modification. Side reactions at the potential reactive sites on the residues of Tyr, Lys, Thr, and Ser were not actually observed (data not shown). In Fig. 2a peptide (2) has the least intense peak before TMPP-Ac modification. In Fig. 2b, however, the modified peptide (2) has the most intense peak after the modification. If a peptide devoid of Arg and/or Lys residue in its sequence is modified with a TMPP-Ac reagent, the signal intensity in MALDI-MS can be enhanced. TMPP-Ac peptide (2) was cleanly recovered from the mixture of TMPP-Ac peptides, as depicted in Fig. 3a. The CID spectrum was acquired (Fig. 3b) for sequencing the TMPP-Ac peptide. Relatively simple fragmentation was observed, which was enough for *de novo* sequencing [17–19]. In addition to the readout of the sequence using a-type ions, several characteristic fragmentations were observed, including d_3/d_7 (-58), d_4/d_8 (-16), and d_5 (-14), which can be very helpful for assigning the residues [20]. The peak of *m/z* 573 is derived from one of the common reagent-related background ions $[TMPP\text{-}CH{=}CO]^+$ [12].

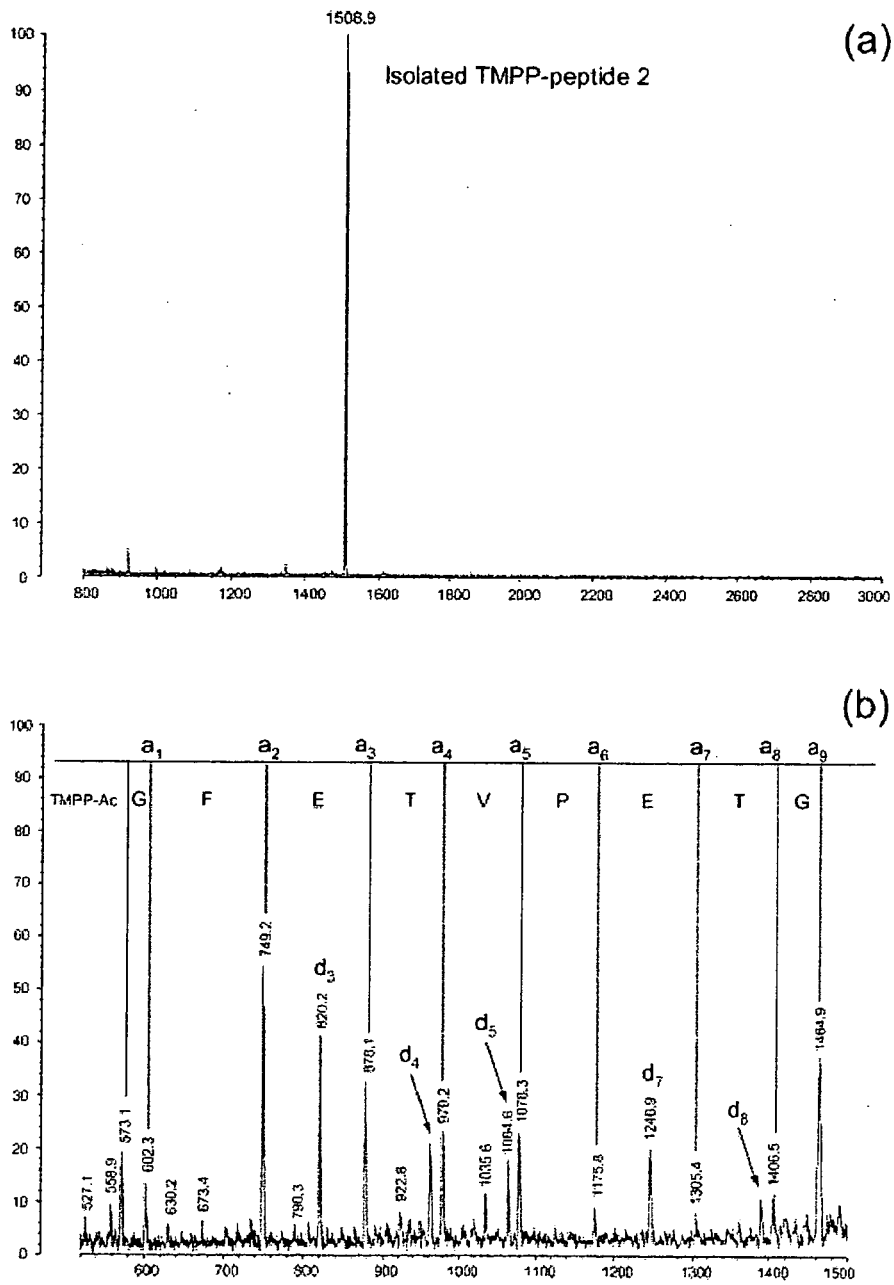

Figure 3. MALDI-TOF mass spectrum of the isolated peptide (2) after DITC resin treatment (panel a) and the CID spectrum (panel b).

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3.2 Model proteins

The model proteins used were lysozyme (chicken), ovalbumin (chicken), cytochrome *c* (horse), and α-casein (bovine). Both the lyophilized powder and the excised gel strip obtained after purification by SDS-PAGE of each protein were used as starting material. For the solution phase experiment, 10 pmol of each LysC digest was used for the TMPP-Ac modification, followed by DITC resin treatment for the selective recovery of the C-terminal peptide. To prepare 1-D-separated gel samples, 30 pmol was applied to the gel, and each stained band was excised after separation. The resulting gel pieces were treated with LysC, TMPP-Ac reagent and DITC resin as described in Section 2.2.1. In this protocol, amino group-containing reagents were avoided because they may consume the NCS group on the resin. Figure 4 presents the MALDI-TOF mass spectra of their isolated C-terminal fragments. Figures 4a and b depict gel-started results, and the other two figures depict solution-started results. Isolation was successful regardless of whether gel pieces or solutions were used as the starting material. Their C-terminal peptides are GTDVQAWIRGC*RL (lysozyme, *m/z* 1530.8: *S-alkylated with iodoacetamide), HIATNAVLFFGRC*VSP (ovalbumin, *m/z* 1787.9: *S-alkylated with iodoacetamide), ANTE (cytochrome *c*, *m/z* 433.2), TTMPLW (α-casein S1, *m/z* 747.4), and VIPYVRYL (α -casein S2, *m/z* 1021.6). The C-terminal peptides of lysozyme, cytochrome *c* and ovalbumin were sequenced in CID mode using a MALDI-TOF mass spectrometer. The spectra are plotted in Fig. 5. Sequencing was performed using mainly a-type ions and using the structural information from characteristic d-type ions that occurred due to the elimination of the side chain from certain amino acid residues. For example, Fig. 5a plotted the CID spectrum of the C-terminal fragment of lysozyme, in which d-type ions are formed on Thr (-16), Asp (-44), Val (-14), Gln (-57), Ile (-28), Arg (-85), Cys (-104: S-alkylated with iodoacetamide), and Leu (-42). The d-type ions are very helpful for *de novo* sequencing [20].

Two peaks were observed for α-casein (Fig. 4d) because it consists of two components (S1 and S2), which indicates that protein mixture (*e.g.* hetero-oligomeric complex consisting of multiple subunits) can be analyzed with this strategy.

The isolation of C-terminal peptide and its *de novo* sequencing were successfully carried out using the four model proteins. Hence, we next used a recombinant protein consisting of three subunits to analyze their C-terminal peptides.

