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Sequencing of Phosphopeptide using Post-Decay Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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A simple method of solid-phase derivatization and sequencing of phosphopeptide has been developed for rapid using post-decay (PSD) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The phosphopeptide are chemically modified by 4-sulfophenyl isothiocyanate. The derivatization traction introduces a negative sulfonic acid group at the N-terminus of a phosphopeptide, which can increase the efficiency of post-decay fragmentation and enable the selective detection of only a single series of fragment ions (y-ions). In this study, we applied this approach to screen Cx31 phosphorylation sites using HT1080 cells stable expressing a myc-tagged Cx31. Serine 234 of the Cx31 was determined to be a site of phosphorylation.

Key Words: 4-Sulfophenyl isothiocyanate, Phosphopeptide, Matrix-assisted laser desorption/ionization-Post-decay, Cx31.

INTRODUCTION

Dynamic post-translational modification of proteins is important for maintaining and regulating protein structure and function. Among the several hundred different types of post-translational modifications characterized to date[1], protein phosphorylation plays a prominent role[2]. Enzyme-catalyzed phosphorylation and dephosphorylation of proteins is a key regulatory event in the living cell. Complex biological processes such as cell cycle, cell growth, cell differentiation and metabolism are orchestrated and tightly controlled by reversible phosphorylation events that modulate protein activity, stability, interaction and localization[2,3]. Perturbations in phosphorylation states of proteins, *e.g.* by mutations that generate constitutively active or inactive protein kinases and phosphatases, play a prominent role in oncogenesis[4]. Comprehensive analysis and identification of phosphoproteins combined with exact localization of phosphorylation sites in those proteins (phosphoproteomics) is a prerequisite for understanding complex biological systems and the molecular features leading to disease[5]. Whereas protein identification is now routinely performed by mass spectrometry combined with bioinformatics techniques[6]. The localization of post-translationally modified residues, including phosphorylation sites, in proteins still remains a challenge[5]. A number of mass spectrometry based methods have been applied for identification and sequencing of phosphopeptides derived from low picomole levels of protein. Phosphopeptide sequencing is commonly performed by tandem mass spectrometry either directly from unseparated peptide mixtures[7,8], after affinity purification[9-13], or after peptide separation by liquid chromatography (LC)[14-17]. Post-source-decay (PSD) analysis of phosphopeptide ions in matrix-assited laser desorption/ionization (MALDI) reflector time-of-flight (TOF) instruments sometime enables phosphorylation site determination[18]. Phosphopeptide characterization by tandem mass spectrometry is typically performed in the size range 500-2500 Da but phosphopeptides with a size of more than 4 kDa have been succesfully analyzed by LC/MS/MS[19].

Solid-phase peptide synthesis (SPPS) is a common protocol for the synthesis of phosphopeptides[20]. Both *t*-butyloxycarbonyl (Boc) and fluorenylmethoxycarbonyl (Fmoc) are effectively used, the latter approach being more realistic for phosphopeptides due to mild reaction conditions used in the deprotection and cleavage steps. However, in addition to the desired peptide, a number of undesired components are also produced because of the possibility of significant undesired side reaction. During the complete cycle of peptide synthesis, the products of incomplete and aberrant reactions continue to accumulate. The presence of impurities may have significant impact on the activity of the target peptide. Purification of the desired product is an essential step of the solid-phase peptide synthesis protocol. Reversed-phase high-performance liquid chromatography(RP-HPLC) is usually applied for this objective[21].

The introduction of an N-terminal sulfonic acid group was applied to successfully enhance fragmentation towards

the peptide-amide bond by Keough and coworkers[22,23]. This reaction is very similar to Edman degradation and the present work can be regarded as an extension of the work of Keough *et al.*[22,23]. Phenyl isothiocyanate is a perfect chemical for amino acid sequencing, so 4-sulfophenyl isothiocyanate (SPITC) would also be well suited to derivatization and fragmentation of peptides in MALDI-PSD sequencing. The work of Gevaert and co-workers verified the feasibility of this theory[24]. The work of Ping Chen and co-workers mended this method.

The objective of this research is to develop a simple and faster approach for analysis of phosphopeptides from solid-phase peptide synthesis products and their byproducts.

EXPERIMENTAL

4-Sulfophenyl isothiocyanate (SPITC) was obtained form Aldrich, α-cyano-4-hydroxycinnamic acid (CHCA) was obtained form Sigma, Peptide synthesis reagents were obtained from ABI, Fmoc-Tyr(PO_3H_2)-OH was obtained from Novabiochem, C18ZipTip and $ZipTip_{MC}$ were purchased from Millipore. All other reagents were analytical reagent grades.

