Evaluation of the New MALDI Matrix 4-Chloro-α-Cyanocinnamic Acid

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MALDI-TOF continues to be an important tool for many proteomic studies. Recently, a new rationally designed matrix 4-chloro-α-cyanocinnamic acid was introduced, which is reported to have superior performance as compared with the "gold standard" α-cyano-4-hydroxycinnamic acid (CHCA).[1] In this study, the performance of this new matrix, using the Shimadzu Biotech Axima TOF[2] (Shimadzu Biotech, Manchester, UK), was investigated. The overall sequence coverage as well as sensitivity of this matrix were compared with CHCA using standard protein tryptic digests. The performance of this matrix with labile peptides, such as phosphopeptides and 4-sulfophenyl isothiocynate-derivatized peptides, to facilitate de novo sequencing was also explored. This matrix was found to be better performing than CHCA in overall sensitivity and showed better sequence coverage at low-digest levels, partly as a result of less of a bias for arginine-containing peptides. It also showed as much as a tenfold improvement in sensitivity with labile peptides on standard stainless-steel targets. In addition, as a result of the much cooler nature of this matrix, labile peptides are readily seen intact with much less fragmentation in mass spectrometry (MS) mode. This matrix was also evaluated in the MS/MS fragmentation modes of post-source decay (PSD) and collisional-induced dissociation (CID). It was found that fragmentation occurs readily in CID, however as a result of the very cool nature of this new matrix, the PSD fragments were quite weak. This matrix promises to be an important addition to the already extensive array of MALDI matrices.

KEY WORDS: α-cyano-4-hydroxycinnamic acid, CID, MS, phosphorylation, PSD, 4-sulfophenyl isothiocyanate, TiO_2, trypsin

INTRODUCTION

As its introduction in the late 1980s, MALDI mass spectrometry (MS) has been an important tool in the analysis of many biomolecules, in particular, proteins and peptides. This has been, in part, facilitated by the discovery that certain organic acids, when cocrystallized with the analyte, allow for the facile ionization of large biomolecules into the gas phase when irradiated by laser light under vaccum.[2,3] Instrumentation has evolved immensely over the past 20 years; however, the types of organic acid matrices used have remained essentially unchanged.

The MS analysis of peptide digests to identify a protein as well as some of its modifications is a well-established proteomic technique. MALDI TOF MS is particularly well suited for the peptide mass fingerprinting (PMF) technique as well as selected fragmentation of various precursors using post-source decay (PSD) and/or collisional-induced dissociation (CID). It was found early on that the organic acid α-cyano-4-hydroxycinnamic acid (CHCA) was one of the best universal matrices for analyzing peptide digests.[4]

Recently, Jaskolla and Lehmann[1] undertook a systematic approach of altering functional groups on the α-cyanocinnamic acid (CCA) core and testing the performance of each of these new matrices. By substitution of the 4-hydroxyl moiety with a chlorine atom in the benzene ring (Fig. 1), a new matrix was found with properties that rival and in many cases, surpass the performance of the "gold standard" matrix CHCA. This new matrix ClCCA was found to have properties quite similar to CHCA in terms of forming uniform cocrystals as well has having an absorption maxima close to the wavelength of the typical nitrogen (N_2) laser used in MALDI TOF MS (337 nm). As a result of the lower proton affinity of ClCCA relative to CHCA, it was hypothesized that this would in turn lead to better proton donation to the analyte, which would result in more intense ion yields as well as a lower bias for peptides with the strongly basic residue Arg.[1] These studies were extended with a comparative analysis, which showed that the sequence coverages with ClCCA as compared with CHCA were even more improved with nontryptic enzymatic digests as a result of enhanced detection of acidic and neutral peptides.[5]

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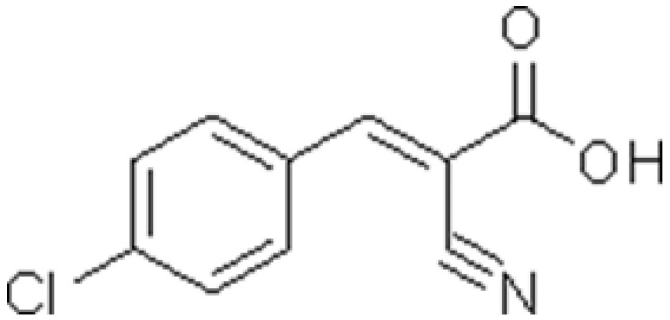

FIGURE 1

Molecular structure of 4-chloro-α-CCA (ClCCA). The chlorine atom is replaced by a hydroxyl group in CHCA.

In this study, the performance of ClCCA, using a modern MALDI TOF instrument (Shimadzu Biotech Axima TOF,[2] Shimadzu Biotech, Manchester, UK), was investigated. Initially, it was difficult to find a commercial source of this matrix, as it is not a commonly used compound. However, it was found that the Sigma Rare Chemical Library (Sigma-Aldrich, St. Louis, MO, USA) had a compound of a different name but same chemical structure. The Sigma compound 3-(4-chloro-phenyl)-2-cyano-acrylic acid has the same chemical structure as ClCCA, which has been described (Fig 1). The material obtained from Sigma-Aldrich was quite crude in appearance and required further purification through a couple of recrystallization steps. In this study, a direct side-by-side comparison of CHCA to ClCCA in the analysis of tryptic digests of the standard proteins, BSA and chicken OVA, was performed. The performance of this matrix with a standard phosphopeptide mixture in a yeast enolase digest before and after titanium dioxide (TiO_2) enrichment was also examined. Finally, the performance of this matrix with the very labile peptides formed after derivitization with 4-sulfophenyl isothiocyanate (SPITC), which has been found to facilitate de novo MS/MS sequencing dramatically, was compared.[6]

MATERIALS AND METHODS

Materials

Ultrapure water and acetonitrile (OmniSolv grade) were from EMD Chemicals (Gibbstown, NJ, USA). CHCA and the phosphopeptide standard were from Waters MassPREP products (Milford, MA, USA). The protein digest standards of BSA and OVA were from Protea Biosciences (Morgantown, WV, USA). Fibrinogen A peptide standard was from the chemically assisted fragmentation MALDI sequencing kit (Amersham Biosciences, Uppsala, Sweden). SPITC and 3-(4-chloro-phenyl)-2-cyano-acrylic acid (ClCCA), sodium bicarbonate ($NaHCO_3$), sodium hydroxide, and ammonium hydroxide (NH_4OH) were from Sigma-Aldrich. Sequencing-grade trifluoroacetic acid (TFA) was from Applied Biosystems (Foster City, CA, USA). TiO_2 NuTips (1–10 μl) for phosphopeptide enrichment were from Glygen Corp. (Columbia, MD, USA). Micro C_{18} ZipTips were from Millipore (Billerica, MA, USA).

Matrix Recrystallization

Approximately 70 mg crude ClCCA in a 1.5-ml Eppendorf tube was dissolved in 300 μl 70% acetonitrile (70:30 acetonitrile:water). The solution was heated to 50°C to facilitate dissolution and then allowed to cool to room temperature. The tube was then placed in an ice bath for a few minutes, upon which, a phase separation was observed. This is most likely a result of residual organic solvents from the synthesis. The lower phase (~50 μl) was withdrawn and discarded, as few crystals grow from this phase. The crystallization process was allowed to continue in the ice bath, at which point, long white crystals begin to grow. Sometimes, a slight warming and then cooling would get them to grow. The crystals were allowed to grow for an additional 30–60 min. The crystals were then spun down at 7–10 g, and the liquid volume above them was withdrawn carefully with a pipette. Final drying of the crystals was accomplished in a Speed Vac. This process was repeated a second time and typically provides 70–80% recovery from crude material.

