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Application of Electrospray Ionization MS/MS and Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry to Structural Analysis of the Glycosyl-Phosphatidylinositol-anchored Protein[1]

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We applied the improved sensitivity and soft ionization characteristics of electrospray Ionization (ESI)-MS/MS and matrix-assisted laser desorption/ionization(MALDI)-time of flight (TOF) mass spectrometry (MS) to analysis of the GPI-anchored C-terminal peptide derived from 5′-nucleotidase. ESI-MS/MS analysis was applied to the core structure (MW, 2,743). In the collision-induced dissociation (CID) spectrum, single-charged ions such as m/z 162 (glucosamine), 286 (mannose-phosphate-ethanolamine), and 447 ([mannose-phosphate-ethanolamine]-glucosamine) were clearly detected as characteristic fragment ions of the GPI-anchored peptide. On MALDI-TOF-MS analysis, heterogeneous peaks of GPI-anchored peptides were detected as single-charged ions in the positive mode. Product ions were obtained by post-source decay (PSD) of m/z 2,905 using curved field reflectron of TOF-MS. Most of the expected product ions derived from the GPI-anchored peptide, containing the core structure and an additional mannose side chain, were successively obtained. Thus, ESI-MS/MS and MALDI-TOF-PSD-MS proved to be effective and sensitive methods for analyzing the GPI-anchored peptide structure with less than 10 pmol of sample. These characteristic fragments or fragmentation patterns seem to be very useful for identification of GPI-anchored C-terminal peptides derived from any kind of GPI-anchored protein.

Key words: ESI-MS, GPI anchor, inositol phosphoglycan, MALDI-TOF-MS, post-translational processing.

A number of membrane proteins anchored by glycosyl-phosphatidylinositol (GPI) have been reported (*1, 2*). Most of these proteins are detected by means of specific release from membranes by bacterial phosphatidylinositol-specific phospholipase C (PIPLC) (*3-6*). The core structure of the GPI-anchored protein (*2, 7*) was first determined for the *Trypanosoma brusei* variant surface glycoprotein (VSG); *i.e.* {ethanolaminephosphate-(mannose)$_3$-glucosamine-*myo*-inositol phosphate} is the core structure attached to the C-terminal GPI-anchored peptides of this protein (*8, 9*). Then, it was revealed that this core structure is also conserved in the mammalian GPI-anchored proteins (*10-12*). In the previous work, we revealed the existence of microheterogeneity in the GPI-anchor structure of bovine liver 5′-nucleotidase by electrospray ionization (ESI)-mass spectrometry (MS) (*13*). Variations observed in additional mannose, *N*-acetylhexosamine, and ethanolaminephosphate residues are responsible for this heterogeneity, as reported previously (*13*).

Analysis by ESI-MS and matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) has been reported to be useful for determining post-translational modification of proteins by glycans or lipids (*14-16*). In this study, we applied ESI-MS and MALDI-TOF-MS to address GPI-anchored structures because of their improved sensitivity and soft ionization characteristics. Further understanding has been gained with ESI-MS/MS and post-source decay (PSD) of TOF-MS.

In this study, we intended to elucidate the characteristic and sequential degradation patterns of GPI-anchored peptides using MALDI-TOF-MS with PSD or ESI-MS/MS. Also we tried to find characteristic fragment ions of GPI-anchored peptides by CID or in-source collision of ESI-MS for systematic identification of GPI-anchored C-terminal peptides by ESI-MS/MS or LC-ESI-MS.

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Abbreviations: GPI, glycosyl-phosphatidylinositol; IPG, inositol phosphoglycan; PI, phosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C; PVDF, poly(vinylidine difluoride); ESI-MS/MS, electrospray ionization MS/MS; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; CID, collision-induced dissociation; PSD, post-source decay; TFA, trifluoroacetic acid; CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; HIC, 1-hydroxyisoquinoline.

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MATERIALS AND METHODS

Chemicals—Affinity adsorbents such as Con A-Sepharose were purchased from Pharmacia, and DEAE-Toyopearl was from Tosoh (Tokyo). Matrix materials, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and 5-methoxysalicylic acid were purchased from Aldrich (Milwaukee, WI). All other chemicals used were of analytical reagent grade unless otherwise stated.

Assaying of 5′-Nucleotidase—Assaying of 5′-nucleotidase was performed by the method of Emmelot and Bos (*17*), with 5′-AMP as the substrate, by determining the amount of inorganic phosphate produced.

Phosphatidylinositol-Specific Phospholipase C—Phosphatidylinositol-specific phospholipase C (PIPLC) was purified in a homogeneous state from culture broth of *Bacillus thuringiensis* IAM 12077, as described in the foregoing report (*18*). The PIPLC assay was performed with phosphatidylinositol (PI) as the substrate as reported previously (*19*).