Synthesis of phosphopeptide: Phosphopeptide was synthesized by the standard Fmoc approach using an Pioneer peptide synthesis system (ABI). The whole sequence (ADpYGEGDFLAEGGGVR) of the peptide is built up starting from the C-terminal amino acid. The following steps were used: (a) The dried resin containing 0.25 mmol of the amino acid was weighed, placed in the reaction vessel and allowed to swell by addition of dimethylformamide (DMF). (b) The N-terminal fluoroenylmethoxy carbonyl blocking group is removed by treating the resin with 10 mL of 25 % piperidine in DMF for 10 min. This step was repeated again. The resin was washed with DMF. (c) The next amino acid was coupled *via* a carbodiimide-activating agent. A 2.5-fold excess each of the next amino acid, diisopropylcarbodiimide in DMF. And 1-hydroxybenzotriazole in DMF was added to the resin and allowed to mix for 1 h. The resin was washed with DMF. By repeating this cycle sequentically the remaining amino acids were combined to provide the desired peptide sequence. The phosphotyrosine was introduced as Fmoc-Tyr(PO_3H_2). It was necessary to repeat the coupling step when phosphotyrosine was introduced into the peptide sequence. (d) Finally, the products of the synthesis were cleaved from the resin by treatment with a cocktail containing 95 % trifluoroacetic acid (TFA), 2.5 % thioanisole and 2.5 % ethanedithiol (both used as scavengers) for 1 h. The contents were filtered into 40 mL of precooled anhydrous diethyl ether. The precipitated peptide was recovered by centrifugation and purified by RP-HPLC.

Phosphopeptide binding to the ZipTip: All derivatization procedures were performed on solid-phase supports, C18ZipTip. A ZipTip was wetted with 50 % acetonitrile/0.5 % trifluoroacetic acid by slowly aspirating and dispensing the solution up and down a few times, then equilibrated with 0.1 % trifluoroacetic acid. The sample (5 μg) to be derivatized was dissolved in 0.1 % trifluoroacetic acid and drawn up and down the ZipTip about 10 times to absorb onto the reversed-phase material. After a wash with 0.1 % trifluoroacetic acid, the sample was ready for derivatization.

N-Terminal derivatization of phosphopeptide with 4-sulfophenylisothiocyanate: 4-Sulfophenyl isothiocyanate was prepared at a concentration of 2.55 mg/mL, in *Tris*-HCl buffer (50 mM, pH 8.2). This solution was aspirated into contact with the bound sample on the ZipTip (making sure no bubbles were present), then left in the ZipTip and incubated at 50 ºC. After 1 h excess reagent was washed out with 0.1 % TFA and the derivatized peptide was eluted in 2.5 μL of 80 % acetonitrile/0.5 % TFA for MALDI analysis.

Mass spectrometry: All mass spectrometry experiment were performed using an Applied Biosystems (Framingham, MA01701,USA) Voyager DE-STR time-of-flight mass spectrometer equipped with a N_2 laser (377 nm, 3ns pulse width, 20 Hz repetition rate). All mass spectra were acquired in the reflectron mode with delayed extraction. External mass calibration was performed with a mixture of peptide standards (angiotensin I, m/z 1296.6853; ACTH fragment 1-17, m/z 2093.0867; ACTH fragment 18-39, m/z 2465.1989) and mass-measurement accuracy was typically ± 0.3Da.

Post-decay fragment ion spectra were acquired for peptides and peptide derivatives after isolation of the appropriate precursor ions by using timed ion selection. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflectron in the following ratios: 1.000 (precursor ion segment), 0.918, 0.830, 0.777, 0.650, 0.483, 0.316, 0.178 and 0.106 (fragment ion segments). Care was taken to adjust the laser power so that unit mass resolution was obtained and 200-300 laser shots were collected for each voltage segment. The individual segment were stitched together using software developed by applied biosystems. The PSD fragment ions were measured as isotopically averaged masses.

The matrix used for all mass spectra was α-cyano-4-hydroxycinnamic acid (CHCA), as a saturated solution in 1 % TFA in acetonitrile/water (1:1, v/v). Samples for mass spectra were prepared by using 2 μL of the charge-derivatized phosphopeptide solution mixed with 5 μL of the matrix solution and applied to the stainless-steel sample plate. The mixture was air dried before insertion into the mass spectrometer.