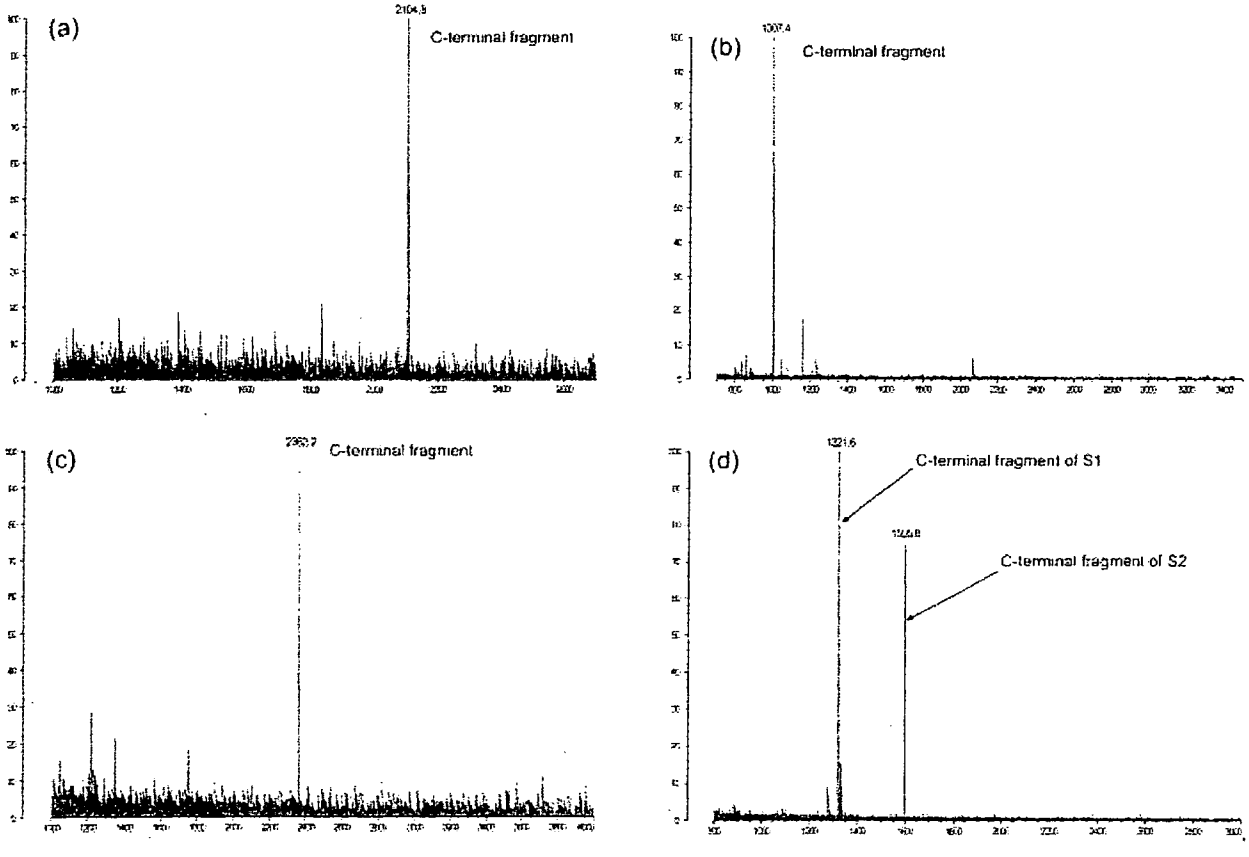

Figure 4. MALDI-TOF mass spectra of the isolated C-terminal peptides from four model proteins. (a) The isolated peptide from lysozyme. (b) The isolated peptide from cytochrome *c*. (c) The isolated peptide from ovalbumin. (d) The isolated peptide from α-casein.

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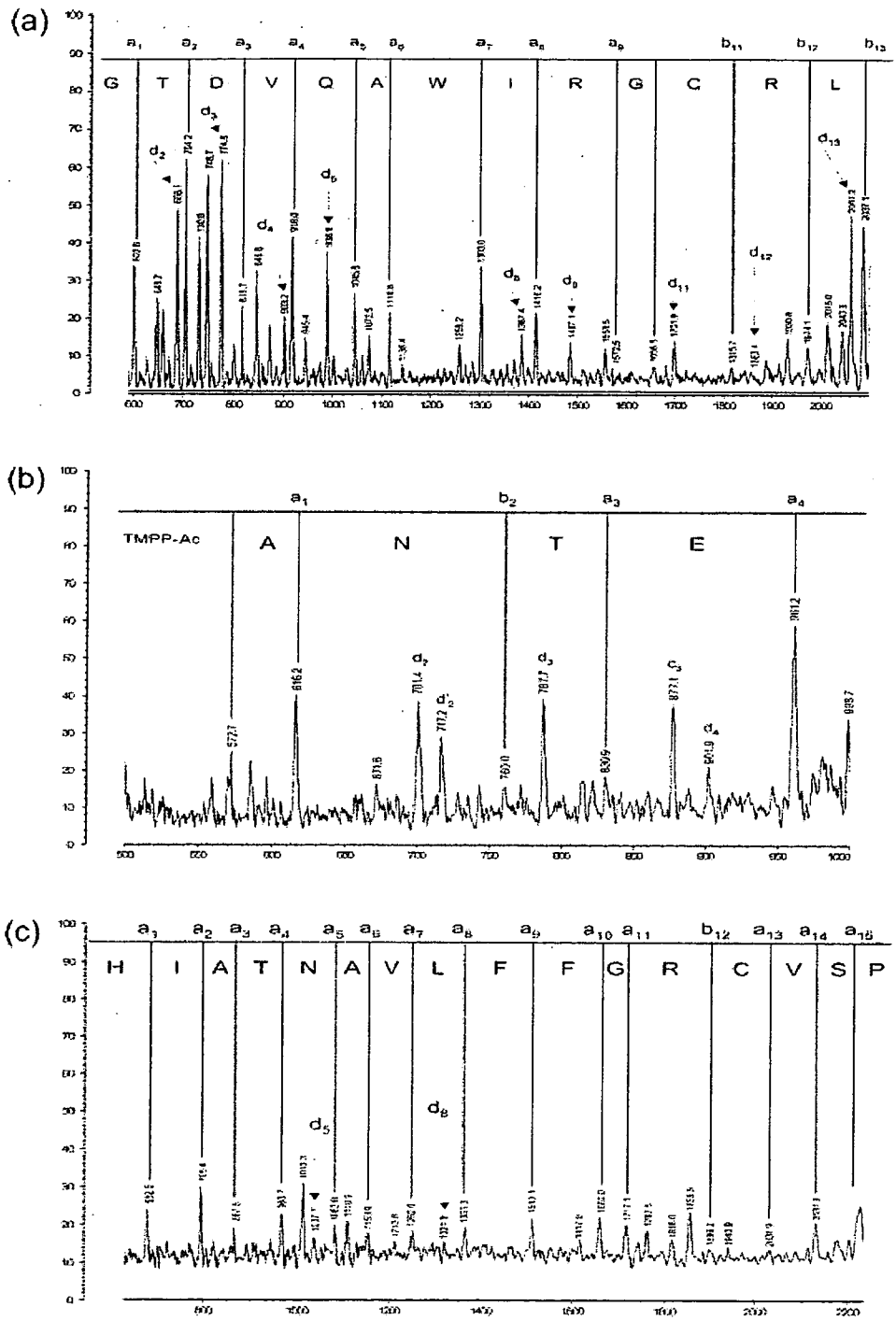

Figure 5. CID spectra of the isolated C-terminal peptides from lysozyme (panel a), cytochrome c (panel b), ovalbumin (panel c).

3.3 Recombinant protein

We then examined the method using a recombinant protein (PfuRPA) [21], consisting of three subunits, RPA14, RPA32, and RPA41. Figures in the abbreviations indicate their molecular weights.

The recombinant protein was first analyzed without separation into subunits. The whole protein (100 μg) was

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processed through reduction and S-alkylation, followed by digestion with LysC (10 μg) in 250 μL of ACN-50 mM NaHCO3 (1:9). An aliquot (50 μL) was then treated with TMPP reagent, and one-tenth of the resulting mixture was added to 5 mg of DITC resin.

The results of TMPP-Ac modification and selective recovery of the three C-terminal peptides are depicted in Fig. 6. Among the many TMPP-Ac peptides that appeared after TMPP-Ac modification (Fig. 6a), the three objective C-terminal peptides were selectively recovered with DITC resin (Fig. 6b).

We next used three subunits of the recombinant protein separated on gel: 25 μg of the protein was applied for the separation on gel. After separation, the excised gel pieces were processed through reduction/S-alkylation, digestion (LysC), TMPP-Ac modification, and isolation with DITC resin. Figure 7 presents MALDI-MS spectra at the stages of TMPP-Ac modification (Figs. 7a, b, c) and isolation (Figs. 7d, e, f). These experiments demonstrated the successful isolation of the C-terminal peptides, regardless of the methods used for sample preparation. The three C-terminal peptides were sequenced by MALDI-TOF-MS. Their CID spectra are shown in Fig. 8. The sequence readouts of the C-terminal peptides derived from RPA14 (Fig. 8a) and RPA32 (Fig. 8b) are consistent with their reported C-terminal sequences. The sequencing was performed using a-type and d-type ion signals (Ile, Leu, Gln, Glu, Arg, Val, Met, etc.). The signals of the d-type ions were quite helpful in the sequencing [20].

