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Analysis of protein digests by transmission-mode desorption electrospray ionization mass spectrometry with ultraviolet photodissociation

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ABSTRACT

Transmission mode desorption electrospray ionization is coupled with ultraviolet photodissociation at 193 nm for the analysis of protein digests. SEQUEST is utilized for data interpretation and database searches. Comparative results are presented for transmission mode desorption electrospray ionization (TM-DESI)-MS/collision induced dissociation (CID) and TM-DESI-MS/ultraviolet photodissociation (UVPD) analyses of five proteolyzed model proteins ranging in molecular weight from 8.5 kDa (ubiquitin) to 66 kDa (bovine serum albumin, BSA). In some cases TM-DESI/UVPD yielded greater confidence in database correlation scores for peptides and comparable protein identification compared to TM-DESI-MS/CID due to the production of an extensive array of sequence ions and the ability to detect low m/z terminal sequence ions and immonium ions.

1. Introduction

The undisputed success of MALDI-MS and ESI-MS methods for proteomic applications has led to increasing emphasis on the adaptation of these methods for higher throughput analysis. There have been significant advances in the development of MALDI-MS for high-throughput applications because of its natural compatibility with the analysis of discrete samples, such as those from a complex 2D-gel separation, robotically deposited on a multi-well plate, with analysis requiring just a few laser shots per sample and detection limits in the sub fmol range [1,2]. For bottom-up proteomics studies, peptide mass fingerprinting (PMF) and now primarily sequencing by tandem mass spectrometry (MS/MS) are the most frequently used methods for protein identification [3,4]. Numerous MS/MS-based strategies have emerged as valuable tools for the analysis of both pure protein samples and complex mixtures and are readily integrated with HPLC–ESI-MS platforms [5–10].

The benefits of high-throughput workflows have motivated the search for new alternatives to MALDI-TOF-MS and HPLC-ESI-MS/MS methods. In this context, ambient mass spectrometry techniques, such as desorption electrospray ionization (DESI) [11] and direct analysis in real time (DART) [12], provide simple yet highly efficient methods for analyzing multiple discrete samples, generating multiply charged analyte ions, and allowing analysis in a high-throughput manner. Ambient mass spectrometry has revolutionized the means by which samples are introduced to the mass spectrometer. Since the introduction of DESI [11], the field of ambient mass spectrometry has grown rapidly due the recognition of the innumerable possible applications that exploit the ability to analyze samples quickly and with minimal sample preparation in a high-throughput manner. As a result, nearly 30 ambient ionization techniques have been introduced in the past six years, as summarized in two recent reviews [13,14].

In addition to touted benefits for desorption and ionization of small molecules, ESI-like ambient ionization techniques are capable of analyzing large non-volatile biomolecules, and a number of recent studies have been reported for the investigation of peptides, proteins and enzymatically digested proteins [15–25]. In one of the first studies, DESI was coupled to an FTICR mass spectrometer for high resolution detection of small proteins (up to insulin, MW 5.8 kDa) [15]. The rapid analysis of tryptic peptides from bovine serum albumin (BSA) without sample pre-treatment or clean-up by DESI-ion mobility mass spectrometry was reported [16], allowing the identification of BSA by a pseudo-peptide mass fingerprint and MASCOT database search strategy. DESI-MS was also utilized to analyze intact proteins up to 18 kDa with ng detection limits with generally less successful detection of much larger proteins (up to 66.4 kDa) [17]. The use of electrospray-assisted laser desorption ionization (ELDI) was demonstrated for the analysis of liquid samples of peptides and proteins up to 66 kDa (BSA) and tryptic protein digests, as well as the combination of ELDI with tandem mass spectrometry for effective MS/MS characterization [18]. In a follow-up study the successful analysis of proteins up to 80 kDa by IR-ELDI was reported [19]. Nano-porous alumina