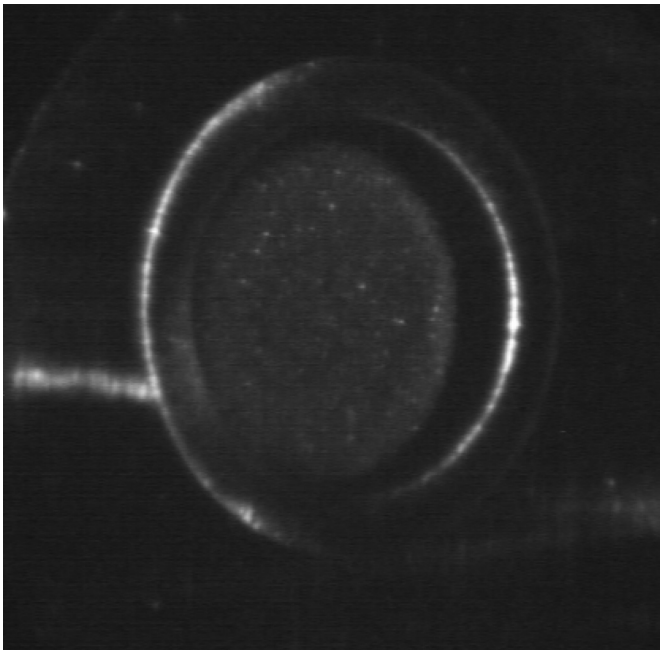

FIGURE 2

Magnified image of a single stainless-steel MALDI target showing a dried ClCCA matrix spot. The diameter of the circular-steel target is approximately 3.0 mm.

TiO_2 Phosphopeptide Enrichment

Phosphopeptides were enriched using a modification of the published method.[7] The dried peptide digest was dissolved in 10 µl binding solvent (75:19:6 acetonitrile:water:TFA). A 1- to 10-µl TiO_2 NuTip was washed two times with 10 µl aliquots of binding solvent. Phosphopeptides were bound by repetitive pipetting (20 times) of the 10-µl sample volume. The NuTip was then washed five times with binding solvent and then five times with 0.1% TFA. Phosphopeptides were then eluted in a separate tube with a 10-µl vol 6% NH_4OH after repetitive pipetting (10 times). A 5-µl aliquot of 5% TFA was then added, and the phosphopeptides were concentrated further and purified prior to MALDI analysis using a micro C_{18} ZipTip.

SPITC Derivatization

Protein digests were derivatized with SPITC as follows.[6] Dried protein digests were dissolved in 8.5 µl SPITC solution (10 mg/ml in 20 mM $NaHCO_3$, pH 9.5). The sample was incubated for 30 min at 55°C in a heating block. The reaction was stopped by the addition of 4.5 µl 5% TFA. Samples were concentrated further and desalted using a micro C_{18} ZipTip prior to MALDI analysis.

MALDI MS Analysis

All spectra were obtained manually on a Shimadzu Biotech Axima TOF^2 (Shimadzu Biotech). The appropriate concentrations of samples were used such that a 0.5-µl aliquot provided the indicated amount on the target for analysis. ClCCA (0.5 µl; 5 mg/ml) in acetonitrile:0.1% TFA (80:20) or CHCA (5 mg/ml) in acetonitrile:0.1% TFA (50:50) was added to the sample and allowed to air dry. Typical spot sizes were about 2 mm on the standard 384 spot stainless-steel targets. No attempts were made to concentrate samples further on target using special anchor chips or drying techniques. All spectra were acquired in the positive ion reflectron mode using a 337-nm N_2 laser. Typically, 250–500 laser shots were acquired for each MS spectra, and spectra were obtained at a laser power, which attempted to maximize resolution and peak intensity. All spectra were obtained from at least two samples to verify the consistency of the

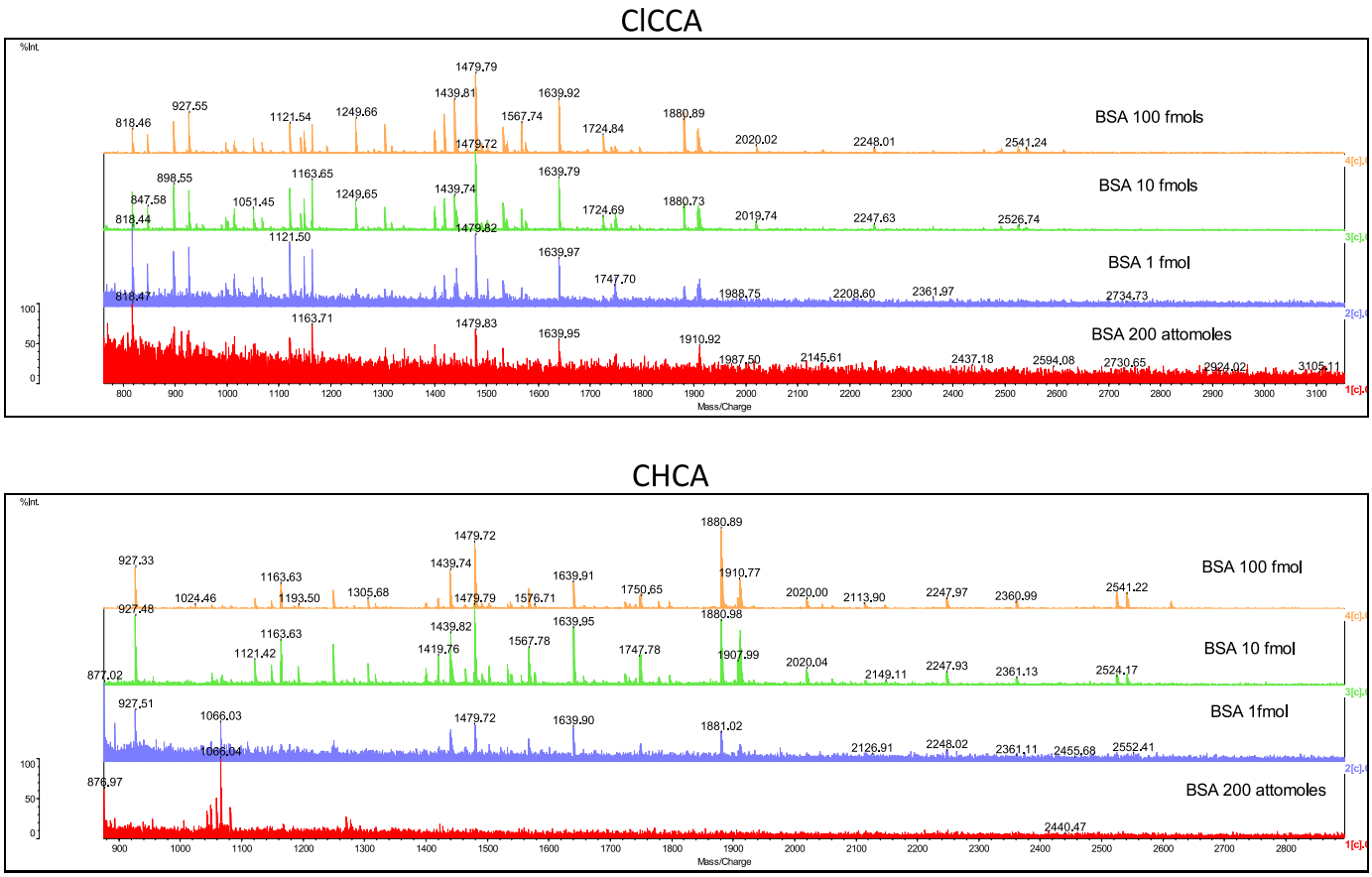

FIGURE 3

PMF of tryptic digests of BSA. The indicated amounts are what were applied to the sample target. Traces for ClCCA are on the upper panel and those for CHCA on the lower panel.