In the case of the C-terminal peptide of RPA41, no significant fragmentation peaks were observed in the CID spectrum (data not shown), hence its sequencing failed. This seemed attributable to the highest basicity of the arginine

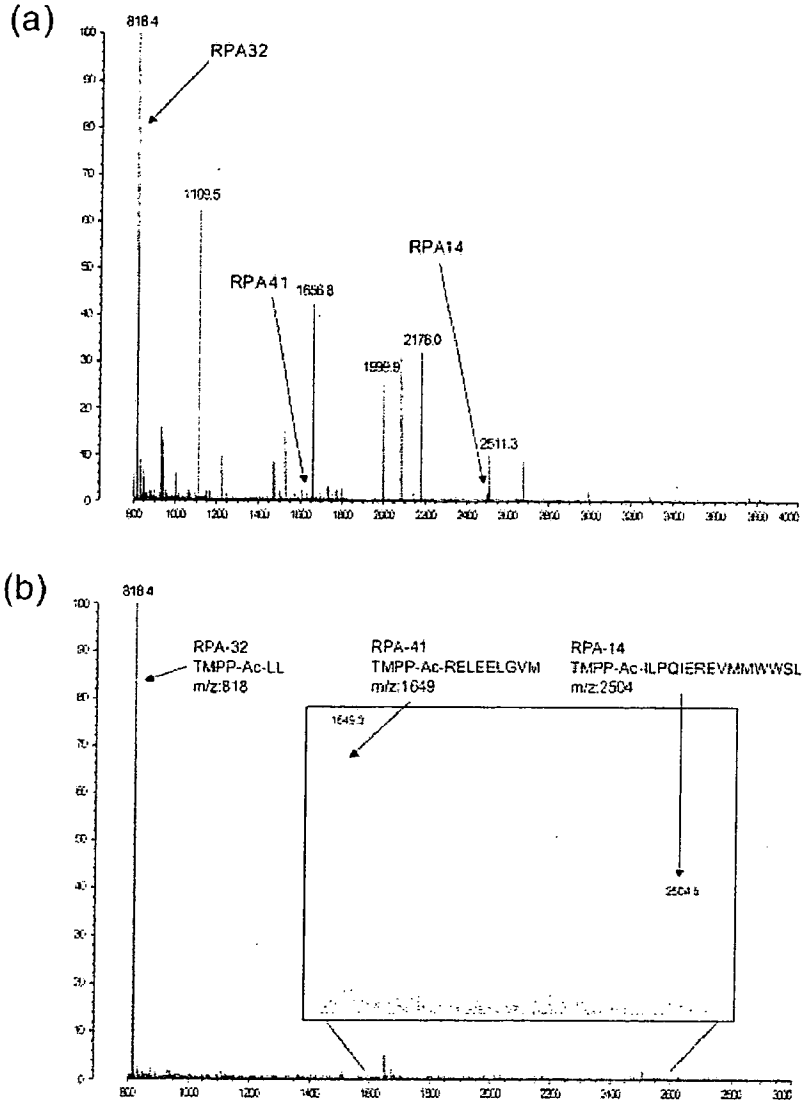

Figure 6. MALDI-TOF mass spectra for C-terminal peptides isolated from PfuRPA protein complex. MALD-TOF mass spectrum (a) after TMPP modification of the LysC digest, (b) after isolation of three C-terminal peptides using DITC resin. Arrows indicate signals of the C-terminal peptides derived from the three subunits, RPA14, RPA32, and RPA41 of PfuRPA.

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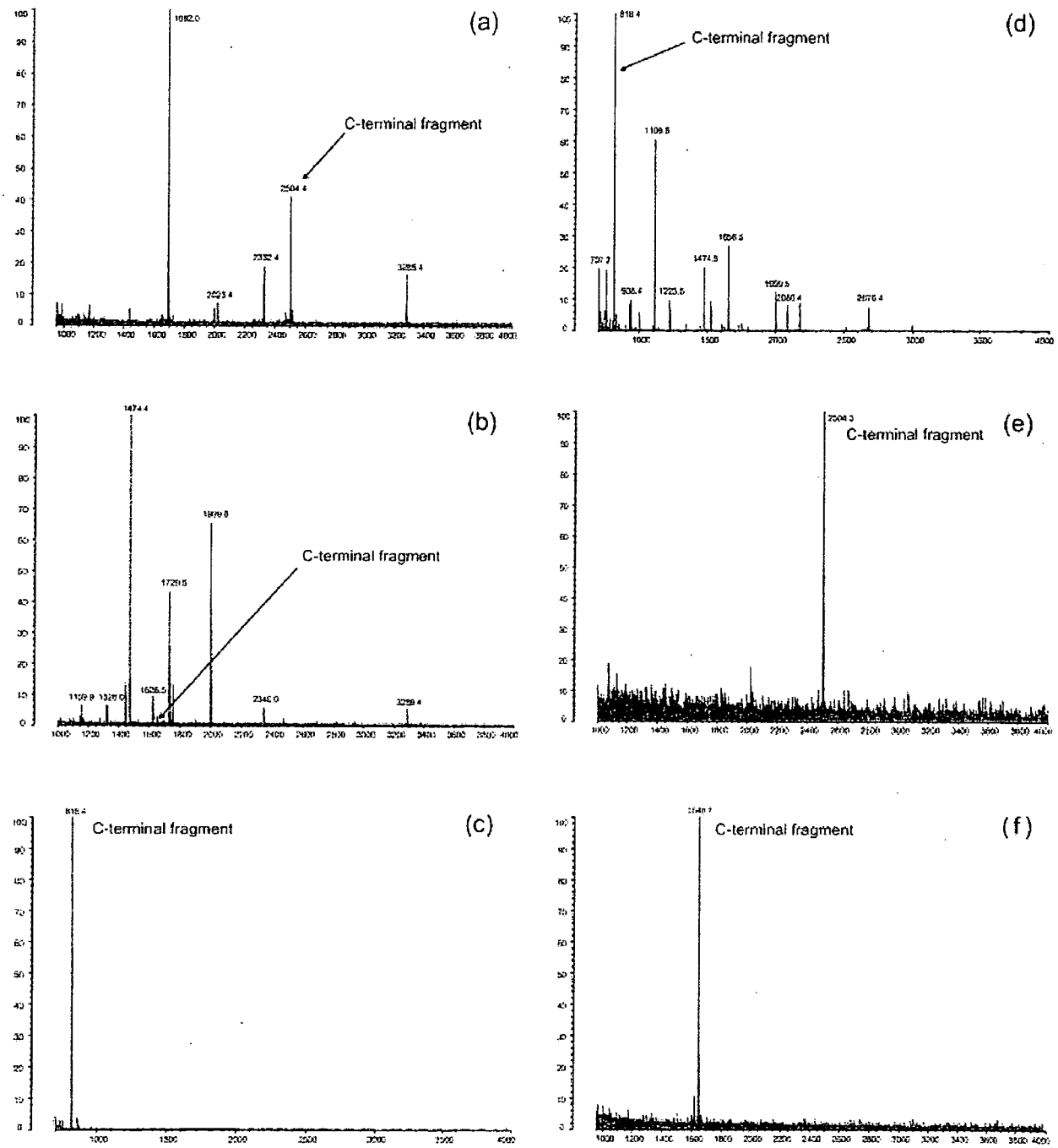

Figure 7. MALDI-TOF mass spectra of two stages of the protocol; TMPP modification (a, b and c) and isolation of the C-terminal peptide (d, e and f) using gel separated subunits of RPA14 (a, d), RPA32 (b, e), RPA41 (c, f) from PfuRPA complex.

residue in the peptide. It has been reported that arginine containing peptides (with and without the TMPP-Ac tag at the α-amino group) exhibit a low degree of structurally informative fragmentation in CID/PSD analysis [22, 23]. Although a technique has been reported for enhancing fragmentation of TMPP-modified peptides [19], it is valid for peptides having arginine residue at their C-terminal position. Arginine is normally protonated to be positively charged because it has the highest basicity among other genetically coded amino acid residues. The localized charge