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surfaces were shown to afford higher ion abundances, greater signal stability, and improved limits of detection compared to other surfaces for DESI analysis of peptides and proteins [20]. Another hybrid method, MALDESI, was demonstrated for the analysis of peptides and proteins ranging from 1 to 17 kDa [21]. In a pair of studies that focused on the use of DESI for direct detection of analytes separated on thin layer chromatography plates, DESI was employed for the analysis of tryptic peptides from cytochrome *c* and myoglobin with sequence coverages of 81% and 74% for cytochrome *c* and myoglobin, respectively, based on data dependent MS/MS [22,23]. DESI was also used for the direct analysis of liquid samples in which amino acids, peptides, proteins up to 66 kDa and tryptic digests were successfully ionized from solutions with pmol detection limits [24]. More recently, DESI-FTICR-MS was successfully utilized for protein sequencing of intact myoglobin and cytochrome *c* by collision induced dissociation (CID) and electron capture dissociation (ECD), requiring approximately 10 nmol for top-down analysis [25].

Due to the inherent complexity of biological samples and transient nature of analyte ion signal generated by desorption ionization methods, it is crucial for ion activation to be fast and efficient in order to maximize the depth of analysis for each sample in MS/MS experiments. Recently, a number of studies [26–32] have shown short wavelength (157 and 193 nm) ultraviolet photons provide fast and efficient activation of peptide ions upon the absorption of a single photon. Ultraviolet photodissociation (UVPD) at 157 and 193 nm produces fragmentation patterns containing *a*, *b*, *c*, *x*, *y*, *z* sequence ions as well as immonium and *v* and *w* side-chain loss ions. In a recent study from our group [32] it was shown that UVPD at 193 nm using a single 5 ns, 8 mJ laser pulse provided comparable and often improved peptide identification by *in silico* database searching for biologically relevant samples. The integration of fast UV photoactivation with the minimal sample preparation, high-throughput capabilities of DESI offers a compelling opportunity for proteomics applications. We have previously reported transmission mode desorption electrospray ionization (TM-DESI) [33,34], a method employing a simplified geometry for DESI experiments in which the sample is placed in-line between the ESI source and the mass spectrometer inlet on a mesh substrate, allowing transmission of the ESI plume through the sample. In the present study, the analysis of proteins is facilitated by the use of TM-DESI-MS/UVPD. CID and UVPD are compared as sequencing methods for confident protein in a TM-DESI-MS mode.

2. Experimental

2.1. Materials

Bovine model proteins α-casein, bovine serum albumin, β-lactoglobulin A, carbonic anhydrase, cytochrome *c*, ubiquitin, proteomics grade trypsin, acetic acid, ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide (IAM) were purchased from Sigma–Aldrich (St. Louis, MO). Chicken lysozyme was purchased from MP Biomedicals (Solon, OH). HPLC grade acetonitrile, methanol and water were purchased from Fisher Scientific (Fairlawn, NJ). Sep-Pak® 1 cm^3 C18 cartridges were obtained from Waters (Milford, MA). Polypropylene mesh with a 149 μm open space and 106 μm strand diameter (PP149) was purchased from Small Parts Inc. (Miramar, FL).

2.2. Sample preparation

Model protein stock solutions were prepared at 100 μM in pH ~8, 100 mM ammonium bicarbonate for trypsin digestion. Sulfhydryl reduction was performed by adding 10 μL of acetonitrile and 5 μL of 100 mM DTT to 50 μL of protein stock in a 2 mL centrifuge tube, then incubated at 40 °C for 1 h. Reduced sulfhydryl groups were then alkylated by adding 4 μL of 500 mM IAM and reacted in the dark for 1 h at 37 °C. Excess IAM was quenched by adding another 15 μL of 500 mM DTT and reacted for 30 min at room temperature. 1 mg/mL trypsin in 1 mM HCl was added to give a protein to trypsin ratio of 50:1. The total volume was increased to 100 μL with water and incubated for 16 h at 37 °C. Protein digests were cleaned-up using C18 cartridges then diluted to 10 μM with 90:10 (v/v) of methanol/water with 1% acetic acid.