Purification of 5′-nucleotidase—The solubilization and purification of 5′-nucleotidase from bovine liver were performed by the methods described previously (*13, 20*). Briefly, bovine livers were cut into pieces and washed with 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 100 μM PMSF. Pellets were obtained by centrifugation at 2,000×*g* for 15 min, suspended in the same buffer and then incubated with 10 milliunits/ml of PIPLC at 37°C for 30 min. The solubilized 5′-nucleotidase was collected in the supernatant by centrifugation at 40,000×*g* for 30 min. 5′-Nucleotidase was purified by precipitation with 40-80% saturated ammonium sulfate, Con A-Sepharose column (60×60 mm) chromatography, DEAE-Toyopearl column (60×60 mm) chromatography, and SDS polyacrylamide gel electrophoresis (PAGE) (*21*). After electrophoresis, protein bands were transferred to an Immobilon PVDF membrane (Millipore) with a Bio-Rad electroblotting system.

Preparation and Purification of CNBr-Cleaved Peptides Derived from 5′-Nucleotidase—The PVDF membrane was stained with Ponceau S after electroblotting. The stained band of 5′-nucleotidase on the PVDF membrane was treated with 1 M CNBr, and then the cleaved peptides were eluted from the membrane with 70% formic acid and dried under N_2 gas. The sample was dissolved in 0.1% trifluoroacetic acid (TFA) containing 10% acetonitrile, and then applied to a C_{18} reverse phase column. Peptides were eluted with a gradient of 0-100% acetonitrile in 0.1% TFA. The peaks of the separated peptides were subjected to further analysis.

ESI-MS Analysis—CNBr-cleaved GPI-anchored peptides were analyzed with an API 300 (Perkin Elmer Sciex) or Quattro II (Micromass) equipped with an electrospray ion-source (ESI). Ten-microliter aliquots of samples (0.1-5 pmol/μl) dissolved in water/methanol (1:1) containing 0.1% acetic acid were introduced by means of a flow injector into the ESI chamber, at the flow rate of 2 μl/min. A unique feature of ESI-MS is the generation of multiple-charged ions which brings the mass (*m/z*) values of large glycopeptides to easily determinable lower mass values. ESI-MS data were analyzed directly or deconvoluted by means of an algorithm to extract molecular weight information from the peak spacings of the multiple-charged ions.

For CID experiments, significantly higher concentrations of the samples (4-10 pmol/μl) were used to get greater signal strength. Ar was used as the collision gas at a collision cell pressure of $3\text{-}5\times10^{-1}$ Pa, and the collision energy of +25 eV was used for obtaining fragment ions from GPI-anchored peptides.

MALDI-TOF Analysis—The instruments used in this study were a Kompact MALDI IV (Kratos-Shimadzu, Kyoto) and a Voyager Elite-DE (Perseptive Biosystems). The Voyager Elite-DE was used in the linear and reflectron modes using delayed extraction (*22*). The Kompact MALDI IV was used for analysis of the post source decay (PSD) spectrum in the curved field reflectron mode (*23*).

The α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix was prepared by dissolving 10 mg α-CHCA in 1 ml acetonitrile/0.1% TFA (1:1 v/v for the Kompact MALDI IV and 7:3 v/v for the Voyager Elite-DE). The 2,5-dihydroxybenzoic acid (DHB) matrix for the Kompact MALDI IV was prepared by dissolving 20 mg DHB in 1 ml acetonitrile/0.1% TFA (7:3 v/v). Also, the DHB-1-hydroxyisoquinoline (HIC) matrix for the Voyager Elite-DE was prepared by dissolving 0.2 M DHB and 0.06 M HIC in 1 ml acetonitrile/water (1:1).

Typically, a 1 μl aliquot of GPI-anchored peptides dissolved in 0.1% TFA at a concentration of 0.1-5 pmol/μl was applied to the probe tip and then dried in air. Then, 1 μl of the matrix solution was added and dried again. In some experiment, 0.5 μl of sample and 0.5 μl of matrix solution were successively put on a sample plate, mixed immediately, and then dried under air. Samples were irradiated with UV light (337 nm) from a N_2 laser. A MALDI spectrum was obtained in the linear or reflectron mode with individual instruments.

As molecular weight standards of peptides, human angiotensin I (MW, 1,296.49), ACTH (18-39clip) (MW, 2,465.70), and bovine insulin (MW, 5,733.53) were used. Two-point external calibration was performed for mass assignment of the ions. Generally, a mass accuracy of <0.05% was achieved in the reflectron mode of operation with external calibration, and a mass accuracy of <0.1% was obtained in the linear mode with external calibration.

For analysis with the Voyager Elite-DE, standard peptides and GPI-anchored peptides were analyzed at 20 kV with a linear (2 m flight path) or a reflectron (3 m flight path) mode in the positive ion mode. The delay time was set at 100 ns, in addition to the inherent 20 ns delay present in the electronics. Typically, spectra from 64-256 laser shots were summed to obtain the final spectrum. The data presented herein are either not smoothed or smoothed using a 19-point Savisky-Golay second order filter. For data acquisition for the GPI-anchored peptides, a low-mass gate was used to prevent the matrix ions from striking the detector plate.

For analysis with the Kompact MALDI IV, the instrument was operated in the positive ion mode at an accelerating voltage of 20 kV; the optimum laser power value in terms of the instrument dial setting (arbitrary units, maximum 180). A MALDI spectrum was generated in the linear or reflectron mode. The product-ions of a major GPI-anchored peptide, obtained by PSD, were analyzed with the Kompact MALDI IV using the curved field reflectron mode (*23*).