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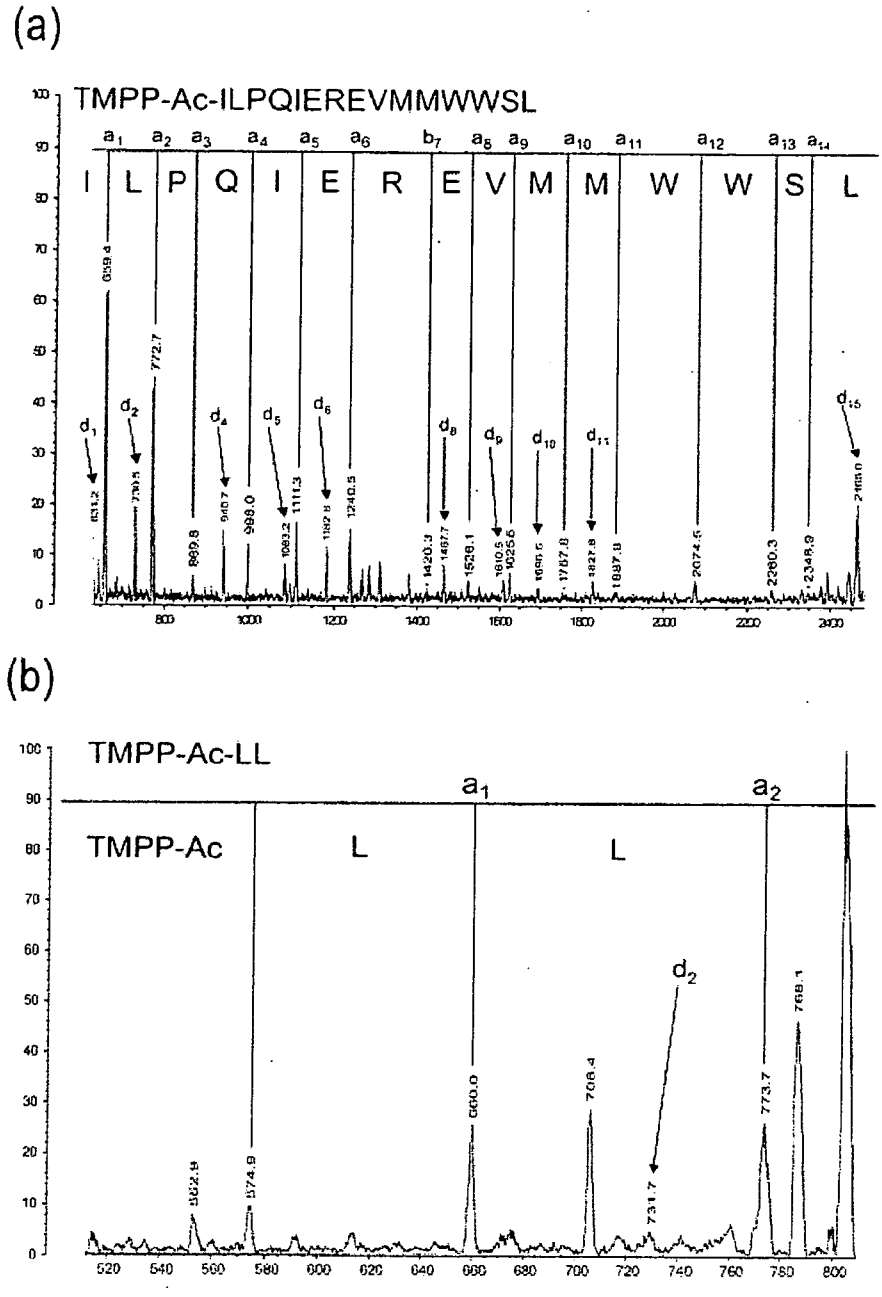

Figure 8. CID spectra of the isolated C-terminal peptide from RPA14 (a) and RPA32 (b).

suppresses random fragmentation of the backbone structure of the peptide. Hence, we reduced the basicity of the arginine residue by modification with acetylacetone (2,4-pentanedione) [22], with which arginine residue was converted to N^{δ}-(4,6-dimethyl-2-pyrimidinyl) ornithine (Pyo) residue. Figure 9 presents the reaction (Fig. 9a) and CID spectrum (Fig. 9b) of the modified C-terminal peptide of RPA41.

The fragmentation efficiency was greatly enhanced to provide sequence information (Fig. 9b). A side reaction is reported to occur at the lysine residue [22]. However, it is not problematic because in our method the isolated C-terminal peptide has no lysine residue. Details of this modification for enhancing fragmentation using TMPP-Ac peptides will be reported elsewhere.

This method thus successfully performed isolation and *de novo* sequencing of the C-terminal peptide, using model peptides/proteins and a recombinant protein, PfuRPA.

A technique using LysC and DITC resin for isolating C-terminal peptide of protein was already mentioned [16]. Comparing our present method with the former technique,

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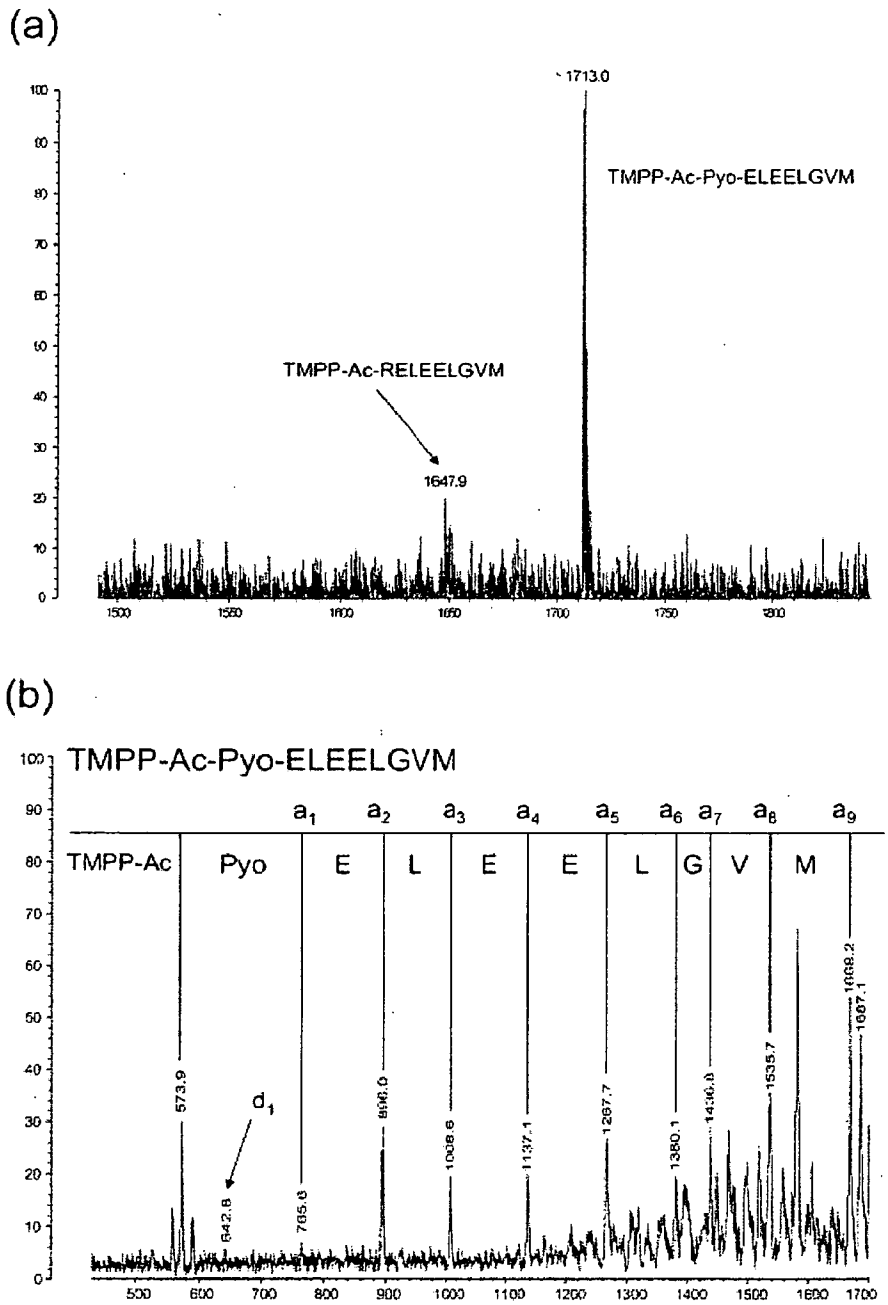

Figure 9. MALDI-TOF mass spectra of the C-terminal peptide of RPA 41. Panel (a) shows the C-terminal peptide of RPA14 after modification with acetylacetone. The modified peptide, TMPP-Ac-Pyo-ELEELGVM is observed along with the unmodified peptide. Panel (b) shows CID spectrum of the arginine modified peptide. The fragmentation is greatly improved to provide sequence infE

advantages lie mainly in that higher sensitivity (10- to 100-fold increase), throughput, and more efficient fragmentation for *de novo* sequencing can be realized by our method because an isolated C-terminal peptide is modified with TMPP-Ac tag. In addition, our method does not use hazardous chemicals such as conc.TFA used in the former method.

