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Methods for on-chip protein analysis

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Abstract

The unambiguous identification of peptides/proteins is crucial for the definition of the proteome. Using ProteinChip Array technology also known as surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS), we developed experimental protocols and probed test conditions required for the protein identification on ProteinChip surfaces. We were able to directly digest peptides/proteins *on-chip* surfaces by specific proteases, such as trypsin, and to obtain the peptide mass fingerprint of the sample under investigation by its direct analysis on a simple laser desorption/ionization mass spectrometer. Furthermore, tandem mass spectrometry was performed on several of the resulting tryptic peptides by using collision quadrupole time of flight (Qq-TOF) MS/MS via the ProteinChip interface, thus allowing the unambiguous identification of the protein(s) within the sample. In addition, we were able to identify the C-terminal sequence of peptides by their digestion with carboxypeptidase Y directly on ProteinChip surfaces coupled with SELDI-TOF MS analysis of the resulting peptide mass ladders employing the instrument's protein ladder sequence software. Moreover, the removal of up to nine amino acid residues from the C-terminal end of a peptide extends the functional range of Qq-TOF MS/MS sequence determination to over 3000 m/z. The utility of these procedures for the proteome exploration are discussed.
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In the postgenomic era, attention has focused on how the data contained in sequence databases generated by genomic sequencing projects might be interpreted with regard to the structure, function, and control of biological systems [1]. Approaches for global analysis of gene expression at the protein level are required to provide more accurate information about biological systems and pathways because the measurements directly focus on the biological effector molecules. Classical strategies for proteome analysis are based on the combination of 2D[1]-PAGE with mass spectrometry or tandem mass spectrometry for the sequence identifica-

tion of separated protein species [2]. However, 2D-PAGE presents several disadvantages such as lack of reproducibility, a failure to resolve most proteins greater than approximately 100 kDa or less than 5 kDa, a failure to routinely detect more than 1000 spots than can be identified by MS, and the inability to adequately separate most membrane proteins [3,4].

To study biological systems at the protein level, efforts have been directed to the improvement in instrumentation and to the development of novel technologies [5–7]. ProteinChip Array technology also known as surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) is a novel approach to the study of complex protein mixtures based on two powerful techniques, chromatography and mass spectrometry [8,9]. It consists of selective protein extraction and retention on chromatographic chip surfaces and their subsequent analysis by a simple laser desorption/ionization mass spectrometer. Such surfaces have been successfully applied to expression and functional proteomics [10–13]. Together with the development of this novel technology, the need for protein

chemistry techniques that are applicable to ProteinChips is fundamental and important to facilitate exploration of the proteome.

In this report, we describe various protein chemistry procedures that can be applied to the identification of proteins directly on the surface of ProteinChip arrays. In particular, we were able to identify proteins by *on-chip* trypsin digestion and subsequent microsequencing of the resulting peptides using a ProteinChip Interface [8] coupled to the tandem MS/MS QStar instrument [14].

We also demonstrate C-terminal enzymatic sequencing on peptides by a combination of direct *on-chip* carboxypeptidase Y (CPD Y) digestion [15,16] with SELDI-TOF mass analysis of the resulting peptide ladders. The *on-chip* CPD Y digestion was found effective under both native and denaturing conditions. The sequence information was obtained in a matter of minutes by using the SELDI-TOF MS instrument's PLS software, which provided a high statistical level of confidence to amino acid assignment. The combination of the SELDI-TOF MS sensitivity with no sample loss upon moving from digestion to analysis allowed the C-terminal sequencing of very few picomoles of total peptide. These procedures together have the potential to be applied to our exploration of the proteome.

Materials and methods

Materials

Sequencing-grade trypsin was obtained from Sigma (St. Louis, MO) and from Roche Molecular Biochemicals (Mannheim, Germany). CPD Y was a product of Carl-biotech (Copenhagen, Denmark). H4 ProteinChips and the calibration standard molecules for the SELDI-TOF mass spectrometer were purchased from Ciphergen Biosystems Inc. (Fremont, CA). α-Cyano-4-hydroxy-cinnamic acid (CHCA), sinapinic acid (SA), iodoacetic acid (IAA), and angiotensin were obtained from Sigma. Magainin II was obtained from Bachem (Philadelphia, PA) and adrenocorticotropic hormone (ACTH$_{(1-24)}$) peptide was a component of the calibration kit from Ciphergen Biosystems. Gross cystic disease fluid protein-15/prolactin-inducible protein (GCDFP-15/PIP) was prepared in laboratory according to the procedure previously reported [17]. Glucocerebrosidase was from Genzyme (Boston, MA). Dithiothreitol (DTT) was from GIBCO BRL (Grand Island, NY). All other chemicals used were analytical grade and the highest purity available.