results. A local external calibrant was used, which consisted of angiotensin II (MH^+ of 1046.54), P14R (MH^+ of 1533.86), and adrenocorticotropic hormone corticotrophin-like intermediate lobe peptide 18–39 (MH^+ of 2465.20). PSD and CID spectra were acquired using a dual-timed ion gate at a laser power approximately 20% higher than MS acquisition. Helium was used as the collision gas in the CID cell. PSD and CID fragments were resolved in a curved-field reflectron, which allowed for seamless, full mass range acquisition of the MS/MS spectra. All spectra were processed with Mascot Distiller (Matrix Sciences Ltd., UK) prior to database searching, which was performed on a local server using the Mascot search engine, using the SwissProt nonredundant protein database. For MS searches, the PMF program was used with a peptide mass tolerance of 150 ppm. For MS/MS searching (PSD and CID spectra), the MS/MS Ion Search program was used with a precursor tolerance of 150 ppm and a fragment tolerance of 1.5 Da. The variable modifications of protein N-term acetylation, carbamidomethylation of Cys, oxidation of Met, and Gln to pyro Glu for N-term Gln were considered. In the phosphopeptide analyses, phospho Ser, Thr, and Tyr were also considered.

RESULTS AND DISCUSSION

Matrix Properties

The crude ClCCA obtained from the Sigma Rare Chemical Library was slightly pink in color and had a chunky appearance. After recrystallization, the material appeared white with splinter-like crystals. The ClCCA matrix formed uniform cocrystals with the peptide samples on the MALDI target (see Fig. 2). Occasionally, a slight crust ring would form at the boundary of the dried spot, but this did not appear to affect the sample signals. ClCCA usually required 10–15% more laser power for threshold signals than what was typically required for CHCA. Unlike CHCA, the ClCCA matrix was also found to be quite volatile under vacuum and would show noticeable spot disappearance and loss in signal intensities after 1 day under vacuum. This therefore requires that samples be analyzed within a few hours after being placed in vacuum or removing the plate

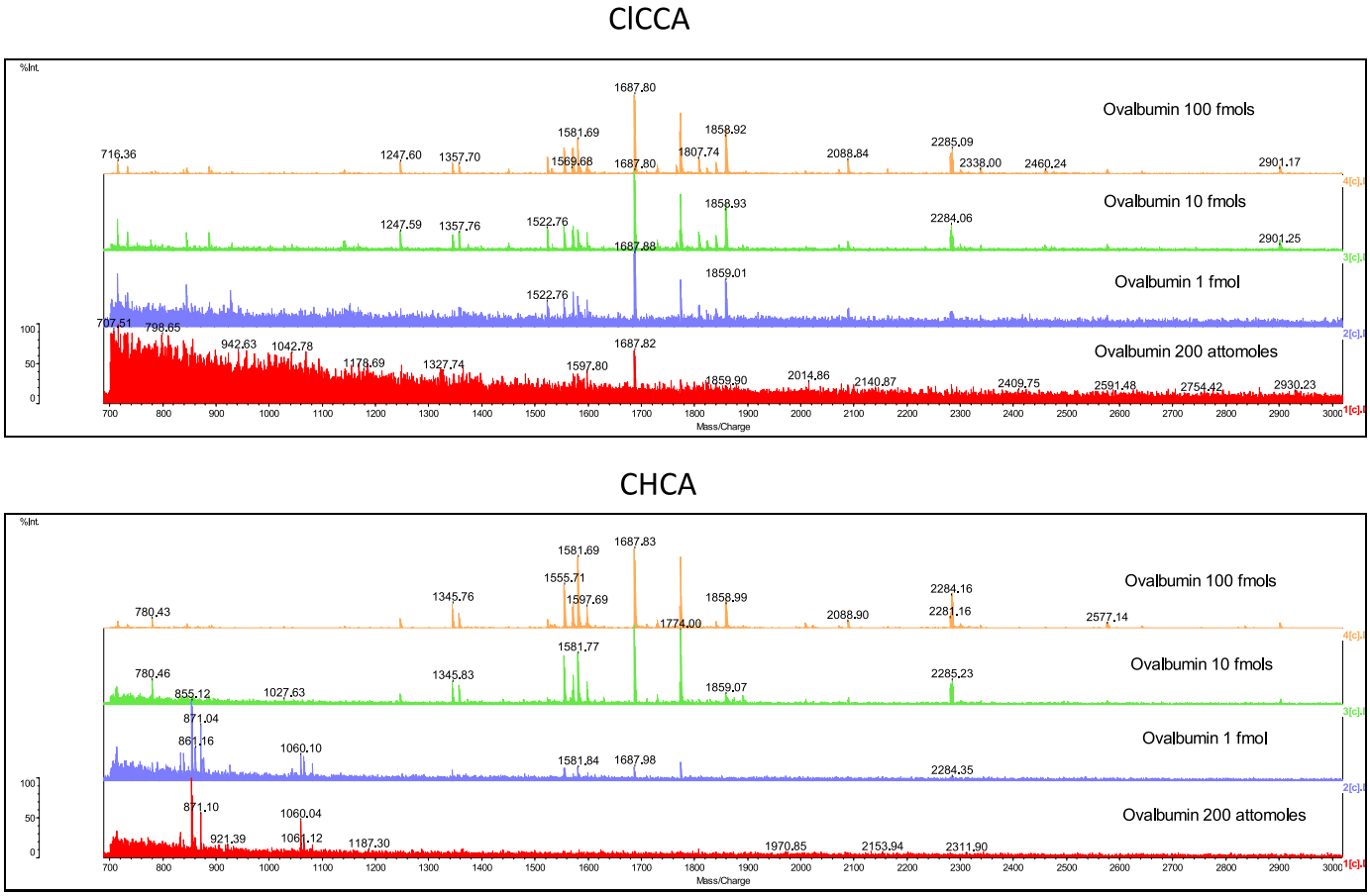

FIGURE 4

PMF of tryptic digests of chicken OVA. The indicated amounts are what were applied to the sample target. Traces for ClCCA are on the upper panel and those for CHCA on the lower panel.