Proteolytic fragmentation was carried out with LysC. The length of C-terminal peptide obtained from proteins used in this successful study ranged from 2 to 16 residues. Even if a C-terminal "peptide" is only one amino acid, it can be analyzed because TMPP-Ac moiety is sufficiently great to convert the tagged molecular mass to m/z 647 (glycine)-m/z 776 (tryptophan). However, if a C-terminal peptide is larger than m/z 3000, the *de novo* sequence analysis may be problematic, though such cases are not many. If *de novo* sequencing proves to be difficult, as described above, the sequence of the C-terminal moiety of a protein can easily be estimated using the observed m/z value of the isolated C-terminal peptide,

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since the predicted, full-length sequence of the protein can be obtained by searching a database such as an MS/MS ion search or PMF.

4 Concluding remarks

We have developed a simple and highly successful method to perform C-terminal sequencing of proteins, using a combination of chemical procedures (LysC digestion, modification of α-amino groups with TMPP-Ac-OSu and isolation of the C-terminal peptide using DITC resin) and *de novo* analysis by MALD-TOF-MS. This methodology was used to determine the C-terminal sequences of three model proteins and three subunits of PfuRPA. The method described here is applicable to high-throughput analysis of proteins separated by 2-D SDS-PAGE.

We are grateful to Professor Yoshizumi Ishino (Kyushu Univ.) for providing the recombinant protein of PfuRPA.

The authors have declared no conflict of interest.

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RCM

Sensitive detection of phosphopeptides by matrix-assisted laser desorption/ionization mass spectrometry: use of alkylphosphonic acids as matrix additives

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been one of the most powerful tools for analyzing protein phosphorylation. However, it is frequently difficult to detect phosphopeptides with high sensitivity by MALDI-MS. In our investigation of matrix/matrix-additive substances for improving the phosphopeptide ion response in MALDI-MS, we found that the addition of low-concentration alkylphosphonic acid to the matrix/analyte solution significantly enhanced the signal of phosphopeptides. In this study, the combination of methanediphosphonic acid and 2,5-dihydroxybenzoic acid gave the best results. In addition to enhancing the signal of the phosphopeptides, alkylphosphonic acid almost completely eliminated the signals of sodium and potassium ion adducts. We report herein sensitive detection of phosphopeptides by MALDI-MS with the use of alkylphosphonic acids as matrix additives. Copyright © 2008 John Wiley & Sons, Ltd.

Phosphorylation is one of post-translational modifications in proteins, which is a ubiquitously found biological event in living systems.[1–7] One-third of all eukaryotic proteins are reported to be phosphorylated,[8,9] and phosphorylation plays a pivotal role in a wide range of important signal transduction pathways and other cellular processes such as growth, metabolism, proliferation, motility, interaction, and differentiation in a living cell.[1–7] Hence, protein phosphorylation has been recognized as one of the most relevant post-translational modifications, and localization of phosphorylation sites in the protein sequence is an important goal for understanding regulation mechanisms.

Although the importance has been highly acknowledged, the characterization of the protein phosphorylation sites in biologically derived proteins is still challenging.

Since its introduction more than 20 years ago, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become one of the most powerful tools for analyzing proteins, peptides, oligosaccharides, and so on, because of its high sensitivity, robustness and easy sample preparation. Hence, phosphorylation has frequently been analyzed by MALDI-MS.

However, although as pointed out above MALDI-MS has many advantages, a serious problem still remains which hampers the analysis of phosphopeptides by MALDI-MS. This is because phosphorylated peptides are frequently more difficult to detect and analyze by MALDI-MS than their non-phosphorylated cognates. The difficulty of detecting phosphorylated peptides arises from their low abundance and intrinsic low ionizability. In addition, ionization is often suppressed in the presence of non-phosphopeptides as in the normal proteolytic digest of a phosphoprotein.

To remove impediments to analysis, there have been, in general, three typical workflows for protein phosphorylation analysis, chemical derivatization,[10–13] selective enrichment,[14,15] and the choice of matrix/matrix-additive system.[16–20] Although these three approaches each have their own advantages, the choice of matrix/matrix additive to enhance the response of phosphopeptides in MALDI-MS is a very appealing approach because no chemical or enzymatic treatments are required nor are selective chromatographic methods necessary.

A few years ago, we reported a versatile dephosphorylation procedure using aqueous HF solution or HF-pyridine to detect phosphopeptides in a digestive mixture,[21] and it has since been used by several groups.[22–25] It is a very efficient and robust measure for cleaving the phosphomonoester linkage in phosphopeptides[22,23,25,26] and phosphoproteins,[24] and facilitates the detection of phosphorylated peptides in a digestive mixture. Comparison of the spectra obtained before and after the treatment with aqueous HF solution or HF-pyridine clearly demonstrates the mass shift (−80 Da) due to removal of the phosphate group. However, phosphorylated peptides themselves are sometimes not discernible in a MALDI mass spectrum because of their low concentration and/or their low ionizability.

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One of our goals has been to directly detect phosphorylated peptides by MALDI-MS. So far, there is no universal methodology for the sensitive detection of phosphopeptides. The goal is to analyze phosphopeptides with high sensitivity and resolution, and it is better to do so using a less cumbersome, less time-consuming approach. Therefore, we chose the last approach (the choice of matrix/matrix additive system) from the viewpoint of readiness of operation, and we undertook this study for more efficient detection of phosphopeptides by MALDI-MS.

Based on the finding of the enhanced response of phosphopeptides by the addition of strong acid,[27] Jensen *et al.* reported that phosphoric acid (PA) was proved to be the matrix additive of choice for the sensitive detection of phosphopeptides.[17] PA works well in enhancing the phosphopeptide signal. However, with use of PA as an additive, some phosphopeptides as well as non-phosphopeptides escape detection by MALDI-MS in the negative ion mode.[17]

This drove us to investigate a more efficient and reliable matrix additive for detecting phosphopeptides by MALDI-MS. In a previous report on matrix selection,[28] we found that the structural similarity between the analyte and matrix might be the key for the proper choice of matrix and/or matrix additive. With this in mind, we started investigating organic and inorganic phosphorous acid as a matrix additive for the enhanced detection of phosphopeptides.

EXPERIMENTAL

Materials

Ovalbumin(chicken egg white), α-casein (bovine milk), albumin (bovin serum; BSA), phosphorylase b (rabbit muscle) and lysozyme (chicken egg white) were purchased from Sigma (St. Louis, MO, USA). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). High-purity MALDI-MS grade 2,5-dihydroxybenzoic acid (DHBA) and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Shimadzu GLC (Tokyo, Japan). BPNA, MPNA, SAA, PNAA and EDTA were purchased from Sigma (St. Louis, MO, USA). The abbreviations cited here indicate matrix additive candidates (see Fig. 1). PNA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PA and MLA were obtained from NACALAI TESQUE, Inc. (Kyoto, Japan). MDPNA, EDSA, NTA, NTMPNA and EDTMPNA were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). EHDPNA and EDPNA were purchased from Alfa Aesar (UK). SCA was obtained from Fluka Chemie AG (Switzerland). Peptides (1P, 2P, 3P and 3PPP; see Table 1) were purchased from AnaSpec, Inc. (San Jose, CA, USA). WAGGDASGE and WAGGDApSGE were purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). TSTEPQYQPGENL and TSTEPQpYQPGENL were purchased from BACHEM AG (Switzerland). GFETVPETG-NH_2 and GFETVPEpTG-NH_2 were synthesized in-house using a model PSSM-8 peptide synthesizer (Shimadzu) by the Fmoc strategy. All other chemicals were analytical reagent grade and used without further purification.

Sample preparation

Three matrix-additive candidates (NTA, EDTA, and EDTMPNA) were dissolved with water to yield saturated solutions. PA and NTMPNA were dissolved with water to give 3% and 1% solutions, respectively (v/v). Other matrix-additive candidates were dissolved in water to 1% (w/w). The matrix solution was prepared by dissolving 5 mg of DHBA in 0.5 mL of 50% aqueous acetonitrile. CHCA solution, saturated in the same solution system as in DHBA, was used as a matrix for the external calibration.

Model peptides were dissolved in water to the concentrations used.

Tryptic digestion was performed in 50 mM Tris-HCl (pH 7.8), 5 mM $CaCl_2$ at an enzyme-to-substrate ratio of 1:20 (w/w) at 37°C overnight. The resulting digests were used with an appropriate dilution.