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RESULTS AND DISCUSSION

ESI-MS Analysis of CNBr-Cleaved GPI-Anchored Peptides—Figure 1 shows the theoretical fragment sizes of a CNBr-cleaved C-terminal peptide bearing inositol phosphoglycan (IPG). This core structure was confirmed by ESI-MS as described in the previous paper (*13*). Figure 2 shows the ESI-MS spectra of CNBr-cleaved IPG-peptides in the ODS fraction. The structures of GPI-anchored C-terminal peptides were determined by examining the *m/z* values of major ions in the ESI spectrum. The heterogeneous components of IPG-peptides were detected mainly as triple-charged positive ions on ESI/MS as reported previously (*13*). After deconvolution, several ion peaks due to microheterogeneity in their structures could be easily assigned. The sizes of major components after deconvolution were 2,743 (core peptide), 2,905 (core+162), 2,946 (core+203), and 3,108 (core+162+203) amu. The mass intervals of 123, 162, and 203 are coincident with those for ethanolamine phosphate, mannose, and *N*-acetylhexosamine, respectively.

ESI-MS/MS analysis was applied to the major compo-

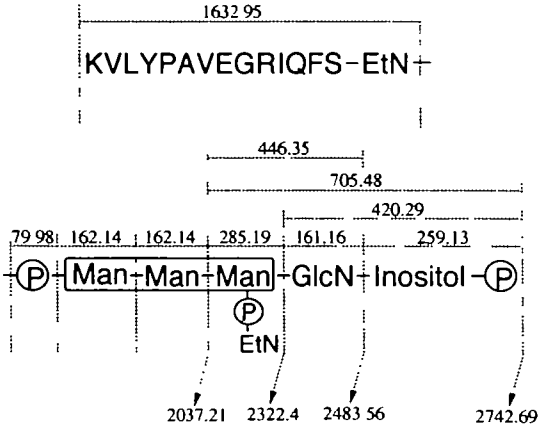

Fig. 1. **Theoretical mass calculation of the core structure of an IPG peptide derived from bovine 5′-nucleotidase.** Mass values are indicated as average mass or nominal mass. Amino acids are denoted as a single letter code. Man, mannose; GlcN, glucosamine; HexNAc, *N*-acetylhexosamine; EtN-P, phosphorylethanolamine; Inositol-P, inositol-phosphate; KVLYPAVEGRIQFS, C-terminal core peptide of 5′-nucleotidase.