Surface-enhanced laser desorption/ionization-time of flight mass spectrometry

The SELDI-TOF mass spectrometer was externally calibrated using the $[M + H]^+$ ion peaks of bovine insulin at 5733.6 *m/z* and human angiotensin at 1296.5 *m/z*. All mass spectra were recorded in the positive-ion mode using a Ciphergen PBS II ProteinChip Array reader, a linear laser desorption/ionization-time of flight mass spectrometer with time-lag focusing [8]. Prior to SELDI-TOF MS analysis, 1 µl of matrix (saturated CHCA in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA)) was added to each feature of ProteinChip surface for the analysis. Raw data were analyzed using the computer software provided by the manufacturer and are reported as average masses.

Reverse-phase HPLC chromatography

Magainin II was further purified on RP-HPLC C18 column (150 × 2.1 mm, 300 Å pore size, Vydac, Hesperia, CA), employing a Beckman system Gold (Fullerton, CA) composed of a Model 168 Diode Array Detector and a Model 126 pump module. A linear gradient from 5 to 65% B solvent over 60 min with 5 min 5% B initial condition (where solvent A was 0.12% aqueous TFA and solvent B was 0.1% TFA in acetonitrile) was applied at a flow rate of 0.2 ml/min. The major peak was manually collected and the volume was reduced by centrifugal evaporation, using a Speed-Vac centrifuge (Savant, Holbrook, NY), and stored at −20 °C until needed.

In-solution and on-chip enzymatic digestion of peptides/proteins

The purified GCDFP-15/PIP protein [17] was reduced with 45 mM DTT in 100 mM Tris buffer, pH 8.6, containing 6 M guanidine for 30 min at 55 °C and then alkylated with 100 mM IAA for 20 min at room temperature in the dark. The excess reagents were removed by dialysis against 50 mM ammonium bicarbonate, pH 7.8, using dialysis membranes with 3500-Da cutoff (Pierce, Rockford, IL) and the volume was reduced by evaporation. An aliquot of the reduced/carboxymethylated protein (approximately 20 µg) was digested with trypsin in freshly prepared 50 mM ammonium bicarbonate, pH 7.8, using an enzyme/substrate ratio of 1:100. The tryptic digestion was carried out at 37 °C overnight. Several aliquots of the reduced/carboxymethylated GCDFP-15/PIP were also spotted onto an H4 ProteinChip surface and subjected to digestion with trypsin. Prior to use, the H4 ProteinChip was prewashed with 10 µl of 0.1% TFA in 50% aqueous acetonitrile. The chip was allowed to dry; then 1 µl of protein solution was applied onto several spots, and the chip was again allowed to dry and then washed with 5% aqueous acetonitrile. The spots were then circled with a PAP pen (Vector Laboratories Inc., Burlingame, CA) to generate a hydrophobic barrier to aid in retention of the digestion buffer; 4 µl of 50 mM ammonium bicarbonate, pH 7.8, and 1 µl (20 ng) of trypsin were used and the reaction

was allowed to proceed for 2–4 h in a humid chamber. After incubation, the chip spots were allowed to dry and CHCA was added for the subsequent SELDI-TOF MS analysis. Glucocerebrosidase was spotted on-chip, allowed to dry, washed with 5% aqueous acetonitrile, and then digested as above.

C-terminal sequencing on-chip

Carboxypeptidase Y digestion was carried out on a number of peptides. They were applied to several spots on an H4 ProteinChip surface as described above for trypsin digestion. The spots were ringed with a PAP pen and 4 μl of 50 mM Mes buffer, pH 6.3, was added to the spots. CPD Y was added, 1 μl from a stock solution of 50 ng/μl in citrate. Reactions were terminated by washing the spots with 5% aqueous acetonitrile, followed by addition of 1 μl of matrix. Prior to a time course analysis an aliquot was digested for 60 min to ascertain the effectiveness of the procedure. In the case of Magainin II, while the digestion appeared to produce lower-molecular-weight species, the major species remained the starting molecular ion. Therefore the digestion was repeated but employing 0.1% SDS in Mes buffer. The resulting peptide ladder was analyzed using the Protein Sequence Ladder software (Ciphergen Biosystem Inc.).

SELDI quadrupole time of flight tandem mass spectrometry

Tandem mass spectrometric peptide sequencing was accomplished using an Applied Biosystems/Sciex QStar triple-quadrupole time of flight instrument (Toronto, Canada) equipped with a Ciphergen SELDI Protein-Chip Interface PCI 1000 [8,15]. The instrument was calibrated externally using an acquired MS/MS spectrum of $ACTH_{(18-39)}$ peptide at 2465.2 m/z, where four fragment ions and the parent were used as calibration points. All mass spectra were acquired in positive-ion mode with a collisional gas pressure of approximately 100 mTorr. MS/MS data were acquired using collision energies following the approximate rule of 50 eV/kDa parent peptide mass. Matrix conditions were identical to SELDI-TOF analysis described previously. Raw data were analyzed using the instrument's Analyst software.