MALDI-MS

An AXIMA-CFR plus mass spectrometer (Shimadzu/Kratos, Manchester, UK) was used to obtain all MALDI-TOF mass spectra. The operating conditions were as follows: nitrogen laser (337 nm); reflectron mode; positive or negative mode. The accelerating voltage in the ion source was 20 kV. A standard stainless steel target plate was used for the analysis. A portion (0.3 μL) of each analyte, matrix, and matrix-additive solution was mixed on the target plate and analyzed after drying. The *m/z* values in the spectra were externally calibrated with angiotensin II (human) and ACTH fragment 18–39 (human) using CHCA as a matrix. All measurements were repeated in at least three independent experiments.

RESULTS AND DISCUSSION

Screening of matrix additives

We used 16 different compounds varying from inorganic to organic acids for screening, as summarized in Fig. 1. To evaluate these compounds as matrix additives, a peptide mixture containing four different phosphopeptides in equimolar amount (final concentration of each peptide was 0.1 pmol/μL) was prepared. The contents were three monophosphorylated peptides, and one triphosphorylated peptide, ranging from *m/z* 1438 to 1880, as listed in Table 1.

We used DHBA, which is known to be a 'cool' matrix, as a matrix in this study. The formed ions remain intact during MALDI mass analysis because they have low internal energy when using DHBA as a matrix. Therefore, DHBA is frequently used for phosphopeptide analysis in MALDI-MS.

The signal intensities of these peptides are listed in Table 2 which were measured by MALDI-MS using DHBA as a matrix with or without the candidate compound as an additive. In this data acquisition, the laser fluence was set identical except for PA and PNA. When using PA or PNA as an additive, higher laser fluence was needed to produce molecular ions.

PA has an enhancing effect for phosphopeptides, and its cognate phosphonic acid (PNA) is comparably effective. Among acidic groups of COOH, SO_3H, and $P(=O)(OH)_2$, the phosphonic acid group is apparently responsible for the enhancement as determined from the comparisons in the group of (MLA, SAA, PAA) and (SCA, EDSA, EDPNA). This

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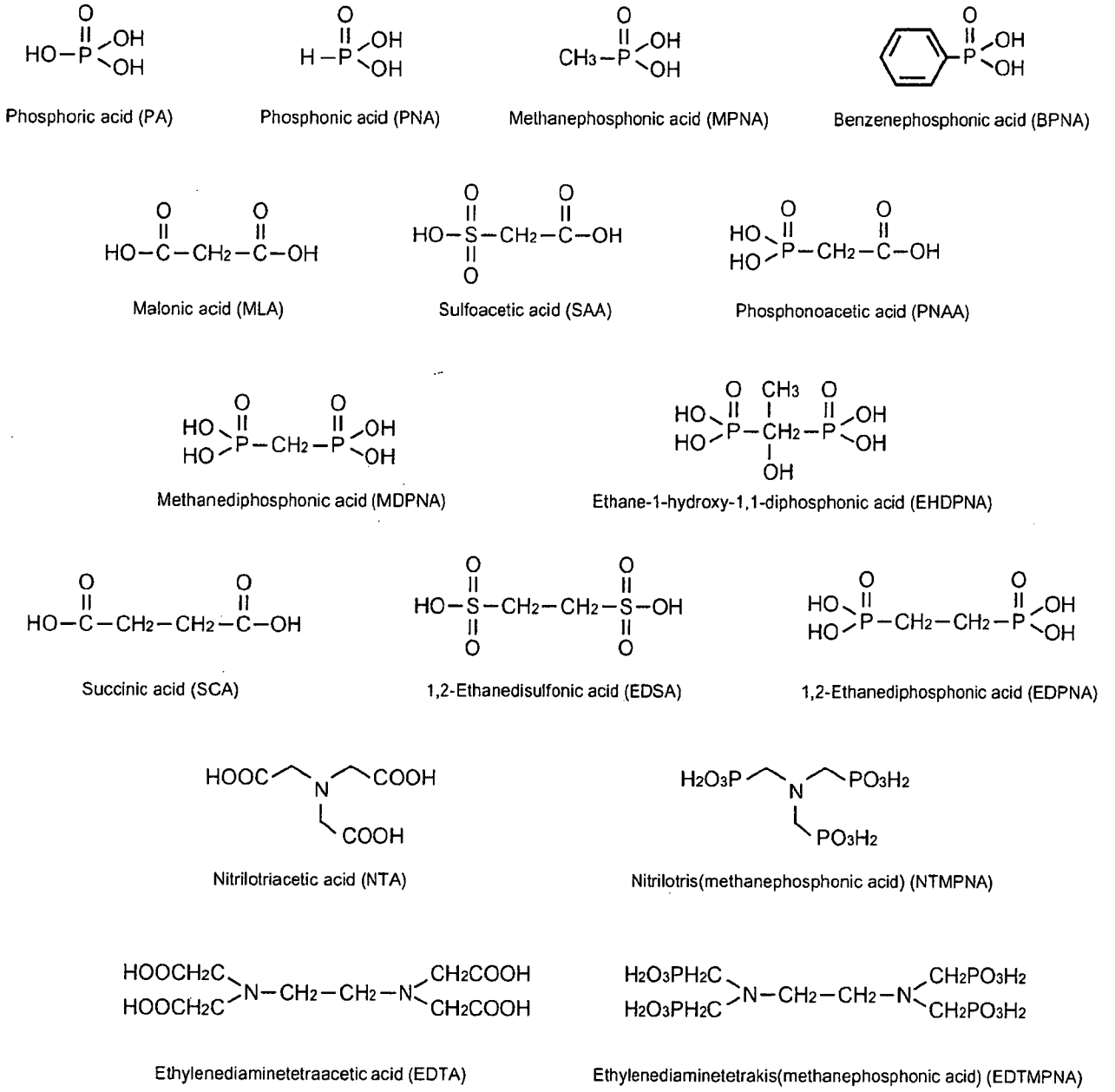

Figure 1. Structures and abbreviations of candidate acids in this study.

might be true for the cases of NTMPNA and EDTMPNA, from the comparison with NTA and EDTA. Multiplicity of the acidic group in one molecule dramatically improves the enhancement (from the data of MPNA, MDPNA, EDPNA and so on). The data for PA and the most promising candidate, MDPNA, indicate that MDPNA addition produces a 5-fold to 34-fold increase in signal enhancement whereas PA addition exhibits a 1-fold to 8.4-fold increase, compared with no addition of matrix additive (DHBA only). As a whole, it is apparent that the phosphonic acid moiety has an enhancing effect and that a molecule incorporating 2 to 4 of the phosphonic acid groups enhances the phosphopeptide signal in MALDI-MS. Among candidates incorporating multiple phosphonic acid groups, MDPNA is the most effective in enhancing the phosphopeptide signal in MALDI-MS.

In the negative mode, the data exhibit a similar tendency in that candidates incorporating 2 to 4 phosphonic acid groups are effective in enhancing the signal intensity of phosphopeptide ions by MALDI-MS, although the enhancing effect is not as high as in the positive mode. MDPNA is the most effective in enhancing the phosphopeptide signal, although the difference between PA and MDPNA becomes smaller.

Among the candidate compounds tested, MDPNA enhances the phosphopeptide signal by MALDI-MS the most in both positive and negative modes. Hence, MDPNA was used as an additive to DHBA for the subsequent experiments.

To optimize the concentration of the MDPNA solution, we tested several points of the concentrations ranging from 0.01% to 10%. At a concentration less than 1% or more than 5%, mass spectra indicated deteriorated signal-to-noise

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Table 1. Phosphopeptides studied in this report*

Peptide ID	$[M+H]^+$	Sequence
1P	1438.6	MHRQEpTVDCLK-NH_2
2P	1880.2	AAKIQApSFRGHMARKK
3P	1702.8	TRDIpYETDYYRK
3PPP	1862.8	TRDIpYETDpYpYRK
OP1	2088.9	EVVGpSAEAGVDAASVSEEFR
OP2	2511.1	LPGFGDpSIEAQCGTSVNVHSSLR
OP3	2901.3	FDKLPGFGDpSIEAQCGTSVNVHSSLR
CP1	769.4	VNELpSK
CP2	1660.8	VPQLEIVPNpSAEER
CP3	1927.7	DIGpSEpSTEDQAMEDIK
CP4	1952.0	YKVPQLEIVPNpSAEER
CP5	2720.9	QMEAEpSIpSpSpSEEIVPNpSVEQK

*Commercially available phosphopeptides are denoted with 1P-3PPP, in which the number of P's indicates the degree of phosphorylation. Phosphopeptides from ovalbumin are presented with OP1-OP3, which are all monophosphorylated peptides. Phosphopeptides from α-casein are presented with CP1–CP5, of which CP-1, CP2 and CP4 are monophosphorylated, CP3 is diphosphorylated, and CP5 is pentaphosphorylated.