2.3. Transmission mode DESI, mass spectrometry, and ultraviolet photodissociation

All experiments were performed using an Omni Spray ion source (Prosolia, Inc., Indianapolis, IN) mounted to a Thermo Fisher Scientific LTQ XL mass spectrometer (Waltham, MA). The Omni Spray source was equipped with a sample holder that permits 0° desorption geometry. The mesh substrate was held between a mask and backing plate made from sheet polyetheretherketone (PEEK). The mask is 1.5 mm thick and has six 4 mm square openings with 2 mm spacing to allow transmission of the electrospray plume through the mesh substrate. The openings in the mask are analogous to the array of wells on a MALDI sample plate. The distance from the electrospray emitter to the mesh was 2 mm, and the distance from the mesh to the mass spectrometer inlet was 8 mm. Samples were deposited on PP149 mesh in 2 μL aliquots and analyzed wet. The electrospray solvent was methanol at a flow rate of 5 μL/min with an electrospray voltage of 4.5 kV. The sample holder was scrolled perpendicularly to the electrospray plume at 250 μm/s and yielded an analysis time of 2.4 min for six sample wells. Nitrogen at a pressure of 100 psi was used as the nebulizing gas and the heated capillary temperature was 250 °C.

193 nm photons were generated by a Coherent Excistar XS excimer laser (Santa Clara, CA). The back plate of the mass spectrometer was modified with a CF viewport flange with a CaF_2 window as described previously [35]. The unfocused laser beam was aligned on axis with the linear ion trap through a 2 mm aperture. XCalibur version 2.2 software package was used to perform data dependent acquisitions. The data dependent acquisition employed the following scan sequence: a full scan mass spectrum over the range of *m/z* 400–2000, then MS/MS spectra for each of the 20 most abundant ions in the full scan mass spectrum. A single 5 ns, 8 mJ pulse and a *q*-value of 0.100 were used during the activation period of each MS/MS scan for UVPD experiments. The commercial LTQ software limited the minimum activation period to 0.03 ms even though the isolated ions were only irradiated for 5 ns. For CID experiments, the standard 30 ms activation period with a *q*-value of 0.250 was used, and the normalized collision energy was set to 35%. An isolation width of 2 *m/z* was used for all MS/MS scan events. For all experiments, automated gain control (AGC) was set to 3×10^4 for MS and 1×10^4 for MS/MS scans. For MS and MS/MS scans the maximum injection times were set to 50 ms and 100 ms respectively. Each MS scan was the average of six microscans and each MS/MS scan was the average of four microscans. MS spectra and CID MS/MS spectra were collected in profile mode. UVPD MS/MS spectra were collected in centroid mode to allow photoionization background subtraction prior to protein database processing. Dynamic exclusion parameters included duration of 45 s, exclusion list size of 50 *m/z* values, and a repeat count of one.

2.4. Background subtraction and protein database searching

UVPD photoionization background peaks possibly arising from pump oil and hydrocarbon impurities in the ion trap helium bath gas were subtracted as previously described [32]. Thermo Fisher Scientific Proteome Discoverer 1.0 software package was used to

Table 1
Results for SEQUEST database search for UVPD and CID.

Protein	MW (kDa)	SEQUEST Score		Unique peptides		Sequence coverage	
		UVPD	CID	UVPD	CID	UVPD	CID
α-Casein	24.5	95 ± 7	85 ± 12	7 ± 1	7 ± 1	37% ± 1	37 ± 4
β-Lactoglobulin A	20	103 ± 10	88 ± 18	9 ± 1	8 ± 1	54% ± 3	52% ± 3
BSA	66	123 ± 22	189 ± 20	21 ± 3	28 ± 2	43% ± 6	54% ± 5
Carbonic anhydrase	29	57 ± 13	76 ± 12	8 ± 2	9 ± 2	47% ± 9	53% ± 7
Lysozyme	14.7	78 ± 19	84 ± 12	10 ± 1	11 ± 2	69% ± 3	75% ± 7

perform SEQUEST database searches for interpretation of MS/MS data. SEQUEST search parameters included a signal:noise ratio of 3, a precursor mass tolerance of 3 Da, fragment mass tolerance of 2 Da, 1 missed cleavage by trypsin, methionine oxidation as a dynamic side chain modification and carbamidomethyl as a static side chain modification of cysteine. Product ion series for CID spectra included *a*, *b* and *y* ions and UVPD product ion series were *a*, *b*, *c*, *x*, *y* and *z* ions. Nonredundant bovine and chicken protein databases from the NCBI were used for proteins form the respective species. Peptide matches were filtered based on charge state and XCorr scores, and protein matches were rejected if they did not receive more than two peptide matches. MS/MS spectra that passed the filters but received probability scores less than 1.00 were manually verified.