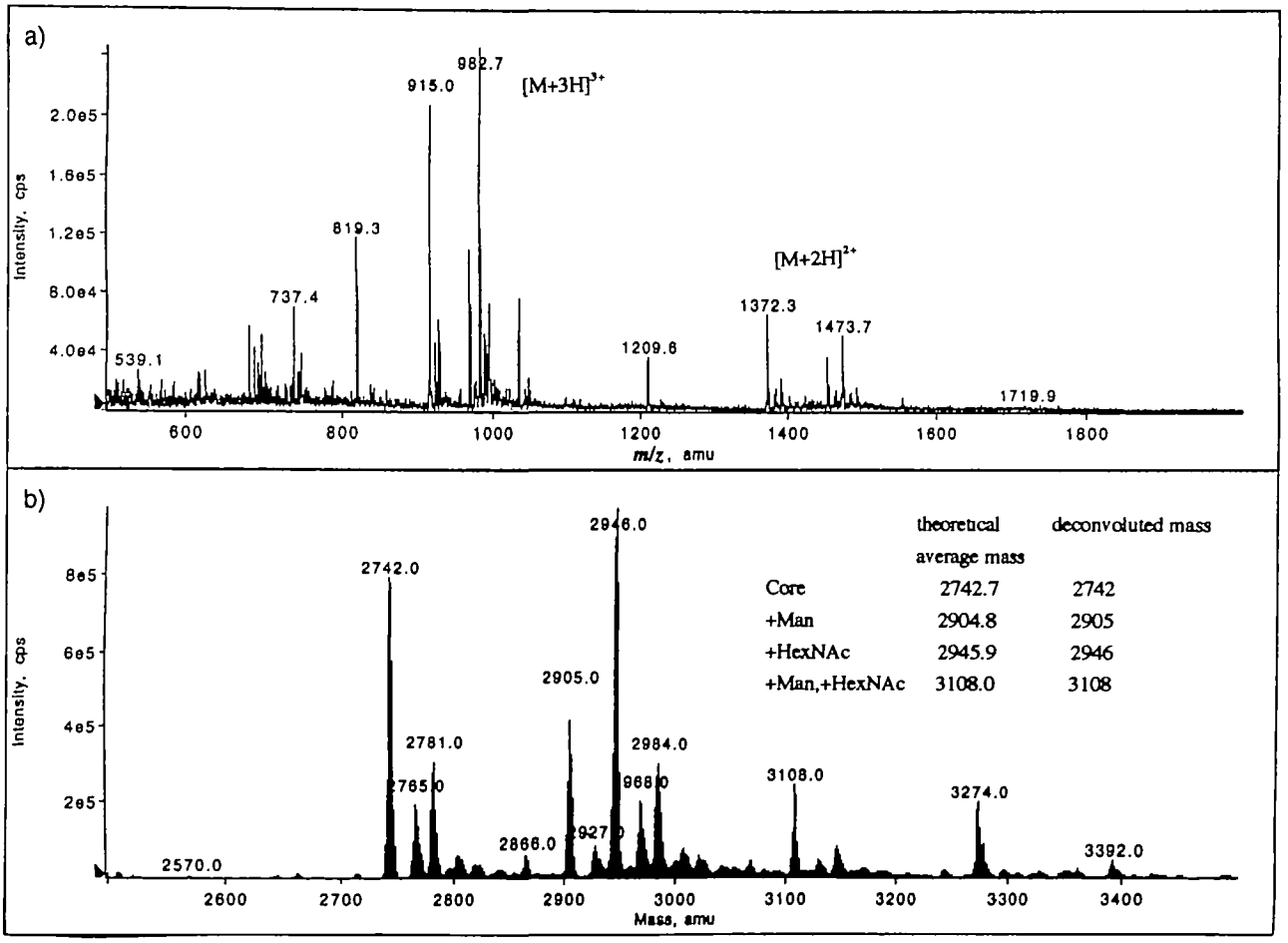

Fig. 2. **Electrospray ionization (ESI)-mass spectrum (MS) of CNBr-cleaved GPI-anchored C-terminal peptides.** CNBr-cleaved IPG-peptides were analyzed with an API 300 (Perkin Elmer Sciex) equipped with an ion spray ion source. Samples dissolved in water/methanol (1:1) containing 0.1% acetic acid were directly injected into the ESI chamber at the flow rate of 1 μl/ml by means of a syringe pump. ESI-MS data were analyzed directly or deconvoluted with an algorithm to extract molecular weight information from the peak spacing exhibited by parent ion multiply charging. Fractions of CNBr-cleaved IPG-peptides were subjected to ESI-MS analysis. (a) ESI-MS spectrum; $[M+3H]^{3+}$ and $[M+2H]^{2+}$ ions were detected for each C-terminal peptide. (b) Deconvolution of the data in (a).

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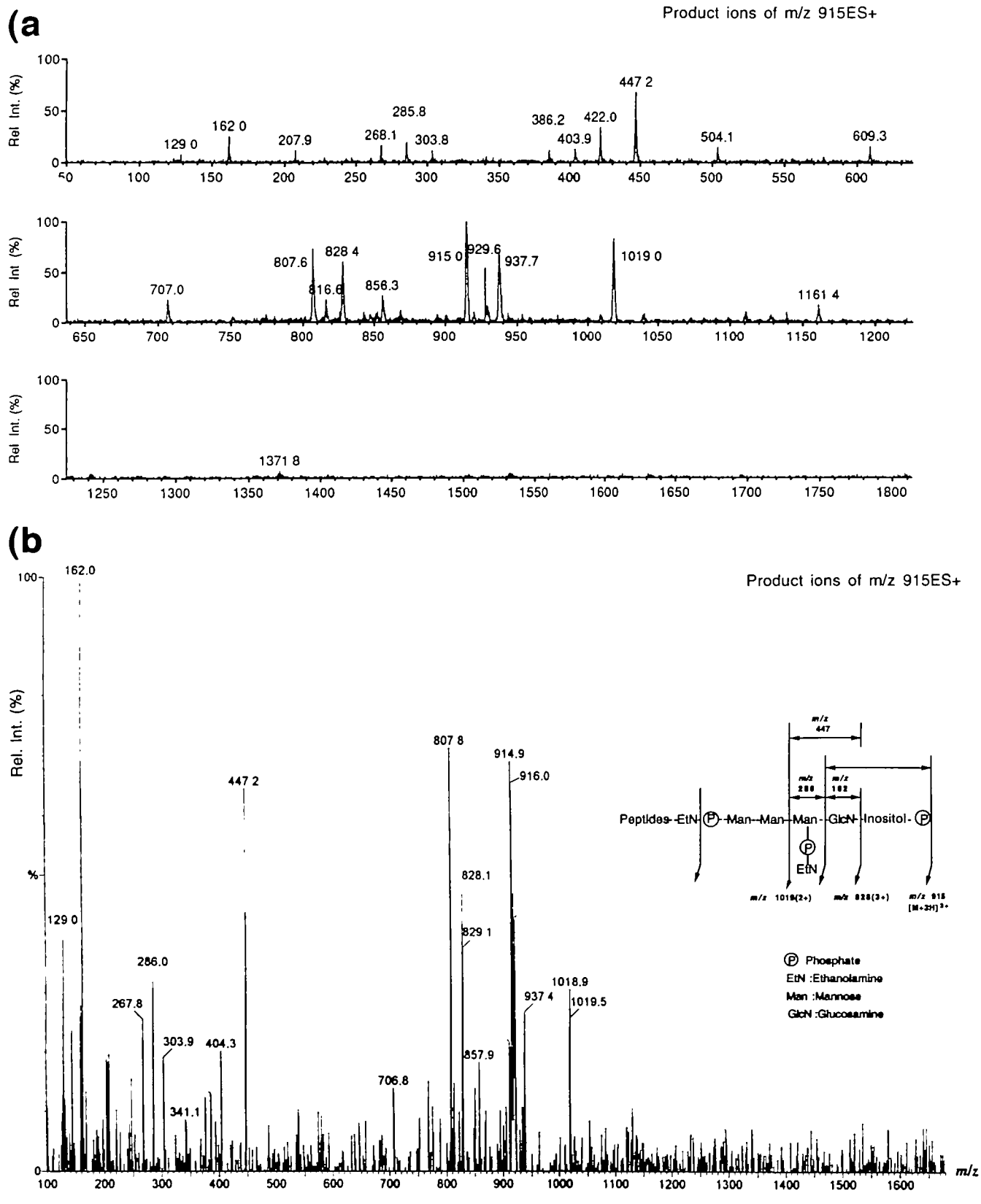