(S/N) ratio (data not shown). However, at a concentration in the 1–5% range, optimized effect was obtained in mass spectra. Hence we chose the lowest concentration in the range, 1%, for the matrix-additive solution.

Effect of MDPNA in eliminating alkali-metal adduct ions

Several additives such as phosphoric acid,[17] diammonium citrate,[16] etc., are reported to reduce or eliminate the signal of the alkali-metal adduct ion as well as to enhance phosphopeptide signals by MALDI-MS. Suppressing adduct formation is of great benefit for MALDI analysis. The signal enhancement of the peptide ion $[M+H]^+$ is supplemented by the reduction or elimination of alkali-metal ion adducts such as $[M+Na]^+$ and $[M+K]^+$. It was reported that these alkali metals may be captured by the additives, which is attributed to the ability of additives to form alkali-metal salts of acids such as carboxylic acid or phosphoric acid ('salting out').[17]

To test the effect of MDPNA, a peptide mixture solution, containing WAGGDASGE ($[M+H]^+$; 849.3), WAGGDApSGE ($[M+H]^+$; 929.3),GFETVPETG-NH_2 ($[M+H]^+$; 935.4), GFETVPEpTG-NH_2 ($[M+H]^+$; 1015.4), TSTEPQYQPGENL ($[M+H]^+$; 1463.6), TSTEPQpYQPGENL ($[M+H]^+$; 1543.6), with a concentration of 1.7 pmol/μL each was prepared.

Figure 2 depicts the elimination effect of alkali-metal adduct signal using MDPNA as an additive to the DHBA matrix. Adduct signals accompany these five peptide signals except for the signal of GFETVPEpTG-NH_2 (m/z 1014.4)

Table 2. Typical signal intensities as well as S/N ratios of the four phosphopeptides (1P, 2P, 3P, and 3PPP) with or without the addition of inorganic/organic acid as an additive to DHBA matrix. All experiments were replicated five times. The signal intensities are averaged of five replicated data, and are relative to those with no additive (DHBA only)

		1P		2P		3P		3PPP	
Comatrix	Ion mode	Signal	S/N	Signal	S/N	Signal	S/N	Signal	S/N
DHBA only	Positive	1.0	56	1.0	93	1.0	16	1.0	38
PA	Positive	1.2	62	3.1	243	8.4	113	1.5	48
PNA	Positive	3.1	42	6.1	200	4.6	84	1.5	18
MPNA	Positive	0.3	65	0.6	179	0.9	124	0.3	72
BPNA	Positive	—	—	—	—	0.1	5	—	—
MLA	Positive	0.2	21	0.3	62	0.3	9	0.1	8
SAA	Positive	1.1	62	1.7	149	6.3	141	1.3	61
PNAA	Positive	3.0	88	5.9	209	12.1	127	2.9	72
MDPNA	Positive	5.4	95	8.3	237	34.2	172	6.7	79
EHDPNA	Positive	1.3	82	2.7	201	10.5	177	2.1	70
SCA	Positive	0.8	28	1.1	65	1.1	11	0.6	14
EDSA	Positive	0.7	68	1.0	167	3.9	111	0.3	19
EDPNA	Positive	3.8	89	7.0	210	24.7	166	3.6	64
NTA	Positive	1.4	76	1.9	167	4.2	64	1.8	66
NTMPNA	Positive	4.2	83	6.9	211	21.6	125	6.2	71
EDTA	Positive	1.2	61	1.9	161	2.4	35	1.9	66
EDTMPNA	Positive	2.1	71	4.0	214	6.8	64	2.2	38
DHBA only	Negative	1.0	57	1.0	62	1.0	23	1.0	12
PA	Negative	1.0	39	3.7	86	7.2	118	1.7	13
PNA	Negative	0.6	25	1.6	68	3.0	52	1.2	10
MPNA	Negative	0.6	44	1.0	73	1.1	33	1.3	19
BPNA	Negative	—	—	—	—	0.1	1	—	—
MLA	Negative	0.1	7	0.2	10	0.2	4	0.1	1
SAA	Negative	0.1	7	0.1	6	0.9	27	—	—
PNAA	Negative	0.7	42	1.7	90	2.8	73	0.9	12
MDPNA	Negative	3.2	76	3.7	87	15.4	140	5.7	26
EHDPNA	Negative	1.8	74	2.2	86	8.8	134	2.2	17
SCA	Negative	0.3	13	0.4	17	0.4	6	0.5	4
EDSA	Negative	—	—	—	—	—	—	—	—
EDPNA	Negative	2.7	62	3.6	78	14.5	144	3.7	16
NTA	Negative	0.6	43	1.0	73	1.3	38	1.2	18
NTMPNA	Negative	2.1	86	2.6	81	8.8	136	4.4	35
EDTA	Negative	1.3	25	2.4	51	2.1	17	4.4	20
EDTMPNA	Negative	1.6	51	2.2	63	3.5	40	2.0	12

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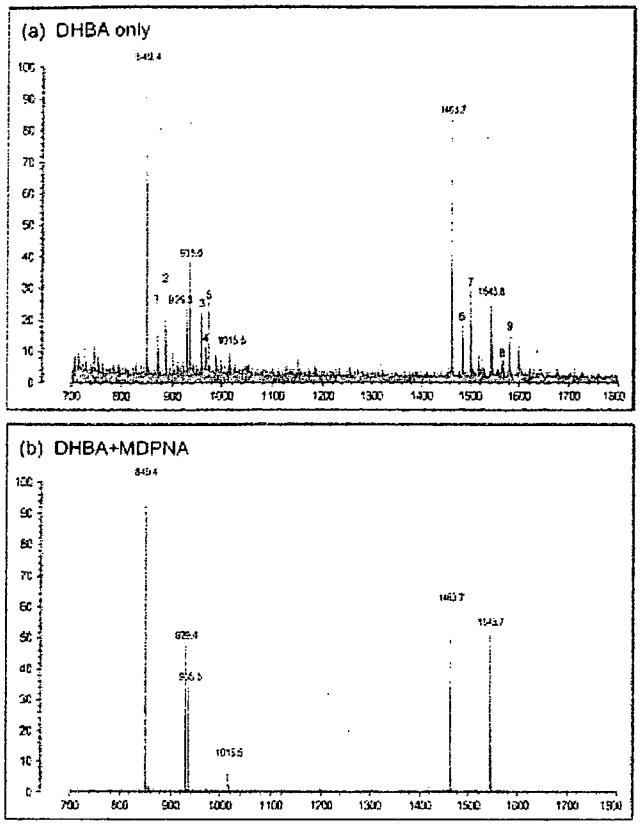

Figure 2. Effect of MDPNA addition in eliminating alkali-metal adduct ions. Intensive adduct formation is observed, except GFETVPEpTG-NH_2 (*m/z* 1014.4), with nothing added to the DHBA matrix (a). However, no adduct signal is observed when MDPNA is added to the DHBA matrix (b). The adduct signals (1–9) in (a) are as follows; 1. *m/z* 871.4 (848.3+Na), 2. *m/z* 887.4 (848.3+K), 3. *m/z* 957.5 (934.4+Na), 4. *m/z* 967.3 (928.3+Na), 5. *m/z* 973.4 (934.4+K), 6. *m/z* 1485.7 (1462.6+Na), 7. *m/z* 1501.6 (1462.6+K), 8. *m/z* 1565.6 (1542.6+Na), 9. *m/z* 1581.6 (1542.6+K).

without addition of MDPNA (Fig. 2(a)). The adduct signals indicated with numbers (1–9) in Fig. 2(a) completely disappeared after addition of MDPNA (Fig. 2(b)).

In this study, significant improvement of sample homogeneity was observed when adding MDPNA to DHBA (data not shown). The quality improvement of the spectrum, as can be seen in Figs. 2(a) and 2(b), may be partly attributed to the reduction of the inhomogeneity causing hot-spot formation.