Results

On-chip protein identification

Identification of proteins is the primary goal in proteomics. In this study, we examined the possibility of identifying peptides/proteins by a novel experimental procedure, using ProteinChip Array technology [8,9].

In particular, we demonstrated the tryptic digestion of peptides/proteins directly on ProteinChip Array surfaces and subsequently analyzed the resulting tryptic peptides by SELDI-TOF mass spectrometry to obtain information on the identity of the sample under examination by a simple mass fingerprint.

To this end, we used the glycoproteins known as GCDFP-15/PIP, a specific apocrine breast cancer marker with molecular mass of about 15,000 Da [17] and glucocerebrosidase with molecular mass of about 60,000 Da (P04062; Swiss-Prot database).

The GCDFP-15/PIP protein was reduced, alkylated, and then subjected to digestion with trypsin in solution and directly on H4 ProteinChip Array surface, as described under Materials and methods. The tryptic digestion was carried out for 2 h *on-chip* and overnight in solution, in 50 mM ammonium bicarbonate, pH 7.8. A blank sample digestion was also done to identify the autoproteolytic products (Fig. 1a).

After *on-chip* hydrolysis, the reaction buffer on each spot of the H4 ProteinChip Array surface was allowed to dry and 1 μl of CHCA was added before the SELDI-TOF MS analysis. The obtained tryptic peptides are shown in Fig. 1b. The cleavage products from the solution reaction were spotted on the H4 ProteinChip Array surface and were examined by SELDI-TOF mass spectrometry (Fig. 1c). The comparison between these spectra showed that the reaction was equally successful when done *on-chip* or in-solution and the masses of obtained peptides matched the theoretically predicted tryptic peptides of GCDFP-15/PIP, as shown in Table 1. The ion of 1997.5 m/z found in the sample was consistent with the partial tryptic product corresponding to the peptide 91–108 of the protein. The spectra presented clearly showed very similar signals for both methods of digestion.

The results of *on-chip* tryptic digestion of glucocerebrosidase are presented in Fig. 2. We subsequently performed a Mascot search of the observed peptide m/z values and confirmed the identification of the sample as glucocerebrosidase (data not shown).

Furthermore, we investigated the possibility of directly analyzing a sample on the ProteinChip surface after tryptic digestion by tandem mass spectrometry, using the ProteinChip Interface [8] on an AB/MDS Sciex Qstar tandem mass spectrometer [15].

We were able to successfully identify a number of the peptides. The MS/MS analysis of one peptide from GCDFP-15 among the various obtained tryptic peptides with the m/z value of 1283.77 is shown in Fig. 3. The collision-induced dissociation (CID) spectra revealed the sequence TVQIAAVVDVIR, which was unambiguosly identified by Mascot search as a peptide from the GCDFP-15/PIP protein.

