following section.

Figure 3 shows the representative (a) CAD and (b) ETD tandem mass spectra for TGQAPGFSYTDANK (retention time (rt) = 25.7 min) from cytochrome c. As expected, quite different fragmentation patterns were observed in these two mass spectra. In the CAD mass spectrum (Figure 3(a)), annotated fragments are all b or y ion-type, while in the ETD mass spectrum (Figure 3(b)), c and z-type ions were the

major fragment ions. Some z-type ions were found also in the form of 'z+1' that are often found in ECD/ETD mass spectra.[28,43,70]

For carbonic anhydrase and bovine serum albumin, similar fragmentation patterns were also observed. As can be seen in Figure 4, c and z-type ions were also the dominant fragment ions in the ETD mass spectra for peptides from carbonic anhydrase (Figure 4(a)) and bovine serum albumin (Figure 4(b)). This fragmentation behavior is consistent with the general characteristic fragmentation patterns of CAD and ETD.[35,38,40,44,71] To determine if there was a correlation between the charge states of the peptides examined and the relative tandem mass spectrometry efficiencies of ETD and CAD, the number of identified peptides was analyzed for different charge states. Figure 5 shows a comparison of the three proteins. For triply-charged (3+) peptides, the number of peptides identified by ETD was consistently higher than that with CAD, which is consistent with previous literature.[37,38,71] However, for doubly-charged (2+) peptides, the efficiency of ETD in comparison with CAD was not consistent. For example, as shown in Figure 5, in the case of BSA, ETD identified more peptides than CAD; however, for carbonic anhydrase, CAD was a little more efficient. For cytochrome c, the identification efficiencies of ETD and CAD were identical. For singly-charged peptides, ETD could not be used to since it requires at least a double charge to operate.

In general, it is known that ETD efficiency is higher for highly charged ($\geq$ 3+) peptide cations under analysis, with some precursor m/z ceiling for a successful ETD event.[37,71] In general, the higher the charge state of the peptide, the better the fragmentation efficiency. Since ETD involves electron transfer reactions between positively-charged peptide ions and negativelycharged anions, peptides of higher charge state tend to more efficiently attract negative ions, thus inducing better ETD fragmentations. However, it should also be noted that non-covalent interaction, particularly for peptide precursors with high residue/charge ratios, may hinder this trend as the cleaved fragments may remain bound
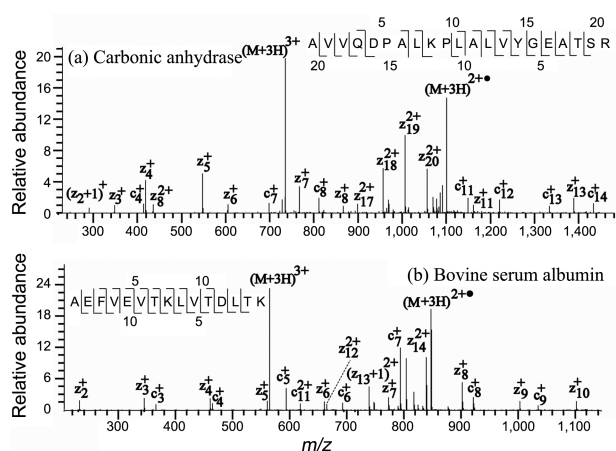

Figure 4. The ETD tandem mass spectra of triply-protonated precursor peptide cations; (a) AVVQDPALKPLALVYGEATSR (rt = 37.5 min) from carbonic anhydrase, (b) AEFVEVTKLVTDLTK (rt = 39.6 min) from bovine serum albumin. The peptide backbone fragmentations are summarized on the linear sequences.
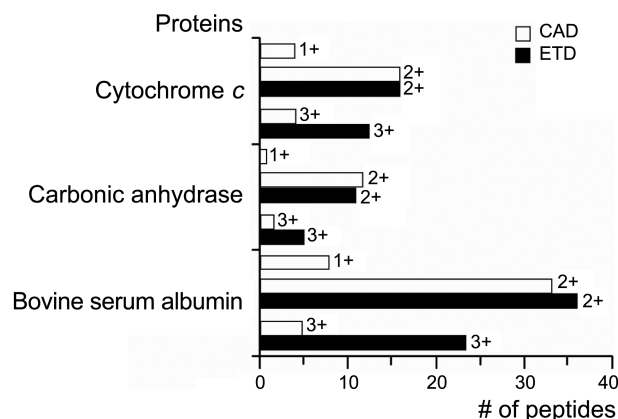

Figure 5. The number of identified peptides summarized based on their precursor charge states. The numbers of peptides identified by ETD are denoted with filled bars, while those by CAD are shown with empty bars.
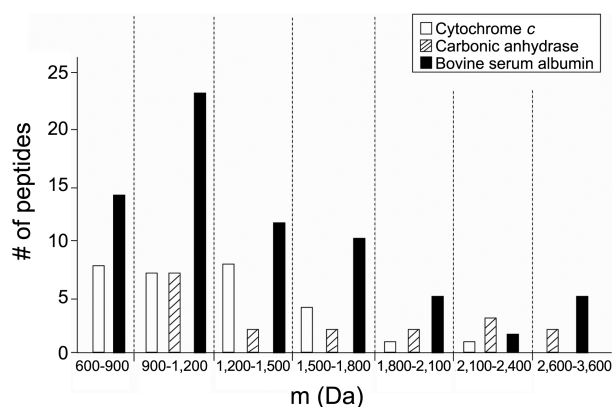
Figure 6. The number of identified peptides as a function of mass values of precursor peptide cations. Note that the different charge states of the same peptides, *i.e.*, those with different *m/z* values, are counted as different entities.