Fig. 3. **ESI-MS/MS analysis of CNBr-cleaved GPI-anchored peptides.** MS/MS analysis of major GPI-anchored peptides was performed. Ar was used as the collision gas at a collision cell pressure $3\text{-}5\times10^{-1}$ Pa, and the collision energy of +25 eV was used to obtain fragment ions from GPI-anchored peptides. The triple-charged ion, m/z 915; $[M+3H]^{3+}$ (MW, 2,743) in Fig. 2a, derived from CNBr-cleaved IPG-peptides, was analyzed by ESI-MS/MS. (a) 10 pmol of sample; (b) 1 pmol of sample.

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nents (MW, 2,743 and 2,946) in Fig. 2a, that is, triple-charged ions m/z 915; $[M+3H]^{3+}$ and m/z 982; $[M+3H]^{3+}$ in Fig. 2a were selected as the precursor ions. Figure 3 shows the CID spectrum obtained, in which m/z 915 was chosen as the precursor ion. The product ions expected from the previous analysis by gas chromatography (GC)/MS and exoglycosidase treatment were effectively detected in the CID spectrum. In particular, single-charged ions such as m/z 162 (glucosamine), 286 (mannose-phosphate-ethanolamine), and 447 ([mannose-phosphate-ethanolamine]-glucosamine) were unambiguously observed as characteristic ions. Ions of high intensity, such as 915; $[M+3H]^{3+}$ (MW, 2,742; the core) and 828 (MW, 2,482; loss of inositol phosphate), were identified as triple-charged ions, while the ion at m/z 1,019 (loss of [mannose-phosphate-ethanolamine]-glucosamine-inositol-phosphate) was assigned as a double-charged ion, as shown in Fig. 3. A fragment ion, m/z 204 (*N*-acetylhexosamine), was detected only in the CID spectrum from the precursor ion of m/z 982; $[M+3H]^{3+}$ (data not shown), *i.e.* not from m/z 915; $[M+3H]^{3+}$. Characteristic fragment ions, such as m/z 162 (glucosamine), m/z 286 (mannose-phosphate-ethanol-