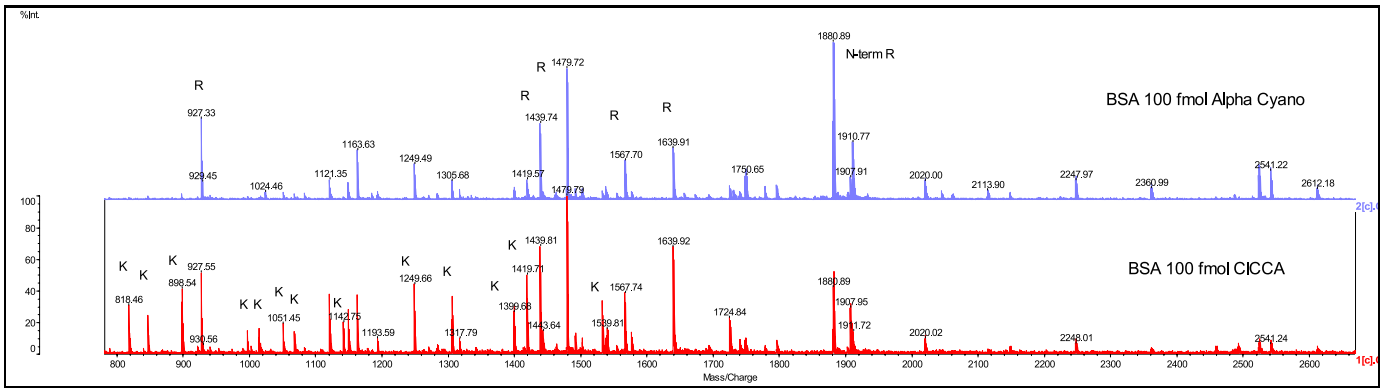

FIGURE 5

A PMF comparison for the 100-fmol load of BSA tryptic digest between ClCCA (lower trace) and CHCA (upper trace). Some of the peptides with C-terminal Lys (K) residues are indicated in the ClCCA trace, and some C-terminal Arg (R)-containing peptides are indicated in the CHCA trace. The 1880.89 peptide contains an N-terminal Arg, which causes its enhanced signal in the CHCA matrix.

completely if samples cannot be analyzed the same day. It was found that the matrix crystals are stable at atmospheric pressure for at least several days. This matrix also exhibits an absorption band, which is shifted down from that of CHCA.[1] This will limit its use with other types of lasers, such as the Nd:YAG at 355 nm.

Tryptic Digests

In an effort to compare the sensitivity and overall sequence coverage obtained with each matrix, quantitated digests of BSA and chicken OVA, which had been reduced and alkylated with iodoacetamide prior to digestion, were obtained. The tryptic peptide mass maps are illustrated in Figures 3–5. If one compares the overall sequence coverage between the two matrices in Table 1, there is a clear trend that indicates that at about the 1-fmol levels and below, there is a clear sensitivity advantage with ClCCA. Overall, sequence coverages were quite comparable at the 100- and 10-fmol level between the two matrices, and ClCCA showed a slightly better coverage in most instances. It has also been observed that ClCCA generally gives cleaner backgrounds than CHCA with less low-mass artifacts, as can be seen in the low-abundance digests of Figures 3 and 4. Figure 5 shows a comparison of the100-fmol level digests of BSA in ClCCA and CHCA. What is most striking about this comparison is the more numerous and intense low-mass peptides found in the ClCCA digest. An examination of the corresponding peptides indicates that most of them end with a C-terminal Lys. Thus, at least for relatively low-mass peptides, it appears that ClCCA gives a more unbiased signal for Arg- and Lys-containing peptides.

TABLE 1

Number of Identified Peptides and Corresponding Sequence Coverage in Percent of the BSA and OVA Tryptic Digests in ClCCA or CHCA Matrix at the Indicated Amounts Loaded on the MALDI Target

BSA	100 fmol	10 fmol	1 fmol	200 attomol
ClCCA	61 (79%)	48 (70%)	32 (52%)	17 (25%)
CHCA	54 (72%)	37 (56%)	9 (18%)	0 (0%)
OVA	100 fmol	10 fmol	1 fmol	200 attomol
ClCCA	28 (69%)	21 (54%)	14 (39%)	8 (23%)
CHCA	24 (65%)	21 (55%)	9 (23%)	0 (0%)

As many proteins cannot be identified by PMF alone, an investigation of the properties of ClCCA in terms of its ability to fragment peptides in PSD and CID modes of operation was undertaken. It became apparent immediately that ClCCA was a much cooler matrix than CHCA. The term cool is often used to refer to the ability of a matrix to impart internal energy to a peptide, which facilitates its further fragmentation in MS mode or various MS/MS modes (CID and/or PSD). Matrices that are very cool, such as 2,5-dihydroxybenzoic acid (DHB), tend to impart less internal energy, and therefore, the peptide has more inherent stability. After fragmenting many peptides from various digests, it became apparent that PSD did not perform nearly as well for ClCCA as it had for CHCA. An example can be seen in Figure 6, showing the PSD fragmentation of the 927 peptide from the 100-fmol level BSA digest. When the CID gas is turned on, however, the fragmentation story becomes quite different. In ClCCA, there is usually a relatively more uniform spectrum of fragments across the mass range with less dominance of low-mass fragments, as is typically seen in CHCA. This is nicely illustrated in the CID spectra of the 1479 peptide from the 100-fmol digest of BSA in Figure 6. Also, as a result of the relatively cleaner backgrounds observed in ClCCA, CID fragmentation