3. Results and discussion

Protein digests were deposited into the wells of the TM-DESI sample holder, then the sample holder was rastered in front of the ESI plume. Full scan mass spectra and MS/MS spectra were acquired with ion activation by CID or UVPD. MS/MS sequencing experiments were performed using a sample well scan rate of 250 μm/s because this rate provided a reasonable compromise between the number of MS/MS spectra acquired per sample well and total analysis time. The total analysis time was 2.4 min (for six wells), and approximately 20 MS/MS spectra could be acquired per sample well. The same sample was deposited in all six sample wells, and

Table 2
Peptide spectral matches for UVPD and CID obtained for five model proteins.

Protein	PSMs	
	UVPD	CID
α-Casein	23 ± 2	26 ± 3
β-Lactoglobulin A	30 ± 4	23 ± 4
BSA	38 ± 7	59 ± 5
Carbonic anhydrase	19 ± 4	20 ± 3
Lysozyme	24 ± 3	23 ± 5

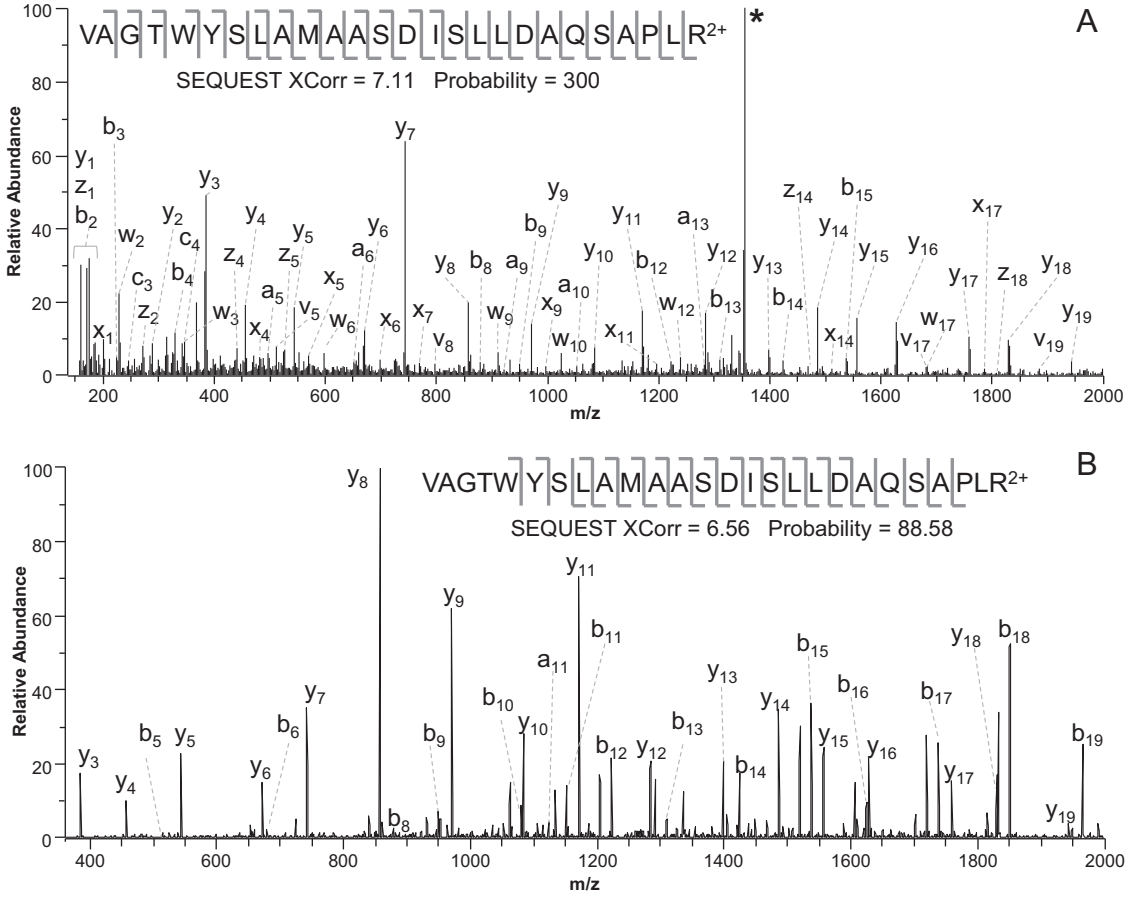

Fig. 1. (A) UVPD (one 5 ns pulse at 193 nm) and (B) CID spectra for β-lactoglobulin A tryptic peptide VAGTWYSLAMAASDISLLDAQSAPLR with sequence coverage, SEQUEST XCorr and probability scores shown below the sequence. The selected precursor ions are labeled with an asterisk.