We observed that the combination of *on-chip* tryptic digestion and MS/MS sequencing procedures on several spots allow us to obtain identification/sequence infor-
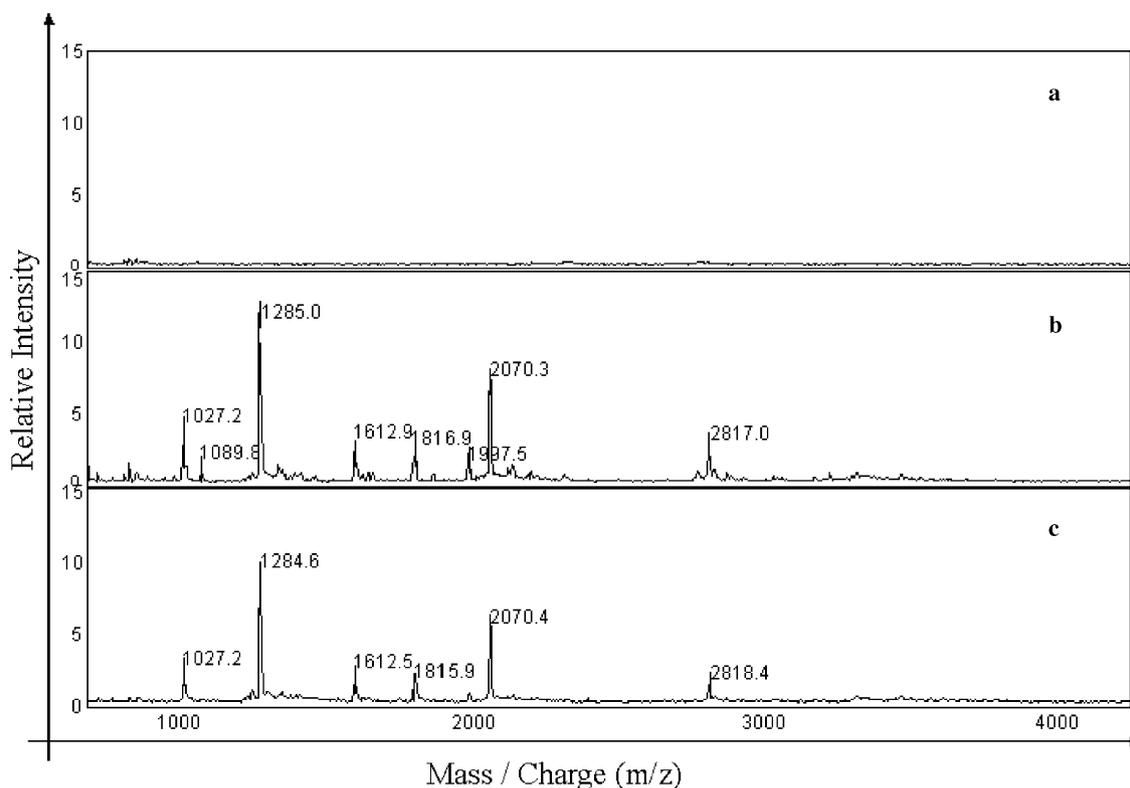
Fig. 1. SELDI-TOF MS spectra of GCDFP-15/PIP after tryptic digestion. Positive-ion mass spectra of peptide products resulting from *on-chip* (b) and in-solution (c) tryptic digestion of GCDFP-15/PIP, as analyzed by SELDI-TOF MS. A blank spot without the sample but having the reaction buffer and trypsin (a) was also carried out. The mass/charge (*m/z*) values of each detected species are shown.

mation on multiple peptides with routine analysis of two independent peptides per spot (data not shown).

On-chip C-terminal sequencing

C-terminal sequencing, either by carboxypeptidases [15,16] or by chemical degradation [18,19], is one of the existing methods for sequence determination together with the N-terminal Edman degradation and MS/MS approaches. We tested the potential of the C-terminal sequence analysis of various peptides by enzymatic digestion with carboxypeptidase Y directly *on-chip*.

To this end the $ACTH_{(1-24)}$ and Magainin II peptides were used. They were initially spotted on H4 Protein-Chip Array surface and analyzed by SELDI-TOF mass spectrometry. The ACTH fragment 1-24 (SYS-MEHFRWGKPVGKKRRPVKVYP) and Magainin II (GIGKFLHSAKKFGKAFVGEIMNS) showed values of 2933.5 and 2466.9 *m/z*, as expected and shown in Figs. 4a and 5a, respectively.

The peptides were then digested directly *on-chip* by adding the buffer (50 mM Mes, pH 6.3) and the CPD Y enzyme as described under Materials and methods. The digestion was carried out for up to 60 min. The ACTH sample *on-chip* was examined after 5, 30, and 60 min of reaction by SELDI-TOF MS and the PLS software was applied to determine the amino acid residues released

during the enzymatic reaction (Figs. 4b–d). The ACTH C-terminal analysis resulted in the release of up to five amino acid residues using the standard Mes buffer and did not require a large number of aliquots to determine the sequence. After 1 h nearly all the parent ion had disappeared and four of the five released amino acid residues were clearly identifiable as shown in Fig. 4.

In the case of Magainin II, the analysis after 60 min revealed that the reaction had progressed to some degree since the vast majority of the parent ion was still intact (Fig. 5b) and the sequence was somewhat difficult to evaluate using the PLS software because of inaccurate masses assigned to very low ion signal. The addition of SDS either to the digestion buffer or to the peptide solution prior to spotting not only allowed the reaction to proceed to a much greater extent but it had no adverse effect on the subsequent collection or analysis of the spectra (Fig. 5c). In this case we observed more complete digestion and we were able to tentatively assign the amino acid residues released.

After 60 min in the presence of SDS the molecular mass of the largest ion was reduced from 2466.9 to 1589.9 *m/z*, corresponding to the removal of eight amino acid residues (Fig. 5d). While the interpretation of the sequence is not simple, the addition of SDS detergent to the digestion buffer has greatly increased the utility of CPD Y digestion for C-terminal sequencing.