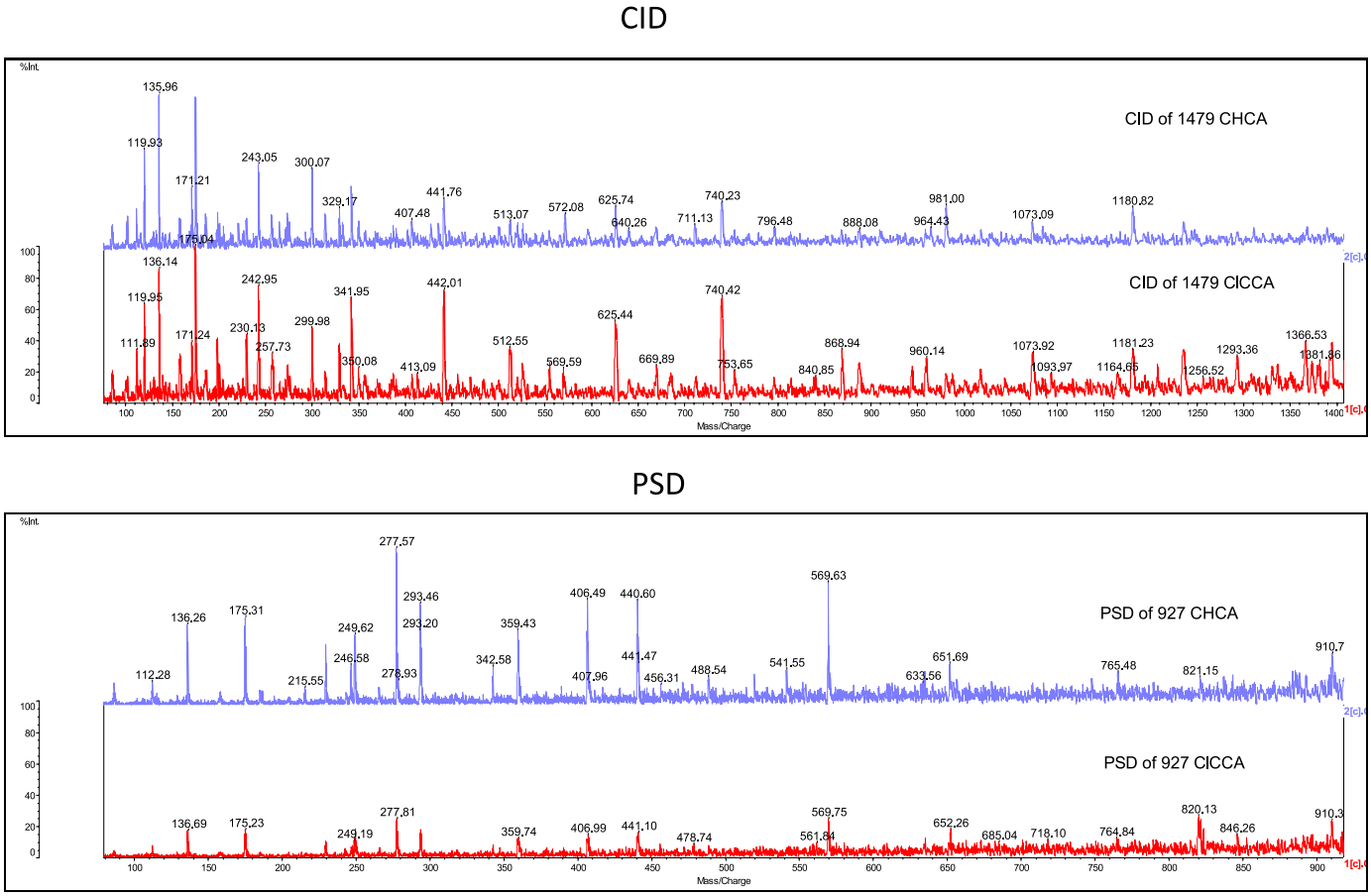

FIGURE 6

Comparison of the CID spectra of the 1479 peptide of BSA in CHCA and ClCCA is found in the upper panel. The lower panel contains the comparison of the PSD spectra obtained for the 927 peptide of BSA in CHCA and ClCCA. These spectra were obtained on the 100-fmol loads.

spectra are easily obtained even at the 1-fmol level for some of the more intense peptide signals. This was not the case at the 1-fmol levels for CHCA, where no discernable spectra could be obtained with PSD or CID.

Typically, many 1-dimensional (1-D) gel bands or 2-D gel spots, which are received by the core facility, are only visible by silver stain, which usually indicates an amount of protein that may fall in the few fmol–few hundred attomole ranges. Having the ability to obtain peptide maps, which easily identify the proteins at these levels, is a definite advantage. Furthermore, having the ability to obtain searchable CID spectra at the 1-fmol and below level allows for identification of proteins that produce too few peaks to make a reliable MS identification.

Phosphopeptide Analysis

Phosphorylation of specific proteins is a key regulator of many biological functions. In an effort toward the identification of phosphorylation sites, the specific phosphopeptides often require further enrichment as a result of their low abundance and ionization potential. Recently, it has been found that TiO_2 supports allow for the selective enrichment of a wide variety of phosphopeptides.[7,8]. Typically, cooler matrices such as DHB show higher sensitivity for phosphopeptides. This is partially a result of the labile nature of the phosphate group, which often results in neutral losses of 98 Da in MALDI MS analyses in hotter matrices such as CHCA. However, DHB tends to form heterogeneous, large crystalline deposits, which necessitate hunting for sweet spots to get good signals.

In this study, a commercially available phosphopeptide standard was used that contained four synthetic phosphopeptides derived from a tryptic digest of yeast enolase. This standard is particularly useful for evaluating the enrichment technique and also peptide ionization efficiency with different matrices. The phosphopeptides (*NPLpYK*, *HLADLpSK*, *VNQIGpTLSESIK*, and *VNQIGTLpSpSIK*) provide for a mixture of a variety of phosphorylation sites, such as singly phosphorylated Ser, Thr, and Tyr or doubly phosphorylated Ser. Amounts ranging from 10 pmols down to 10 fmols phosphorylated enolase digests were

enriched for the phosphopeptides using the TiO_2 NuTips. In this study, the actual amount was enriched instead of a larger amount that could be diluted down further. This allows for a more accurate expectation when dealing with real-life samples. Figure 7 shows the comparison from the 1-pmol enrichment to the 10-fmol enrichment for ClCCA and CHCA. It is obvious immediately that after enrichment, the ClCCA has a clear sensitivity advantage of nearly two orders of magnitude. This sensitivity difference was not observed in the analysis of the unenriched tryptic peptide digests, as even at the 10-fmol level, the CHCA and ClCCA digests showed the MH^+ signals for the 1368.6 and 1448.6 phosphopeptides, although the signals were much weaker for CHCA (data not shown). This suggests that the recovery from the TiO_2 NuTips is far less than quantitative. There may be other factors that contribute to this disparity as well. One such factor could be that CHCA is more sensitive to artifacts from the TiO_2 enrichment that causes ion suppression. This is evident by the contaminate peaks between 800 and 900 MH^+ for the CHCA digests, which were not observed in the ClCCA digests (Fig. 7). Other factors include the fact that all of the phosphopeptides have a C-terminal Lys, which may limit their overall sensitivity in the more Arg-biased CHCA matrix. Clearly, the two larger phosphopeptides (MH^+ of 1368.6 and 1448.6) are more prominent in the unenriched enolase digest for the 1-pmol ClCCA spectra than the corresponding spectra for CHCA (Fig. 7). Also, the ClCCA matrix shows negligible, neutral loss of the phosphate group in MS mode. This is not particularly obvious for the CHCA-enriched digest as a result of the low intensities of the phosphopeptides; however, neutral losses become much more obvious as the amounts increase substantially. The smaller phosphopeptides of *NPLpYK* (MH^+ of 813.39) and *HLADLpSK* (MH^+ of 863.40) showed weak signal intensities in the unenriched and enriched digests. This