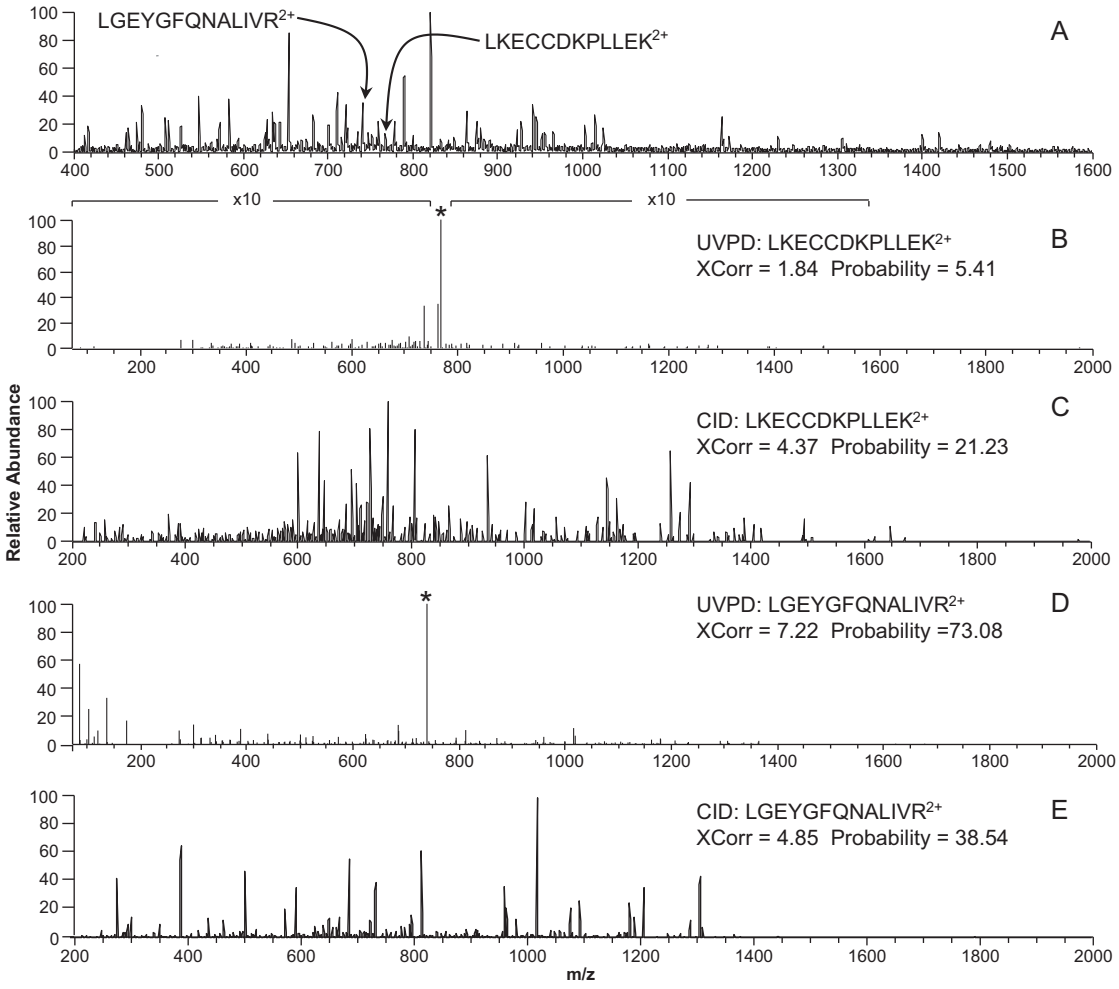

Fig. 2. A representative survey scan (A) of a BSA tryptic digest, data dependent CID (C and E) and UVPD (B and D) spectra of the peptides LKECCDKPLLEK and LGEYGFQNALIVR. Asterisks indicate the precursor ions.

SEQUEST scores were generated from the combined spectra from all six sample wells.

Five model proteins ranging in molecular weight from 8.5 kDa for ubiquitin to 66 kDa for BSA were analyzed by both CID and UVPD. Fig. 1 shows UVPD and CID spectra for one representative tryptic peptide from β-lactoglobulin A, VAGTWYSLAMAASDIS-LLDAQSAPLR. UVPD generated a significantly larger array of sequence-informative ions (e.g., *a*, *b*, *c*, *x*, *y*, *z*) compared to CID (predominately *b* and *y* type ions) as well as greater sequence coverage. The ability to operate at lower *q*-values for UVPD also allowed detection of terminal sequence ions that fall below the low mass cut-off in the corresponding CID spectrum. For some of the peptides analyzed, the greater variety of sequence ions and the ability to detect low mass terminal sequence ions led to greater confidence in the peptide identification as reflected by the higher XCorr and probability scores for UVPD. Ten replicate experiments were performed for each of the five model protein for both UVPD and CID. Table 1 summarizes the overall SEQUEST search results for both UVPD and CID sequencing experiments. The overall results for CID and UVPD are similar with respect to number of unique peptides, protein score, and sequence coverage for the proteins analyzed in this study. CID marginally outperforms UVPD for three of the five proteins analyzed, an outcome attributed to the decreased sensitivity