Table 1
Theoretically predicted peptides of GCDFP-15/PIP after tryptic digestion using the PeptideMass software available on http://www.expasy.ch website
QDNTRKIIIKNFDIPKSVRPNDEVTAVLAVQTELKECMVVKTYLISSIPLQGAFNYKYTACLCDDNPKTFYWDFYTNRTVQIAAVVDVI
RELGICPDDAAVIPIKNNRFYTIEILKVE

Theoretical mass (observed mass)	Position	MC	Artificial modification(s)	Peptide sequence
3040.4751	42–68	1	Cys_CM: 61, 63 **3156.5485**	TYLISSIPLQGAFNYKYTACLCDDNPK
2820.3601	79–105	1	Cys_CM: 95 **2878.3968**	TVQIAAVVDVIRELGICPDDAAVIPIK
2785.1695	11–35	1		NFDIPKSVRPNDEVTAVLAVQTELK
2760.2365	17–41	1	Cys_CM: 37 **2818.2732**	SVRPNDEVTAVLAVQTELKECMVVK
2679.0499	69–90	1		TFYWDFYTNRTVQIAAVVDVIR
2637.8999	58–78	1	Cys_CM: 61, 63 **2753.9733**	YTACLCDDNPKTFYWDFYTNR
2505.9926	36–57	1	Cys_CM: 37 **2564.0293**	ECMVVKTYLISSIPLQGAFNYK
2070.3503 (2070.3, 2070.4)	17–35	0		SVRPNDEVTAVLAVQTELK
1939.2365 (1997.5)	91–108	1	Cys_CM: 95 **1997.2732**	ELGICPDDAAVIPIKNNR
1816.1064 (1816.9, 1815.9)	42–57	0		TYLISSIPLQGAFNYK
1554.8414	91–105	0	Cys_CM: 95 **1612.8781**	ELGICPDDAAVIPIK
1413.5312	69–78	0		TFYWDFYTNR
1411.6433	106–116	1		NNRFYTIEILK
1284.5414 (1285.0, 1284.6)	79–90	0		TVQIAAVVDVIR
1255.4963	109–118	1		FYTIEILKVE
1243.3914	58–68	0	Cys_CM: 61, 63 **1359.4648**	YTACLCDDNPK
1201.4942	7–16	1		IIIKNFDIPK
1027.2482 (1027.2, 1027.2)	109–116	0		FYTIEILK
761.8125	1–6	1		QDNTRK
733.8419	11–16	0		NFDIPK
708.9089	36–41	0	Cys_CM: 37 **766.9456**	ECMVVK
633.6384	1–5	0		QDNTR
614.8491	6–10	1		KIIIK

The complete amino acid sequence of GCDFP-15/PIP is shown above the table. The maximum number of cleavages (MC) was set to one. The variations in the masses of the peptides as a consequence of reduction and alkylation of the protein are also reported in boldface under artificial modifications. The observed mass of peptides deriving from *on-chip* and in-solution digestion are reported in parentheses, respectively.
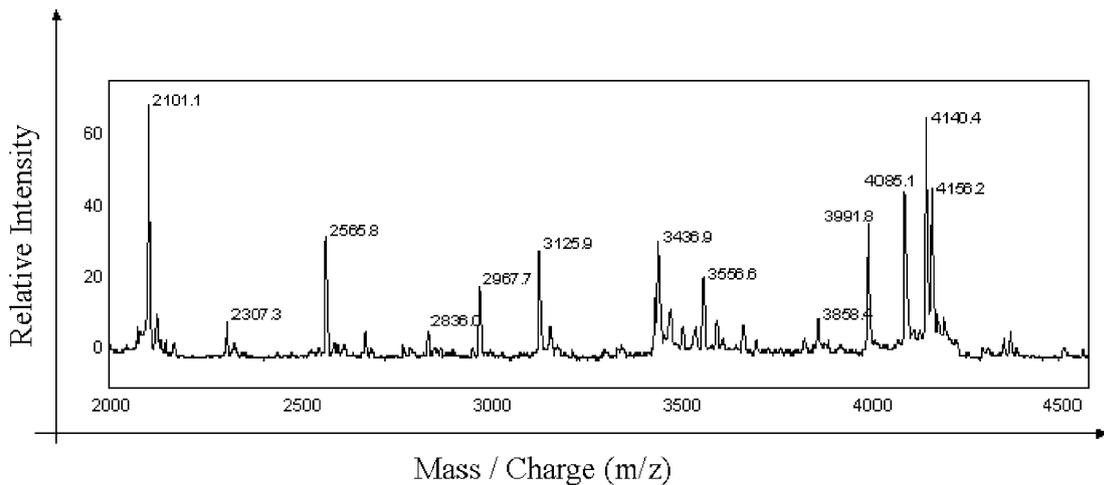
Fig. 2. SELDI-TOF MS spectra of glucocerebrosidase after tryptic digestion. Positive-ion mass spectra of peptide products resulting from *on-chip* tryptic digestion of glucocerebrosidase, as analyzed by SELDI-TOF MS. The mass/charge (*m/z*) values of all detected species are shown.

We have also employed octyl-beta glucoside with similar results (data not shown).