Construct of chimeric Cx31/myc: Cx31 gene sequence was produced by PCR from cDNA library. The PCR was done under the following conditions: 5 min at 95 ºC; 30 cycles of 20 s at 95 ºC, 30 s at 62 ºC and 45 s at 72 ºC; 10 min at 72 ºC. We used the following primers for amplification of the connexin31 insert: upstream primer 5' CG GAA TTC[1] GGC GCC ATG GAC TGG AAG ACA CTC CA 3'; downstream primer 5' CCAAGC TT[3]G GGA TGG GGG TCA GGT TGG G 3' (primers were synthesed by Shanghai Bioasia). And then PCR products were cloned into a TA cloning vector, pGEM-T vector (Promega). The Cx31/pGEM-T were digested by two restrict enzymes (EcoR1 and SalI, TaKaRa, linked with pcDNA3.1/myc/his B (-)). All constructs were verified by DNA sequencing.

Transfection with chimeric Cx31 constructs: Transfection was carried out with Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instructions. Generally, a 1:2 (μg:μL) DNA: Lipofectamine 2000 ratio was used for HeLa cells and HT1080 cells. 24 h Post-transfection, cells were harvested for western blotting, immunoprecipitation or fixed

with cold methanol for fluorescence staining. To select stable-expressing WT-Cx31 HeLa cell lines, cells were passaged at 1:10 and G418 added (800 μg/mL) after 24 h post-transfection. The selective medium with G418 was renewed every 4 days. After 2 or 3 weeks, single cell clones may be obtained. For Cx31/myc, cell clones are picked out to culture for confirmation.

Extraction the Cx31 protein: The transfected cells were washed three times with PBS and lysated in RIPA (containing protease inhibitor (sigma), 2 mmol/L Na_3VO_4, 50 mmol/L NaF) on ice. Insoluble cellular debris was cleared by centrifugation at 16,000 g at 4 ºC for 20 min. The supernatants were precleared by incubation with 100 μL protein A agarose(KPL) for 2 h on a Mixer Genius. Anti-Cx31 polyclonal antibody (8 μg) and protein A agarose (80 μL) were added to lysates and incubated at 4 ºC overnight on a mixer genius. Following precipitation, protein-antibody-beads complexes were washed at least ten times with RIPA. The proteins were separated on 12 % SDS-PAGE under denaturing conditions and then the gel was stained with coomassie brilliant blue. Exciseed band, cut as close to the band as possible to minimize excess gel material and cut into 1 mm^3 cubes and the sample was destained with 50 % acetonitrile. The sample was alkylated by using iodoacetamide, dried and enzymed by using the sequencing grade trypsin at 37 ºC for 12 h. Sample binding to the ZipTipMC and modification of Lys-terminated peptide and N-terminal derivatization of peptide with SPITC. The modified peptides were collected and analyzed with PSD-MALDI mass spectrum (ABI STR™).

RESULTS AND DISCUSSION

The present solid-phase derivatization worked well with synthesized phosphopeptide product and its byproduct (Fig. 1), an arginine-terminated peptide (Arg-phosphopeptide: ADpYGEGDFLAEGGGVR, $[M+1]^+$ at m/z 1691.8270) (Fig. 2) and its byproduct ($[M+1]^+$ at 1932.3920) (Fig. 3). A recent publication suggests that the intensity of the detected ions is biased toward ions with an arginine residue over those with a lysine residue. Guanidination of the ε-amino group of lysine side chains is a simple and useful way to solve the sensitivity problem associated with lysine-terminated tryptic phosphopeptides. Salts of O-methylisourea have been shown to specifically react with thee-amino group of lysine side chains even in the presence of free primary amines at the N-termini of tryptic peptides. In present experiments, the Arg-terminus phosphopeptide was selected for the method.