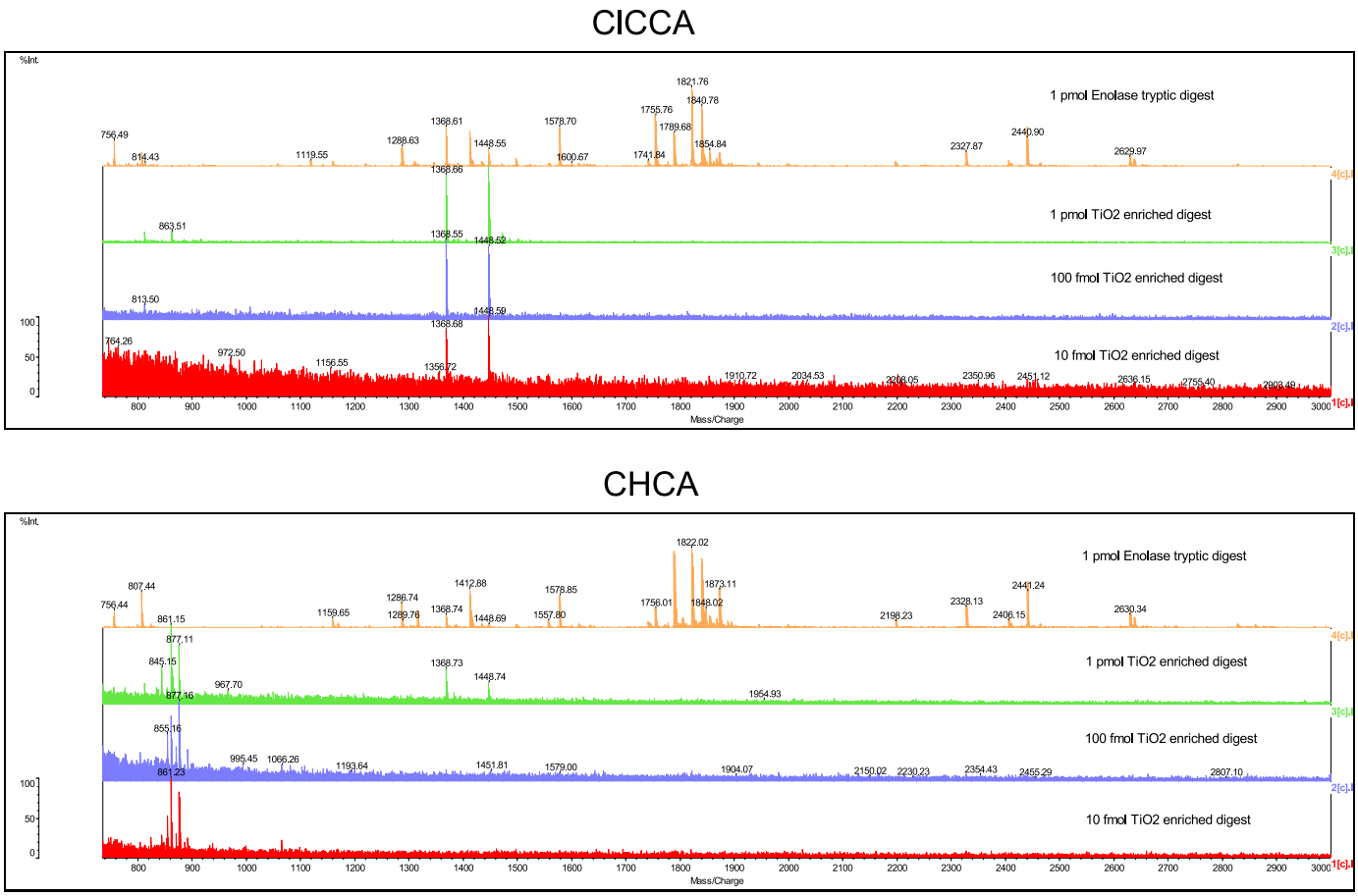

FIGURE 7

Comparisons of the TiO_2-enriched enolase tryptic digests for ClCCA (upper panel) traces and CHCA (lower panel) traces. The top trace in each panel is a 1-pmol load of an unenriched enolase digest containing the four reference phosphopeptides. The amounts indicated on the TiO_2-enriched traces are the amounts of digest that underwent enrichment. Enriched phosphopeptides are *NPLpYK* (MH^+ of 813.39), *HLADLpSK* (MH^+ of 863.40), *VNQIGpTLSESIK* (MH^+ of 1368.68), and *VNQIGTLpSpSIK* (MH^+ of 1448.64).

TABLE 2

Mascot MS/MS Ion Search Scores with an Identity Threshold of >29 for the Four Phosphopeptides Found in the Enolase digest. The Individual Peptide Ion Score for ClCCA and CHCA (in Parenthesis) Is Reported for the Indicated Amount That Was Enriched with TiO_2 Prior to Being Loaded on the MALDI Target

MH+	10 pmol	1 pmol	100 fmol	10 fmol
813.39	24 (20)	25	–	–
863.40	29 (14)	19	–	–
1368.68	61 (29)	65	52	–
1448.64	57 (33)	62	26	–

could be a result of weaker ionizing potentials for these short phosphopeptides. In addition, poorer recovery from the TiO_2 NuTips or the C_{18} ZipTips used for sample desalting and concentration could also be a factor.

Once phosphopeptides are observed in MALDI MS, it is necessary to verify their identification further and localize the site of modification using MS/MS analysis. To this end, all of the observed, enriched phosphopeptides were fragmented using CID in the case of the ClCCA matrix or PSD using the CHCA matrix. PSD was used in the CHCA matrix as a result of overall better sensitivity and less dominance of the neutral loss peak. Table 2 shows Mascot MS/MS Ion Search scores for the four phosphopeptides. In all cases that the peptides were identified, the Mascot search engine also assigned the sites of phosphorylation correctly. Although enriched peptide signals were observed for CHCA for the 1-pmol digest, the PSD was too weak to provide searchable spectra. Figure 8 shows the corresponding CID spectra obtained in the ClCCA matrix for the two phosphopeptides at MH^+ of 1368.68 and 1448.64 at the 1-pmol level of enrichment. What is most striking about these CID spectra is that the neutral loss peak intensities at MH^+ of 1271 and 1253, respectively, are relatively comparable with other fragment peak intensities and do not show the domination, as is the case for the CHCA matrix, where they are often larger than the parent peak (data not shown). Thus, ClCCA shows a clear advantage over CHCA in the analysis of TiO_2-enriched digests in sensitivity and also, in the ability to generate searchable fragmentation spectra.

SPITC-Derivatized Peptide Analysis

De novo sequencing of peptides continues to be a challenge in proteomics. When an organism's genome does not exist in a database, then standard MS/MS correlation analysis typically does not work. Without a reference database for comparison, these spectra are difficult to assign a sequence to. This is a result, in part, of the fact that many MS/MS spectra are an incomplete representation of the peptide sequence. The use of de novo sequencing software algorithms such as PEAKS (Bioinformatics Solutions, Waterloo, ON, Canada) is strongly dependent on instrument accuracy and the richness of the MS/MS spectra and often leads to multiple possibilities for a sequence. Attempts at enhancing the de novo interpretation have been addressed by isotopic-labeling approaches. One such approach used ^{18}O isotopic labeling of tryptic peptides, which allowed for discrimination between C-terminal y-type ions from N-terminal b-type ions.[9] MALDI PSD has been applied to the sequencing of peptides that have been N-terminally derivitized with negatively charged reagents.[6,10,11] This has been found to be particularly useful for the sequencing of tryptic peptides as a result of the dipolar nature of the derivatized peptide, facilitating a more mobile ionizing proton, which results in a more uniform fragmentation of the peptide backbone.[10] The fragmentation spectra formed from such reagents typically only show a y-type ion series, which is