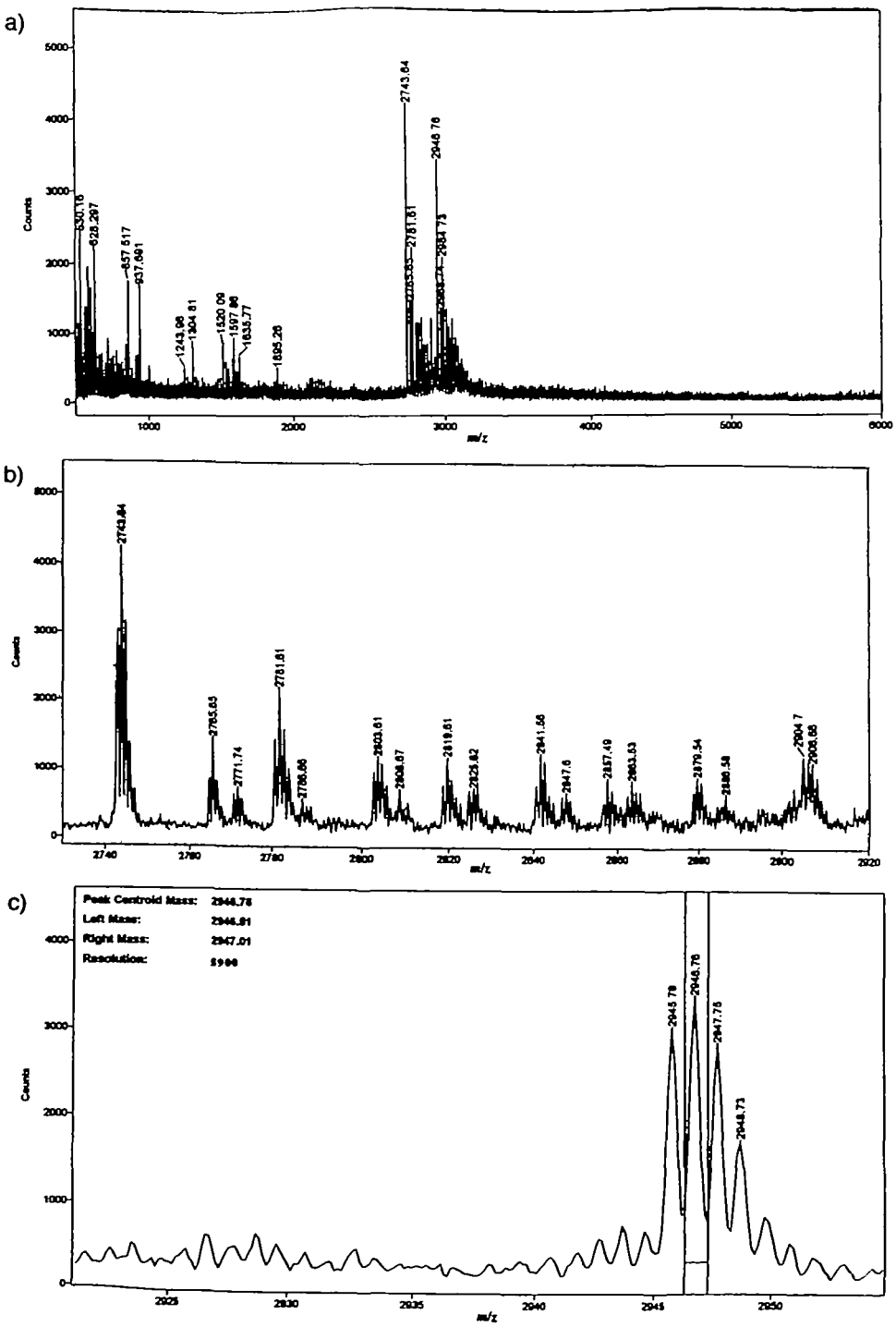

Fig. 4. **Delayed extraction MALDI-TOF mass spectrum of GPI-anchored peptides of 5′-nucleotidase.** Instrument, Voyager Elite-DE; accelerating voltage, 20,000; matrix, DHB/HIC; reflectron mode; grid voltage, 60.0% of the accelerating voltage; guide wire voltage, 0.05% of the accelerating voltage; delay, 50 ns; laser step, 2,250; scan average, 256; and in positive ion mode. (a) Mass spectrum of GPI-anchored peptides in the mass range of m/z 500-6000; (b) expansion of data in (a) between m/z 2,730-2,920; (c) expansion of data in (a) between m/z 2,920-2,955. Core structure, m/z 2,743; core+mannose, m/z 2,905; and core+*N*-acetylhexosamine, m/z 2,946.

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amine), and *m/z* 447 ([mannose-phosphate-ethanolamine]-glucosamine), in the CID spectrum were also useful for distinguishing an IPG-peptide from other peptides by LC-ESI-MS. In this case, in-source collision is also