Discussion

The identification of the proteins is a major challenge in proteomics. Several procedures and novel technolo-

gies have been developed, although they still need improvements [5–7]. They basically consist of separation of proteins, digestion with specific proteases such as trypsin, and identification of the resulting tryptic peptides by tandem mass spectrometry [20].

Here, we report novel experimental methods to identify peptides/proteins by using ProteinChip Array technology [8]. Although this technology was
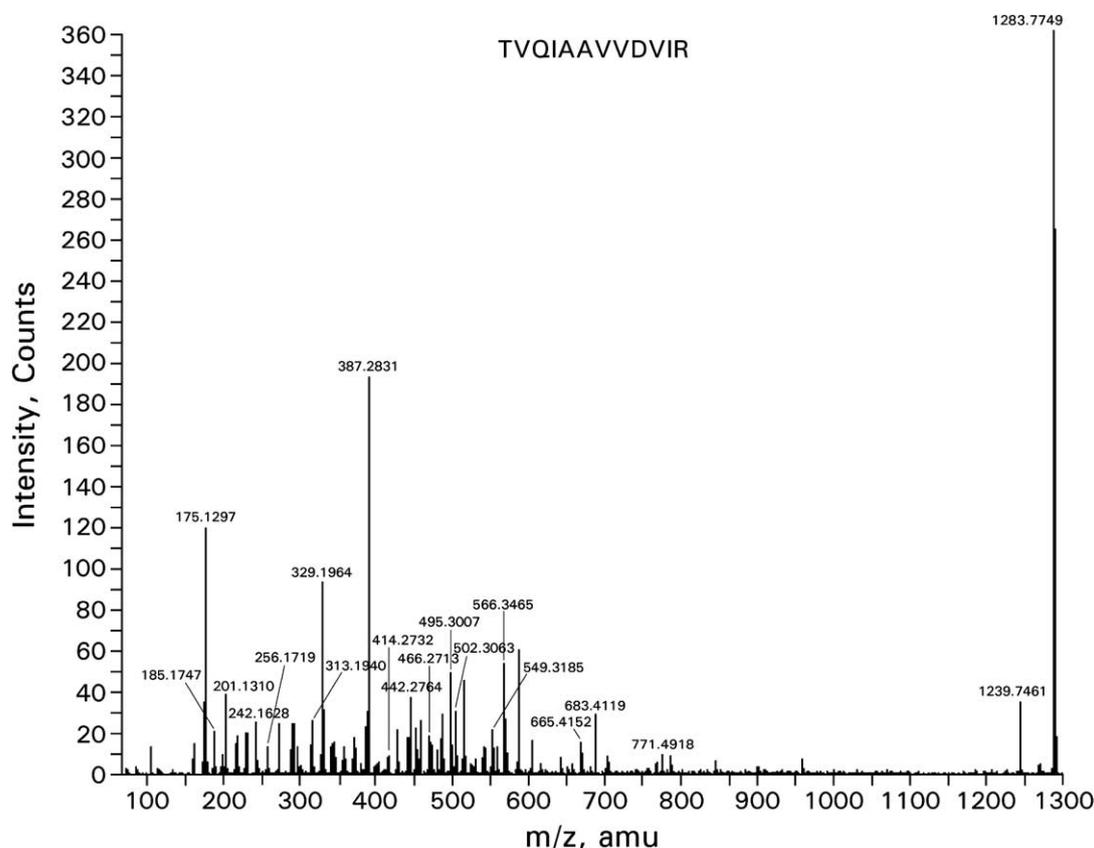
Fig. 3. CID spectra of 1283.7 *m/z* GCDFP-15/PIP derived tryptic peptide. Collision-induced dissociation spectra from 1283.77 (*m/z*) molecular species. The determined sequence is shown.

used successfully for expression and functional proteomic studies [10–13], the development of protein chemistry methods on microchromatography ProteinChip surfaces is fundamental for the application of this new technology to exploration of the proteome.

To this end, we demonstrated the feasibility of generating peptide maps by direct digestion of proteins on ProteinChip Array surfaces. We found that using ProteinChip surfaces we were able to reduce reaction times for enzymatic reactions compared to solution digestion using microscale amounts of sample.

Furthermore, the minichromatographic ProteinChip surfaces provided the best surfaces for enzymatic digestions for several reasons: they retained the substrate, trapped the reaction products, facilitated sample cleanup, and allowed the sample to be placed in the PBS II or QSTAR (via the interface). In addition, only minor modifications of standard solution methods were required when using these surfaces.

The peptide maps generated following these *on-chip* reactions could be read by a simple linear LDI-TOF mass spectrometer, facilitating sample protein identification. The combination of ProteinChip surfaces, SELDI-TOF, and Qq-TOF MS/MS analysis (via the ProteinChip interface) minimized sample loss, enabled sensitive detection and identification, and provided a complete experimental platform for reaction, detection, and identification.