of UVPD due to secondary dissociation of product ions and lower fragmentation efficiency and product ion abundance compared to CID. It has been shown that fragmentation efficiency for UVPD at 193 nm is greater for peptides with amino acids containing aromatic side chains (e.g., F, W and Y) [32]. Peptides containing zero aromatic residues showed fragmentation efficiencies of approximately 50%, and peptides containing multiple aromatic residues yielded fragmentation efficiencies of greater than 90%. However, due to the large number of fragmentation pathways accessible by 193 nm UVPD, the product ion current is spread between a large number of product ions and thus the individual abundances of many of the product ions are low. As a result UVPD may not perform as well for low abundance peptides, leading to fewer peptide spectral matches (PSMs).

Table 2 displays the average number of PSMs obtained by CID and UVPD for each of the model proteins. For α-casein, β-lactoglobulin A, carbonic anhydrase and lysozyme, UVPD and CID perform very similarly. However, in the case of BSA, CID produced on average 21 more PSMs than UVPD which led to the identification of seven more unique peptides on average. These observations can be rationalized by the effects of ion suppression during ESI and the greater average sensitivity of CID compared to UVPD. The molecular weight of BSA is more than twice that of the next largest protein analyzed and produces 46 tryptic peptides (assuming zero missed cleavages and minimum peptide length of six residues). Since the digests are analyzed without prior chromatographic separations, some peptides will experience significant suppression during ESI relative to others.