Figs. 4 and 5 show MALDI mass specrum of the synthesized phosphopeptide product and byproduct modified by 4-sulfophenyl isothiocyanate (SPITC). The modified synthesized phosphopeptide product and byproduct are shifted to m/z 1906.6375 (Fig. 4) and 2147.4581 (Fig. 5) as a result of adding the phenylsulfonate groups (215Da) to the native phosphopeptide. The precursor ion selector was set at m/z 1691.8270, 1906.6375 and both derivatized and underivatized phosphopeptides were subjected to PSD sequencing. The PSD spectra of intact Arg-phosphopeptide and Arg-phosphopeptide modified by SPITC are compared in Fig. 6a. The spectrum obtained from the intact Arg-phosphopeptide demonstrates a complex fragmentation pattern consisting of incomplete series of N-terminal a- and b-type ions, C-terminal y- and z-ions

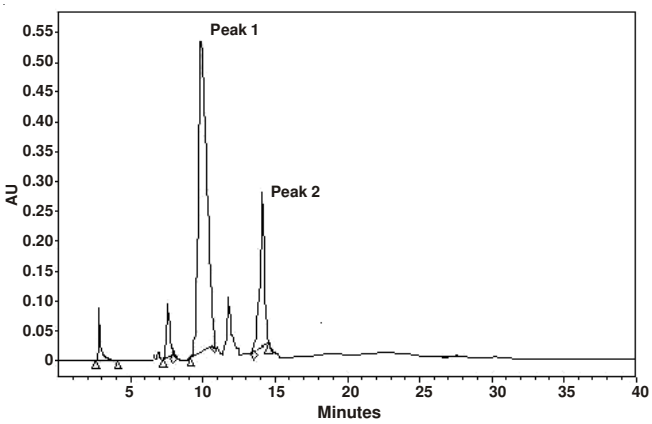

Fig. 1. Synthesized phosphopeptide purification result

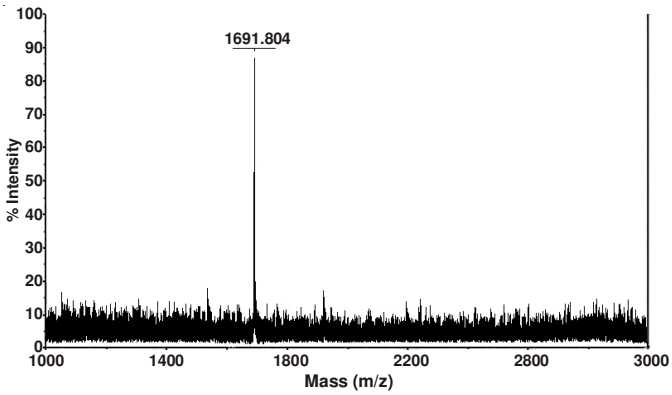

Fig. 2. MALDI-TOF mass analyses the product

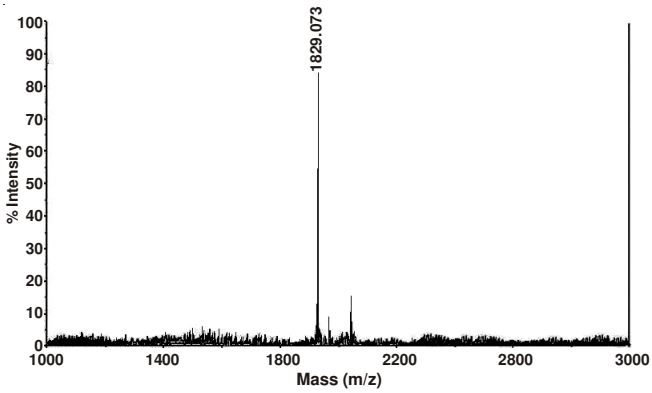

Fig. 3. MALDI-TOF mass analyses the byproduct

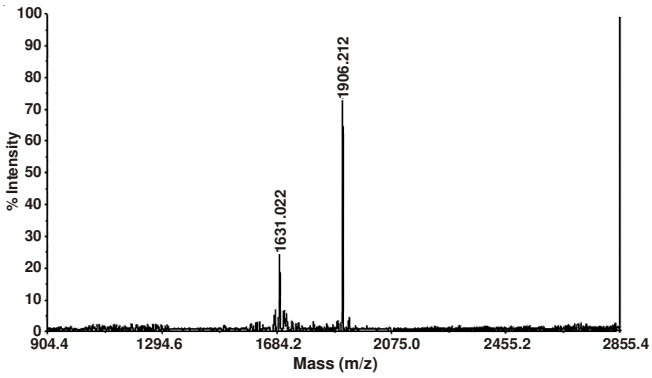

Fig. 4. MALDI mass spectrum of the sulfonated product

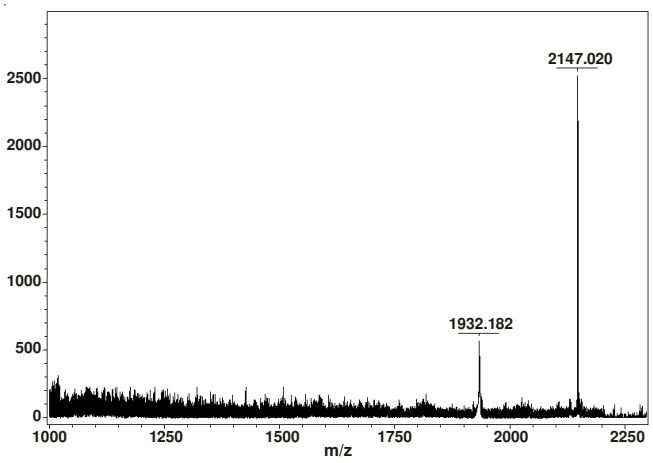

Fig. 5. MALDI mass spectrum of the sulfonated byproduct

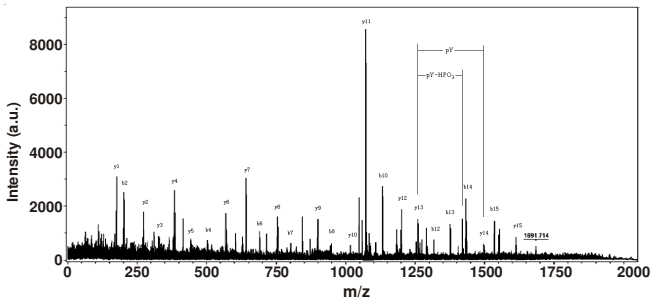

Fig. 6a. MALDI-PSD analysis of the product

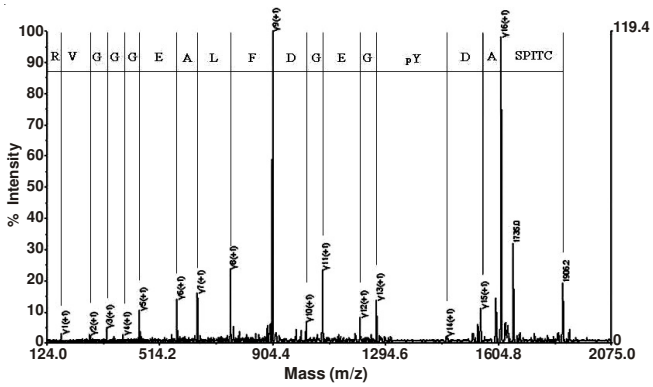

Fig. 6b. MALDI-PSD analysis of the sulfonated product. The result show that the phosphorylated tyrosine at position 3 remained intact with a mass difference of 243 (163 tyrosine + 80 HPO_3) between y14 and y13