Together with the MS/MS approach directly on ProteinChip Array surfaces, we tested the possibility of obtaining C-terminal sequence information from peptides/proteins, by enzymatic digestion *on-chip*, using the carboxypeptidase Y. This enzyme belongs to the family of the carboxypeptidases able to sequentially cleave the amino acid residues from the C terminus of peptides/proteins [21]. Compared to the serine carboxypeptidases used over the past decades, CPD Y is able to cleave all residues from the C terminus, including proline [21]. We were able to digest different peptides with CPD Y directly *on-chip* and to determine the sequence by using the PLS software. Our preliminary results with the C-terminal sequencing of known peptides by their *on-chip* digestion by CPD Y has the potential to be applied to unknown peptides. The sequence determined for ACTH, P-Y-V-K/Q-V, illustrates one of the minor difficulties with MALDI/SELDI where the amino acid pairs I/L and K/Q possess the same molecular mass. On the other hand, difficulty in interpreting the sequence for Magainin II was caused by two other problems. The first was the inability of CPD Y to effectively release amino acids from the peptide without addition of denaturant and
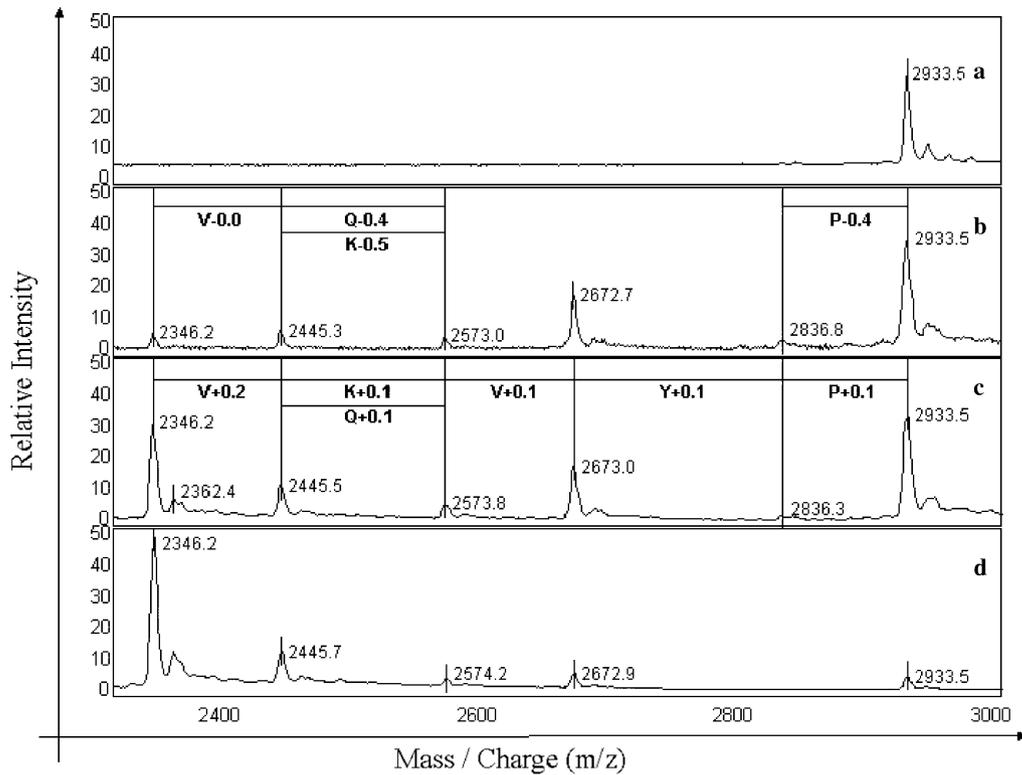
Fig. 4. SELDI-TOF MS spectra of ACTH before and after CPD Y digestion. Positive-ion mass spectra of ACTH peptide (a) and its cleavage products deriving from *on-chip* CPD Y digestion after 5 min (b), 30 min (c), and 60 min (d). The amino acid residues detected by PLS software are shown.
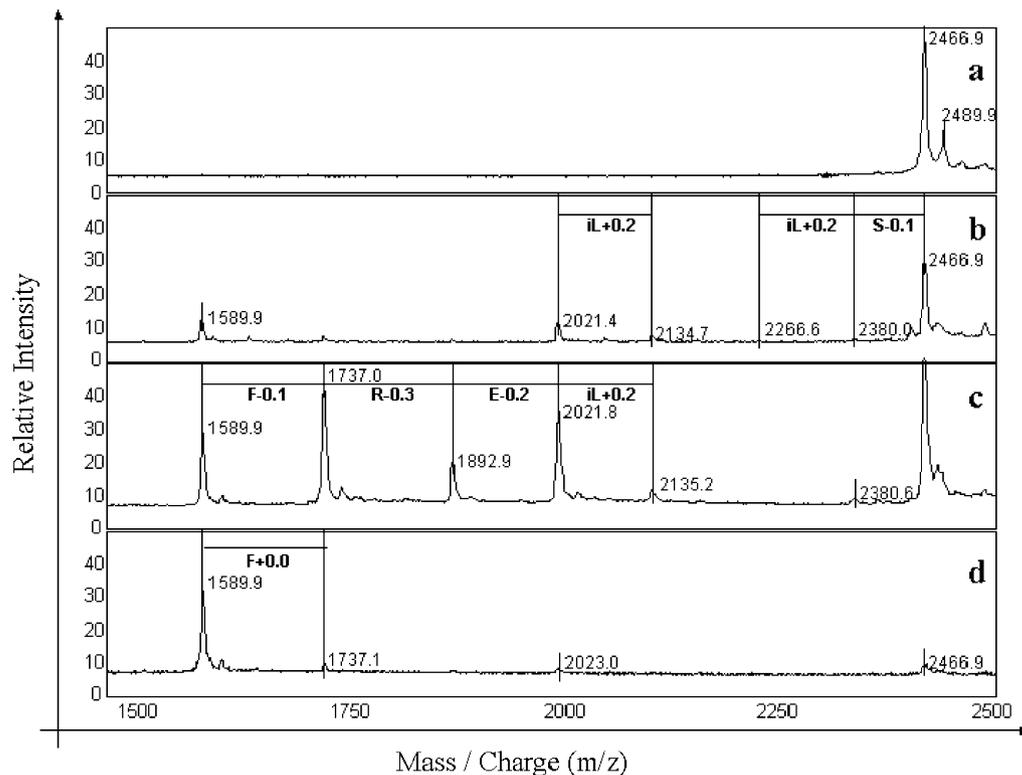
Fig. 5. SELDI-TOF MS spectra of Magainin II before and after CPD Y digestion. Positive-ion mass spectra of Magainin II peptide (a) and its cleavage products deriving from *on-chip* CPD Y digestion in Mes buffer after 1 h (b), and in Mes buffer containing 0.1% SDS after 30 min (c) and after 1 h (d). The amino acid residues detected by PLS software are shown.