through noncovalent interactions, *i.e.*, nondissociated electron-transfer product species, $[M+nH]^{(n-1)+\bullet}$.[37,72,73] To overcome the decrease in sequence information caused by the generation of charge-reduced species, the so-called ETcaD approach, in which gentle collisional activation is applied to the charge-reduced species, has been used.[37,44,71] Based on these unique properties of ETD and CAD, a decision tree-driven tandem mass spectrometry approach, in which, for 2+ peptides, only CAD is applied and, for 3+ and higher charged states, ETD/CAD are co-implemented, has previously been introduced.[38]

Statistical Analysis. Figure 6 shows the analysis results for the masses of the identified peptides. The peptides with masses between 900 and 1,200 Da were most frequently found, although the number of peptides with masses less than 900 Da was also significant. For peptides with high masses, *e.g.*, > 1,500 Da, the presence of the charge-reduced species ($[M+nH]^{(n-1)+\bullet}$), which can be found intact even after backbone cleavage by ETD, were carefully searched; however, the populations of these reduced species were not significant. These results clearly show that the enzyme activity of trypsin immobilized within the capillary was well conserved.

The sequence coverages by CAD and ETD were also compared for the three proteins. For cytochrome *c* with 11.7 kDa, both CAD and ETD covered almost 100% of the sequence. However, for carbonic anhydrase (29.1 kDa),

CAD and ETD independently identified 56% and 68% of the total sequence, respectively, and when the two methods were combined, 72% of the total sequence was covered. ETD sequence coverage was a little higher than that of CAD. For BSA (69.3 kDa), ETD was significantly more efficient than CAD. The sequence coverages by CAD and ETD were 49% and 68%, respectively, and a total 73% sequence coverage was achieved. The reason why ETD sequence coverage was noticeably higher for the larger BSA proteins than that of CAD is speculated as follows. In general, the larger proteins are likely to be less denatured compared with the smaller proteins, particularly under our IMER experimental conditions. Therefore, the larger BSA proteins are more likely to produce longer peptides with a number of possible cleavage sites, such as lysine and arginine, being skipped when subjected to tryptic digestion. For longer peptides, it is well known that ETD is more efficient than CAD in identifying peptides.

Furthermore, although direct comparison is not appropriate due to differences in used sample amount, elution flow rate, and length of the reactor, our sequence coverage for BSA was significantly higher than that of Ma *et al.* in which CAD was solely used for peptide identification; 42% *versus* 73%.[62] Figure 7 shows Venn diagrams representing the number of peptides identified by CAD and ETD, respectively. It was found that a significant number of peptides were uniquely identified by either CAD or ETD; 23.3%, 26.4%, and 50% for cytochrome *c*, carbonic anhydrase, and bovine serum albumin, respectively. This indicates that CAD and ETD provide significant complementary sequence information. Overall, the above analysis results clearly demonstrate that CAD and ETD tandem mass spectrometry used in combination provide excellent sequence coverage, which is in agreement with previous reports.[37,44]

Conclusions

A combination approach utilizing an online trypsin-immobilized enzyme reactor (IMER) and CAD/ETD dual tandem mass spectrometry was demonstrated for some model proteins. In particular, the organic and inorganic hybrid monolithic IMER was utilized for better performance for online trypsin digestion. This combination approach provided the advantages of both trypsin-IMER and CAD/ETD dual tandem mass spectrometry applications: rapid digestion,
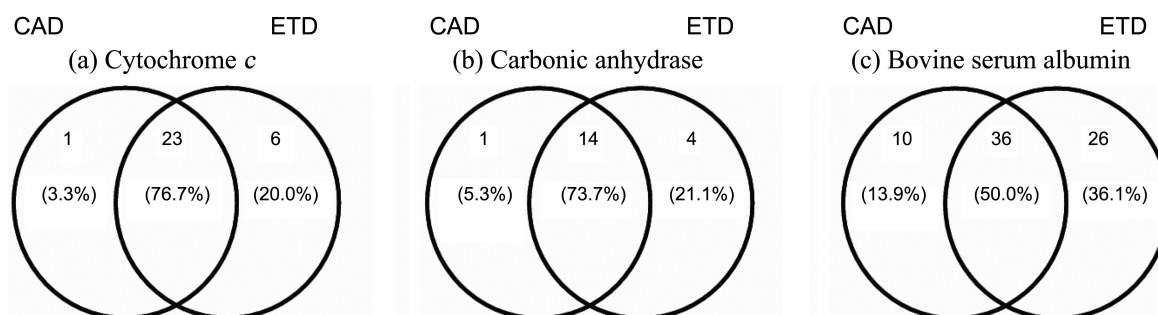

Figure 7. The Venn diagrams representing the number of peptides identified by CAD and ETD, respectively.

good digestion efficiency, online coupling of trypsin IMER and liquid chromatography, and high sequence coverage. It was previously reported that immobilized trypsins in the trypsin-IMER showed good substrate-enzyme affinity (*e.g.*, K_m value in the Michaelis-Menten analysis) similar to free trypsins and a 6,600 times higher maximum velocity, V_m, than in-solution trypsin digestion.[62] The latter advantage was attributed to the high concentration of trypsin in the limited reactor space, the partitioning effects of the immobilized trypsin, and the enhanced mass transfer in the monolithic support. In the present study, it was demonstrated that the advantages of the trypsin-IMER can be enhanced when it is combined with CAD/ETD dual tandem mass spectrometry. As previously demonstrated by other mass spectrometry practitioners, dual tandem mass spectrometry application of CAD and ETD is a very powerful approach for the comprehensive characterization of complex protein mixtures, in particular, for trace-level characterization of proteins with post-translational modifications.[37,44,74] We believe that the combined approach introduced in the present study holds great promise for further improving the automation of LC-MS/MS analysis of complex protein mixtures.

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