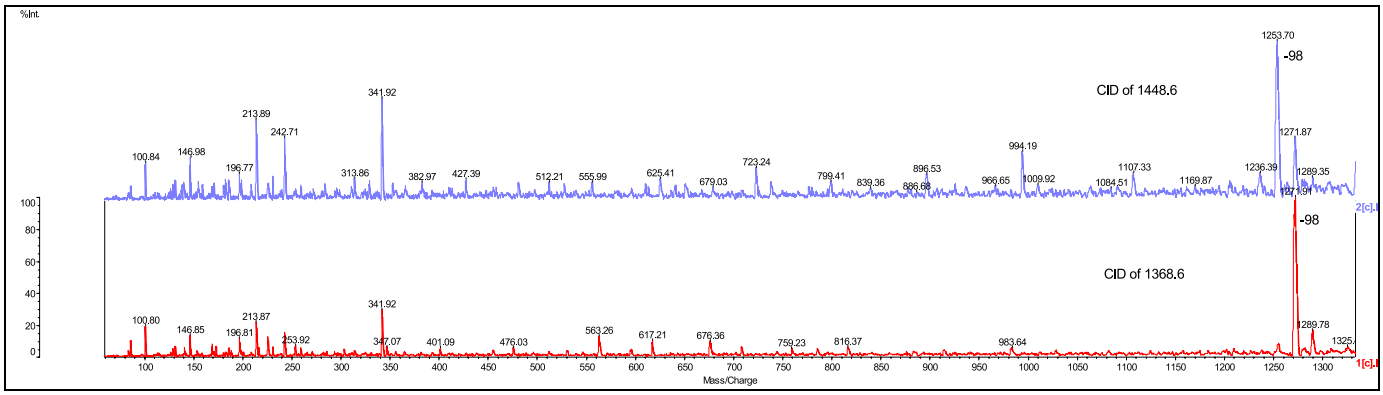

FIGURE 8

CID spectra of the doubly phosphorylated peptide at MH^+ of 1448.6 (*VNQIGTLpSpSIK*, upper trace) and singly phosphorylated peptide at MH^+ of 1368.6 (*VNQIGpTLSESIK*, lower trace) obtained for the 1-pmol enrichment in the ClCCA matrix. Indicated are corresponding phosphate neutral loss peaks (−98 Da).

enhanced as a result of the suppressed ionization of N-terminal b-type ions by the negatively charged reagent in positive-ion mode. Recently, a robust and facile procedure for derivatizing peptides with SPITC has been developed for the sequencing of peptides with MALDI PSD.[6] The previous studies have used CHCA predominately as the ionizing matrix for these derivatized peptides. My laboratory has successfully used this method for many years in the sequencing of novel tryptic peptides derived from organisms with uncharacterized genomes. It has been found that the derivatized peptides are fairly labile with this reagent and partially degrade to generate the underivatized peptide along with some of its fragments in the MS mode. Also, as a result of the negatively charged nature of the reagent, the derivatized peptides often show up with much weaker signals or disappear completely in positive ion mode. As the new ClCCA matrix exhibited a much cooler nature than CHCA and also had much greater sensitivity, it became obvious to apply it to the analysis of SPITC-derivatized peptides. In this study, a control peptide of fibrinogen peptide A (*ADSGEGDFLAEGGGVR*) and a tryptic digest of OVA were derivatized with SPITC and analyzed in CHCA and ClCCA. Amounts ranging from 3 pmols down to 3 fmols were derivatized for the fibrinogen peptide A and 1 pmol down to 10 fmols for the tryptic digest of OVA. No attempts were made to block the Lys residues from further reaction with the reagent, thus restricting sequencing to only peptides that contained C-terminal Arg. Figure 9 shows the MS spectra and PSD spectra of the control peptide derivatized with SPITC, which adds 215 Da to the mass of the peptide (MH^+ of 1751.77). What is compared is a 3-fmol amount in ClCCA with a 30-fmol amount in CHCA. Clearly, the peptide shows cleaner spectra in ClCCA than CHCA, despite a much lower amount. This is most likely a result of the labile nature of the derivatized peptide, which becomes more apparent in the hotter

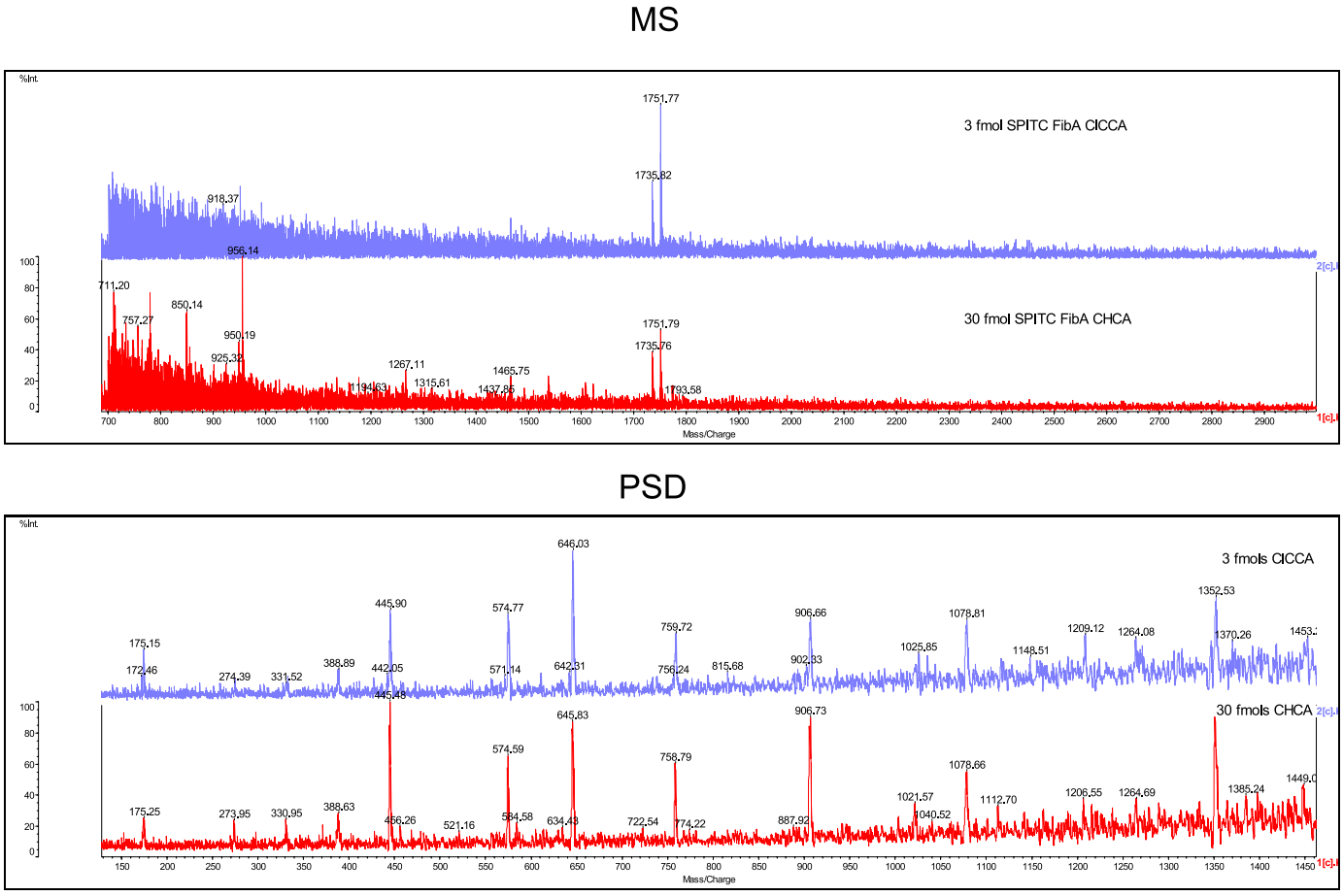

FIGURE 9

MS and corresponding PSD spectra of the SPITC-derivatized fibrinogen peptide A (*ADSGEGDFLAEGGGVR*) found at MH^+ of 1751.77. The spectra represent 3 fmols derivitized for ClCCA and 30 fmols for CHCA, which gave comparable signals in MS (upper panel) traces and PSD (lower panel) traces. The fragment ions represent a y-type ion series.