the second was the rapid release of amino acids where the observed molecular ion differences represent two residues (Fig. 5b) where the PLS software identified the G-V as R. We have been unable to modify the protocol to secure the peptide ion that would be representative of a single amino acid release. However, with the residues identified or suggested by the PLS software and manual examination and assessment, a tentative sequence is available by combining three of the panels in Fig. 5, i.e. S-N(I/L)-M-I-E(K/Q)-R(G-V)-F-A. Obviously, the combination of *on-chip* CPD Y digestion and tandem mass spectrometry by the ProteinChip Interface should provide the unambiguous C-terminal sequence of most peptides examined using this method.

In addition, for peptides with molecular weights just out of the range of MS/MS sequencing, this procedure would allow the removal of up to 500–700 mass units and permit sequence determination of the resulting N-terminal peptide while providing additional C-terminal sequence to aid in the unequivocal determination of the peptide sequence when the MS/MS CID spectra are submitted to Mascot or other search engines, thus expanding the existing limits of MS/MS microsequencing.

The *on-chip* C-terminal sequencing is a novel alternative to the classical enzymatic sequencing method, based on digestion of peptides/proteins by carboxypeptidases and the subsequent analysis of the released amino acid residues from the C terminus of peptides/proteins [22]. The classical reaction was normally evaluated by amino acid analysis (AAA) which was time consuming and rate limited and could be adversely affected by amino acid contaminants in the enzyme and peptides/proteins solution [22]. Multiple amino acid residues when released from a peptide even with defined timed aliquots overlapped and made interpretation impossible. Analysis by MS has obviated this overlap problem since analysis takes advantage of the resulting shortened peptide and has allowed use of picomole amounts of substrate in lieu of nanomoles for AAA. Although, the carboxypeptidase Y sequencing utilizing MALDI-TOF analysis has been reported previously [23], we have introduced the use of denaturants [21] to aid in the digestion and have shown that the reaction can be done and monitored *on-chip* directly with no interference from buffer salts or the denaturant. The use of the ProteinChip allowed C-terminal analysis of real peptide samples and not just standards since the chip surfaces (in this case hydrophobic) retained the peptide, while contaminants such as salts, detergents, and buffer were washed away prior to digestion avoiding the need for Zip-tip cleanup as generally used for direct digestion on MALDI plates [23]. The latter procedure is generally accompanied by losses of starting material.

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