and even internal cleavage products. The spectrum would be difficult to accurately interpret de novo. Fig. 6b shows the spectrum obtained by analysis of the sulfonated Arg-phosphopeptide. This sulfonated Arg-phosphopeptide produces mainly y-type fragment ions with excellent S/N ratios and clear background under MALDI-PSD conditions. This result may be attributed to introduction of a negative charge (sulfonic acid group) at the N-terminal of the phosphopeptide. After derivatization, the formation of a positively charged ion (net charge) would require two protons to be introduced into the phosphopeptide. One of these protons will primarily reside in the basic C-terminal side chain but the other has a higher degree of freedom to mobilize along the phosphopeptide backbone, assisting fragmentation to mainly b- and y-fragments. However, in view of the negative charge (sulfonic acid group) at the N-terminal, the b-fragments are overall neutral resulting in a spectrum in which only the y-ions are observed.

The MALDI-PSD mass spectrum obtained from a sulfonated arginine-terminated phosphopeptide byproduct is shown in Fig. 7. The fragment ion peaks of derivatized arg-phosphopeptide byproduct represent the complete y1 to y16 series plus the labeled precursor ion (+215Da), making the amino acid sequence easily interpretable by manual calculation of the differences between the adjacent y-ion fragments. The results show that the phosphorylated tyrosine at position 3 modified with an unknown chemical group a mass difference of 484 (163 for tyrosine + 80 for HPO_3 + 241 for an unknown chemical group) between y14 and y13. When we synthesized the phosphopeptide, the phosphotyrosine was introduced as Fmoc-Tyr(PO_3H_2). The modified phosphorylated tyrosine may be produced in which the products of the synthesis were cleaved from the resin by treatment with a cocktail.