Fig. 2 shows worst case and best case scenarios for UVPD relative to CID. Fig. 2A is a representative DESI-MS survey scan from one BSA analysis. The ion of *m/z* 767 corresponding to doubly charged

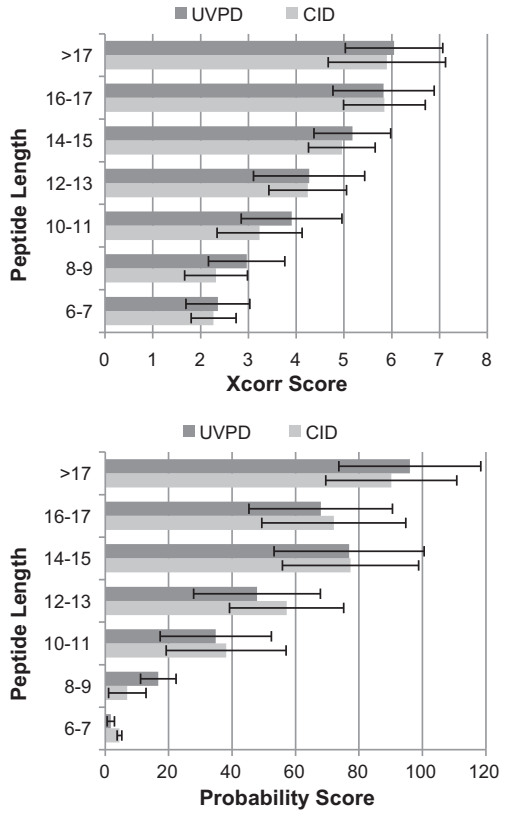

Fig. 3. Average peptide XCorr (A) and probability (B) scores of the top scoring PSMs of the peptides identified by both CID and UVPD as a function of peptide length.

LKCCDKPLLEK was chosen for MS/MS analysis and representative UVPD and CID are shown in Fig. 2B and C, respectively. The abundance of the LKCCDKPLLEK peptide is low (~10% relative abundance), and this peptide does not contain any aromatic residues, moderating its native absorption cross-section at 193 nm. The XCorr and probability scores for the CID spectrum are significantly higher and produce a confident identification whereas the UVPD spectrum does not pass the database search results filtering. Fig. 2D and E are UVPD and CID spectra, respectively, from the same BSA analyses of doubly charged LGEYGFQNALIVR. The peptide LGEYGFQNALIVR of moderate abundance (~40% relative abundance) contains two aromatic residues. In this case UVPD significantly out-performs CID as indicated by the greater XCorr and probability scores. Once again, the diverse array of product ions produced by UVPD at 193 nm and the ability to detect low mass immonium and terminal sequence ions lead to greater confidence in the peptide match.

From the five model proteins, 67 unique peptides were identified by both UVPD and CID. CID identified 10 unique peptides that were not identified by UVPD and seven additional unique peptides were identified by UVPD only. Fig. 3 displays the average peptide XCorr (A) and probability (B) scores of the top scoring PSM for the unique peptides identified by both CID and UVPD as a function of peptide length. The results are similar with neither method displaying a distinct advantage for any particular length of peptide. In general, both the XCorr and probability scores increase with peptide length. This trend is due to the additive nature of their calculations, thus spectra of larger peptides will score higher than smaller peptides even for MS/MS spectra of similar quality [36]. The significance is that there are only marginal differences in the peptide XCorr and probability scores produced by CID and UVPD for each peptide length, and overall both methods are very comparable. The key advantage of UVPD is the rich fragmentation information and very short activation time (i.e., a single 5 ns pulse) compared to the standard 30 ms activation period for CID, making the latter less efficient for high-throughput applications.

4. Conclusions

This study demonstrated the utility of TM-DESI for MS/MS sequencing of protein digests. The coupling of TM-DESI with UVPD at 193 nm allowed rapid MS/MS analysis of protein digests and confident protein identification through SEQUEST database searches. In some cases, UVPD at 193 nm provided greater confidence in peptide identification compared to CID due to the extensive array of sequence ion types produced and the ability to detect terminal sequence ions at reduced q-values. Design and production of sample well masks analogous to a 96 well MALDI target would further increase the throughput of TM-DESI experiments for MS/MS sequencing.

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