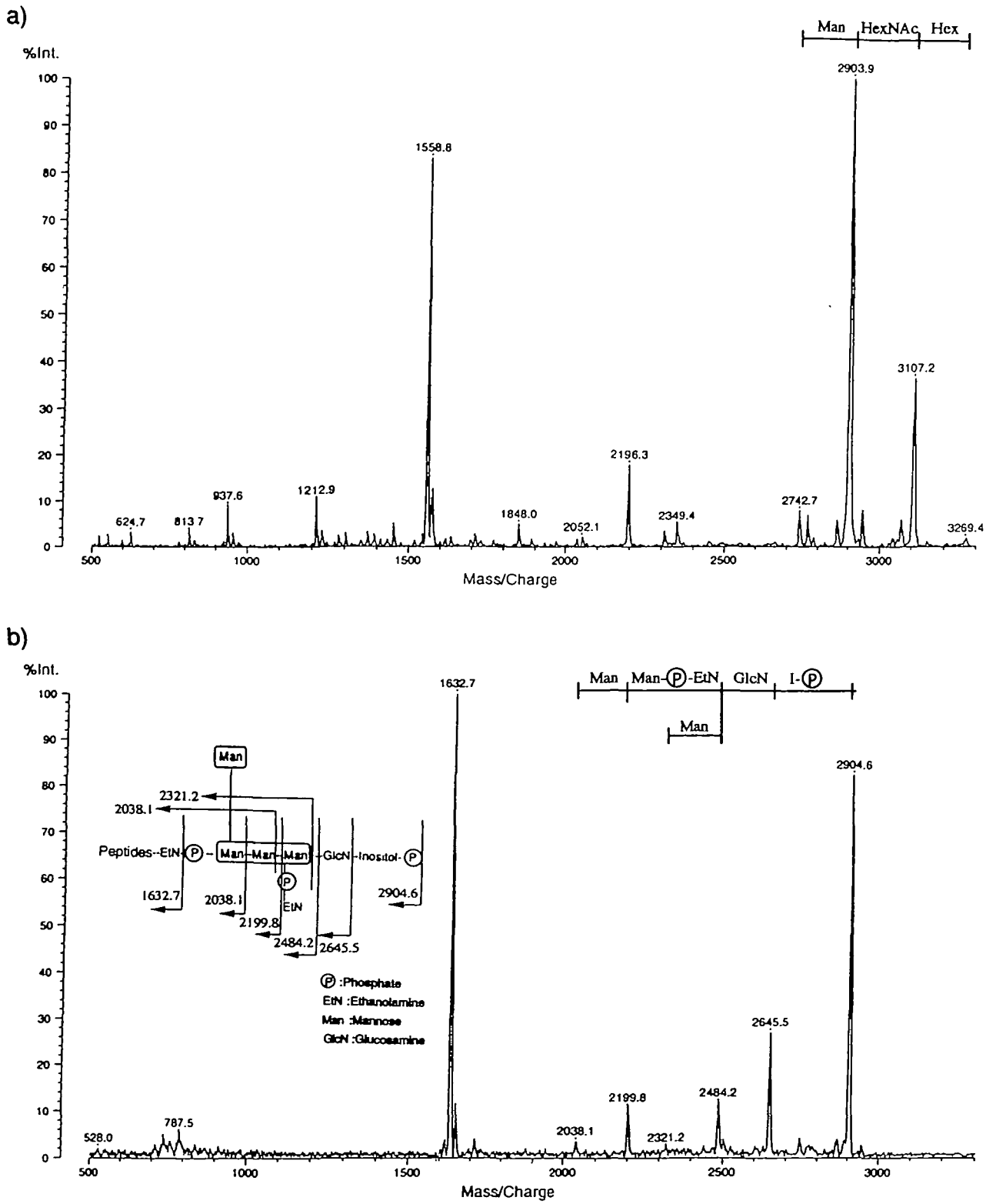

Fig. 5. **The PSD spectrum of TOF-MS of GPI-anchored peptides of 5′-nucleotidase.** Instrument, Kompact MALDI IV (Kratos-Shimadzu, Kyoto); matrix, α-CHCA; accelerating voltage, 20,000; (a) the reflectron mode; the core, *m/z* 2,742.7, the core plus mannose, *m/z* 2,903.9, and molecules containing one more mannose as well as *N*-acetylhexosamine (HexNAc) as an additional glycan in the side chain, *m/z* 3,107.2, are also indicated in the figure. (b) The post-source decay mode; the precursor ion, *m/z* 2,904.6.

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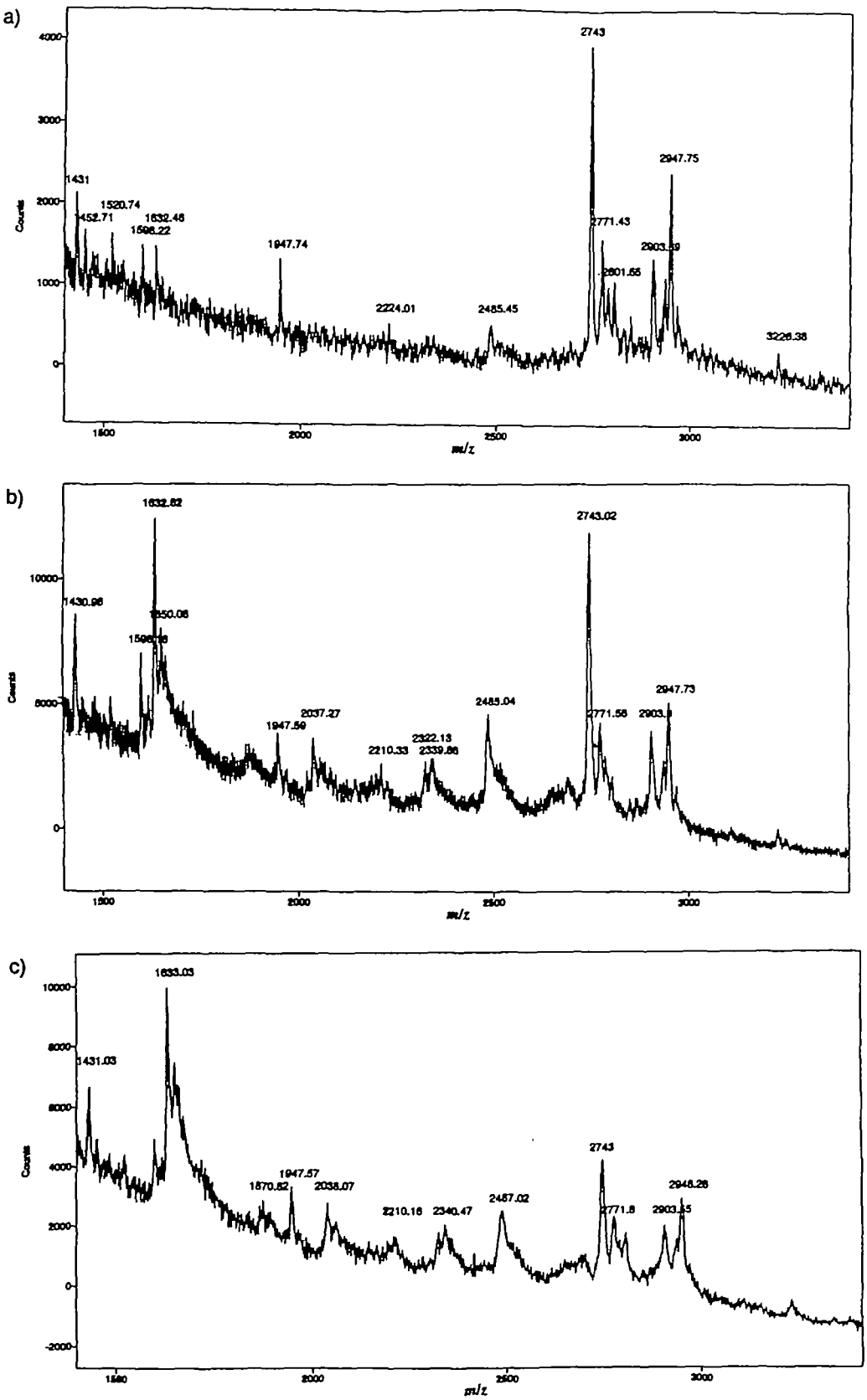

Fig. 6. **Application of in-source fragmentation by means of increased laser voltage in linear delayed extraction MALDI-TOF mass spectrometry for structural study of IPG-peptides of 5′-nucleotidase.** Instrument, Voyager Elite-DE; matrix, α-CHCA; accelerating voltage, 20,000; the linear mode; grid voltage, 94.0% of the accelerating voltage; guide wire voltage, 0.3% of the accelerating voltage; delay, 100 ns; scan average, 128; and in the positive ion mode. (a) Laser step, 2,000; (b) laser step, 2,300; (c) laser step, 2,400.