CHCA matrix. As was mentioned previously, the PSD of tryptic peptides was generally found to be weaker in ClCCA than in CHCA (Fig. 6). Derivitized peptides, however, show a much more enhanced PSD spectra than their corresponding, underivatized peptide. It was thus reasoned that this lower yield in ClCCA would be overcome by the enhanced fragmentation of the derivatized peptide. As can be seen in the lower panel of Figure 9, the order of magnitude-sensitivity improvement in MS is preserved in the PSD fragmentation of the peptide in ClCCA. Even at the 3-fmol level, the complete sequence of the fibrinogen peptide A could be read out from the spacing of the y-type ions. In the analysis of tryptic digests, the labile nature of the derivatized peptides can complicate the MS spectra further, making precursor selection difficult. MS spectra of the tryptic digest of OVA after derivatization are illustrated in Figure 10. Again, an order of magnitude sensitivity improvement is observed in the ClCCA matrix. In addition, the MS spectrum in CHCA is complicated further by many fragment ions from the derivatized peptides. One of the derivatized peptides (MH^+ of 1902.9) was selected for fragmentation. This peptide corresponds to residues 128–143 (*GGLEPINFQTAADQAR*) of OVA. A PSD spectrum obtained with the 10-fmol-derivatized digest in ClCCA is compared with the same spectra for a 100-fmol-derivatized digest in CHCA. Both spectra represent 13 residues of contiguous sequence read from the C-terminus of the peptide. No derivatized peptides were observed in CHCA at the 3-fmol level for the fibrinogen peptide A or for the 10-fmol level of the OVA tryptic digest. The ClCCA matrix not only provides an order of magnitude improvement in sensitivity over CHCA but also provides less-complicated spectra for precursor selection as a result of its cooler nature.

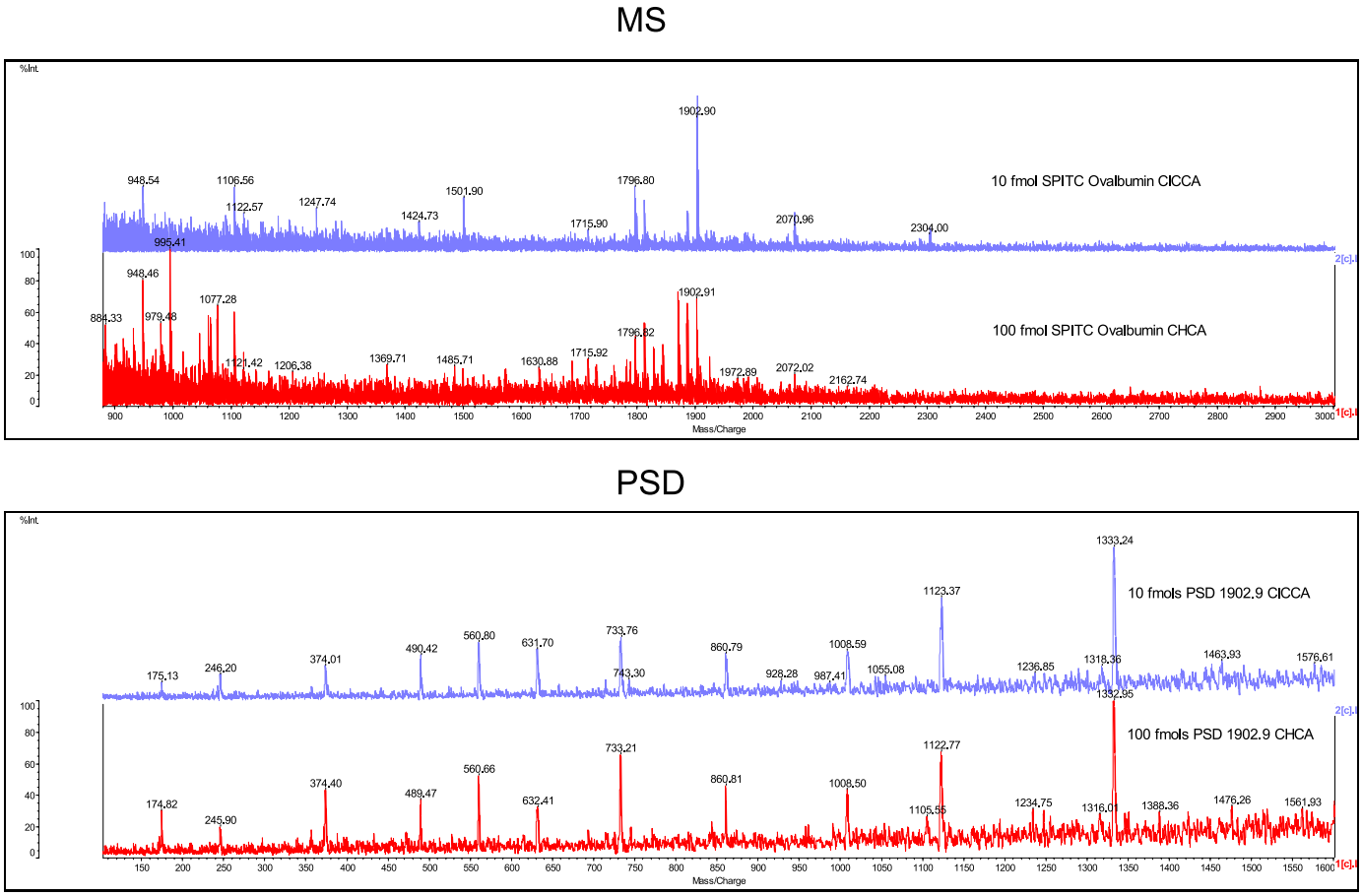

Figure 10

FIGURE 10

MS spectra of 10 fmols and 100 fmols derivatized OVA tryptic digest in ClCCA (upper, blue trace) and CHCA (lower, red trace). The lower panel represents the PSD spectra of the derivatized peptide found at MH^+ of 1902.9 (*GGLEPINFQTAADQAR*) for 10 fmol digest in ClCCA (upper, blue trace) and 100 fmol digest in CHCA (lower, red trace). The fragment ions represent a y-type ion series.

CONCLUSION

The new, rationally designed MALDI matrix ClCCA performed much better than initially expected. In the Axima TOF,[2] its performance far exceeded that of the more traditional CHCA matrix. In the laboratory, this matrix is also being used currently with the Axima QIT (Shimadzu Biotech), which is a hybrid MALDI ion trap TOF instrument. It offers significant advantages over DHB, which was the matrix of choice previously for this instrument. The ClCCA matrix significantly improves the sensitivity obtained with the current MALDI instrumentation. This matrix also shows cleaner backgrounds at low peptide levels as compared with CHCA. In addition, as a result of the cooler nature of this matrix as compared with CHCA, labile peptides are better preserved in the MS mode. This should be particularly useful for the analysis of fragile side-chain modifications. Currently, there are no commercial sources of highly purified matrix-grade ClCCA, but with minimal effort, adequate purity can be obtained from crude material obtained from the Sigma Rare Chemical Library.

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