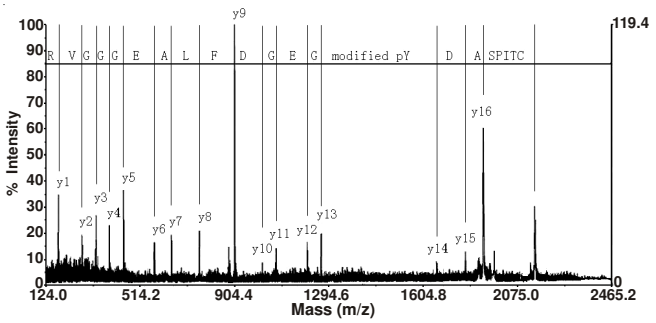

Fig. 7. MALDI-PSD analysis of the sulfonated byproduct. The result show that the phosphorylated tyrosine modified by an unknown chemical group. Between y14 and y13

The modified enzymed phosphopeptide of Connexin31 is shifted to m/z 1809.1260 (Fig. 8) as a result of adding the phenylsulfonate groups (215Da) to the native phosphopeptide. The precursor ion selector was set at m/z 1809.1260 and modified phosphopeptide was subjected to PSD sequencing. The MALDI-PSD mass spectrum obtained from a modified phosphopeptide of Connexin31 is shown in Fig. 9. The fragment ion peaks of modified phosphopeptide of Connexin31 represent the complete y1 to y16 series plus the labeled precursor ion(+215Da), making the amino acid sequence easily interpretable by manual calculation of the differences between the adjacent y-ion fragments. The results show that the phosphorylated serine at position 10 between y7 and y6. Phosphorylation

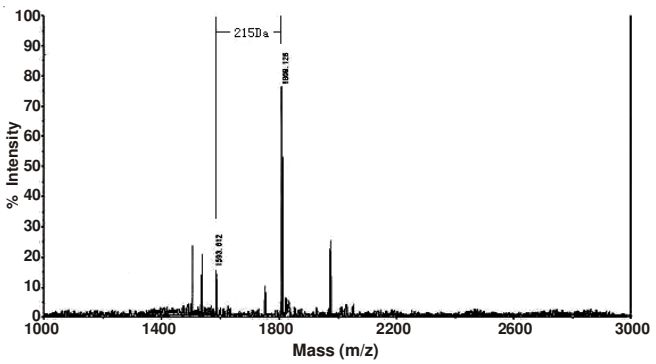

Fig. 8. MALDI mass spectrum of the sulfonated phosphopeptide from the Cx31

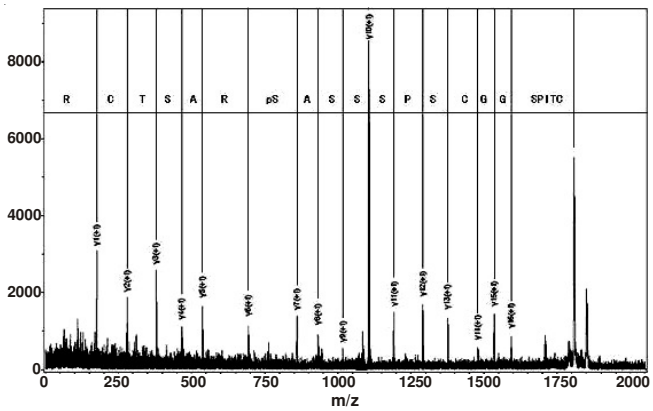

Fig. 9. MALDI-PSD analysis of the sulfonated product. The result shows that the phosphorylated serine at position 10

of Connexin31 may be regulates the kinetics of protein trafficking, gating and turnover. We will have to test this hypothesis by determination of connexin31 phosphorylation sites followed by site-directed mutagenesis, transfection and analysis of connexin trafficking, channel gating, gap junction assembly and turnover.

Conclusion

The solid-phase sulfanation procedure provides a powerful method for high-sensitivity de nono peptide sequencing using MALDI-PSD. The labeling with a sulfonic acid group by 4-sulfophenyl isothiocyanate (SPITC) enhance PSD sequencing of phosphorylated peptide, producing mainly y-type fragment ions with excellent signal-to-noise ratios and clear background under MALDI-PSD conditions and the spectra can be readily interpreted de novo. Thus the amino acid sequence can be simply calculated from the mass differences between the adjacent y-ions and advanced interpretation software is not required. The combination of peptide mass fingerprinting (PMF) and PSD sequencing described here could become a powerful and rapid method for peptide sequence and phosphorylation sites.

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