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available in place of the product or parent ion scanning method (*24*).

MALDI-TOF-MS Analysis of GPI-Anchored Peptides of Bovine 5′-Nucleotidase—The instruments used in this study were a Kompact MALDI IV (Kratos) and a Voyager Elite-DE (Perseptive Biosystems). As reported in the previous paper, various forms of IPG-peptides derived from C-terminal GPI-anchored peptides of bovine 5′-nucleotidase exist. Figure 4 shows the spectra of IPG peptides obtained with the Voyager Elite-DE in the reflectron mode with delayed extraction. As shown in Fig. 4b, a monoisotopic peak of each microheterogenous structure, and the Na^+ or K^+ adducts were detected. The theoretical average mass of the core structure of the IPG-peptides is 2,742.69, and thus the average of a single-charged ion $[M+H]^+$ is *m/z* 2,743.70. The experimental data shown in Fig. 4 coincide well with this value. From the mass differences between the major peaks in this spectrum, additional moieties to the core strucutre such as hexose (+162.1) or hexosamine (+203.2), were evident. As indicated in Fig. 4c, the resolution of the peak, *m/z* 2,946.76 (core structure+*N*-acetylhexosamine), was more than 5,900. Resolution in the monoisotopic ion range of the reflectron mode with delayed extraction of TOF-MS is useful for structural analysis of biomolecules having molecular weights of 5,000 or so.

As shown in Fig. 5a, several IPG-peptide structures were detected; *m/z* 2,742 for the core structure, *m/z* 2,904 containing an additional mannose, *m/z* 3,107 containing additional mannose and *N*-acetylhexosamine, and *m/z* 3,269, which is hypothetically a new component containing two additional mannoses as well as one *N*-acetylhexosamine. The existence of these components was also reported in the previous paper (*13*). These observed mass values were approximately one mass unit lower, respectively, than the theoretical values of these components. This ODS fraction is particularly enriched in species containing a mannose in addition to the core structure (MW, 2,904), which is one of the major components of GPI-anchored peptides detected on ESI-MS analysis. Product ions (metastable ions) obtained by PSD from the precursor ion of *m/z* 2,904.6 were analyzed using the curved field reflectron mode of MALDI IV. In this mode, the whole product ion spectrum was assembled without the need to combine pieces of the reflectron spectrum in different mirror ratios. As shown in Fig. 5b, the theoretically expected product ions were successively detected in this PSD spectrum. These data are also considered to be useful for determining the site to which an additional phosphorylethanolamine or mannose is attached.

The application of in-source fragmentation to structural analyses of sub-picomole samples of GPI-anchored peptides was studied. Here, the linear delayed extraction mode was coupled with varying laser power conditions at a higher voltage to induce selective in-source collision at the glycan parts of GPI-anchored peptides. As shown in Fig. 6a, several molecular-related ions due to microheterogeneity of the sugar side chains were clearly observed on analysis at low voltage, while the core peptide-ethanolamine ion (*m/z* 1,633) gradually increased in intensity in parallel with increasing laser power, as shown in Fig. 6, b and c. In the mass range from *m/z* 1,633 to 2,743, several characteristic peaks, such as *m/z* 1,871, 2,038, 2,322 and 2,485, were also evidently observed at a relatively high voltage, such as 2,300 or 2,400 V. Calculation of the mass values of fragment ions showed that these peaks can be ascribed to extended or partial structures of the GPI-anchored core peptide. We concluded that these ions were derived from the GPI-anchored core peptide itself, and those with an additional moiety, such as *m/z* 2,743, 2,905 and 2,947, by in-source collision at higher laser voltage. Application of in-source collision with high laser voltage in place of PSD seems to be very useful, especially for structural studies on post-translational modification with small amounts of proteins. This is because additional sugar and phosphate are bound to the peptide backbone more loosely than the peptide bond, and these moieties are selectively cleaved at higher laser power.

As described in this paper, ESI-MS/MS and PSD by MALDI-TOF-MS proved to be very useful and sensitive methods for analyzing GPI-anchored peptides with a sample size of less than 10 pmol. These characteristic fragment ions or fragmentation patterns seem to be very useful for identification of GPI-anchored C-terminal peptides derived from any kind of GPI-anchored protein (*24*).

A part of the ESI-MS data used here were obtained with an API-300 by Dai Miyagi, MS Application Laboratory, Takara Co. Ltd., Kusatsu. A part of the MALDI-MS data were obtained with a Kompact MALDI IV by Koichi Tanaka, Analytical Department, Shimadzu Co., Ltd., Kyoto.

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