Efficient detection of phosphopeptides using MDPNA

It was mentioned that fewer peptide ions were produced in negative MALDI-MS mode in the low *m/z* region.[17] In our study, we observed that peptide signals with or without phosphorylation sometimes escaped detection even in positive mode when using PA as an additive. However, this phenomenon was not observed with the use of MDPNA. Figure 3 graphs the spectra of a tryptic digest of α-casein as an example in the range of *m/z* 600–1000; arrows in the spectrum (Fig. 3(b)) indicate the non-detected signals when

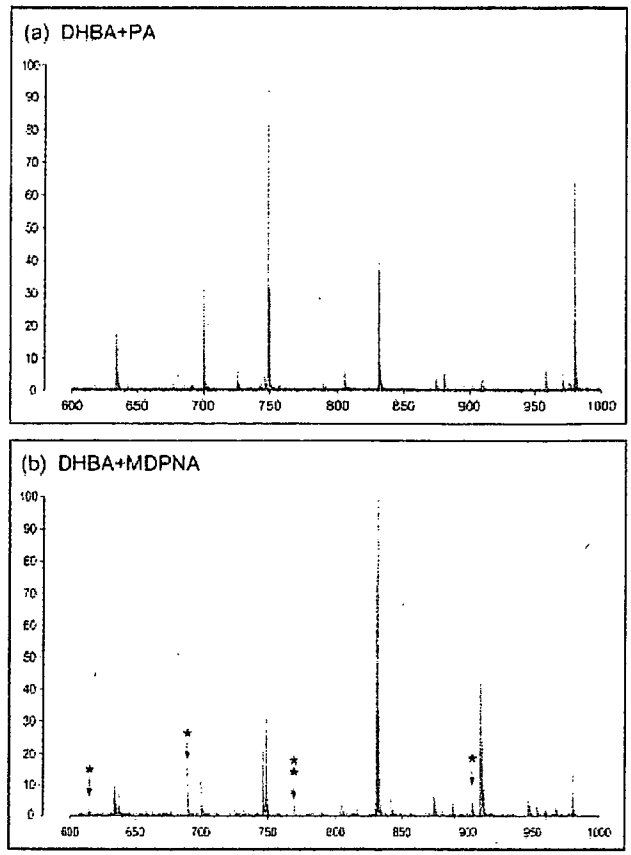

Figure 3. Efficient detectability of phosphopeptides using MDPNA as an additive to DHBA matrix. (a, b) MALDI mass spectra of α-casein digests. Signals that escaped detection using PA as an additive (a) are discernible when using MDPNA as an additive (b).

using PA (Fig. 3(a)). The phosphorylated peptide signal is indicated with a double asterisk; a single asterisk indicates the non-phosphorylated peptide signal.

Phosphopeptide mapping by MALDI-MS

To investigate the general applicability of MDPNA to phosphopeptide analysis by MALDI-MS, we attempted to analyze phosphopeptide fragments contained in a digest of a model phosphoprotein, ovalbumin, and a four-protein mixture (BSA, phosphorylase b, lysozyme, and α-casein).

1. Phosphopeptide mapping of ovalbumin

The tryptic digestion of ovalbumin generates three phosphopeptides (OP1, OP2, and OP3; see Table 1) as well as numerous non-phosphorylated peptides up to *m/z* 3000. Figure 4 presents six panels of mass spectra in three sample preparation conditions with addition of no acid (a, d), PA (b, e), and MDPNA(c, f) and with two sampling amount of ovalbumin digest (30 fmol/well for a,b, and c; and 3 fmol/well for d, e, and f). The phosphopeptide OP1 was observed in the three conditions employed at 30 fmol/well, though the signal from the phosphopeptide was rather low (a, b). The ion signals of OP2 and OP3 were, at 30 fmol/well sampling, not detected under the conditions of no acid (a) (DHBA only) and PA (b). However, these two signals as well as the OP1

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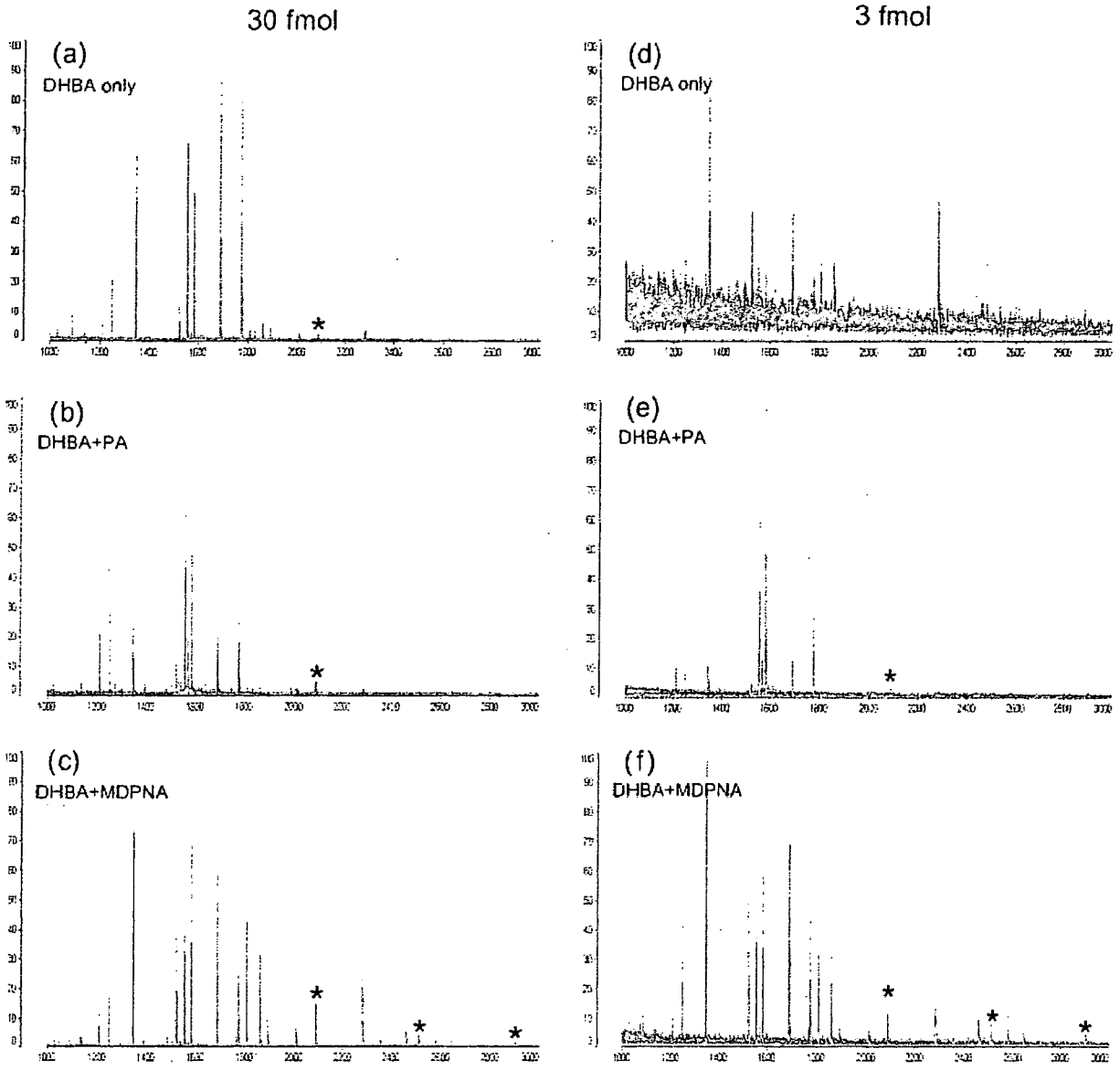

Figure 4. MALDI mass spectra of tryptic digest of ovalbumin. The tryptic digest was diluted with water to adjust the concentrations to 90 fmol/μL or 9 fmol/μL, from which a portion (0.3 μL) was applied to a target plate with or without an additive: no additive (a, d), PA as an additive (b, e), MDPNA as an additive (c, f). Signals with an asterisk represent phosphopeptides of OP1 ($[M+H]^+$: 2088.9), OP2 ($[M+H]^+$: 2511.1), and OP3 ($[M+H]^+$: 2901.3).

signal were clearly detected when MDPNA was used as a matrix additive (c). At 3 fmol/well, no signals from these phosphopeptides were detected with use of DHBA only (d), and one signal from the OP1 phosphopeptide was observed, although OP2 and OP3 escaped detection using PA (e). However, using MDPNA as an additive to DHBA, these three phosphopeptides were clearly observed even at 3 fmol/well sampling (f).

2. *Phosphopeptide mapping of a four-protein mixture*

We next tried a four-protein mixture containing BSA, phosphorylase b, lysozyme, and α-casein. Of the four proteins α-casein is a typical phosphoprotein, from which phosphopeptides generated after proteolysis were analyzed. For testing MDPNA as a matrix additive, two types of solution were prepared; (1) 2.1 ng/μL for BSA, phosphorylase b, lysozyme, and α-casein; (2) 2.1 ng/μL for BSA, phosphorylase b and lysozyme, and 0.21 ng/μL for α-casein. In the solution of (1), the concentration of α-casein was 90 fmol/μL and in (2), 9 fmol/μL. For MALDI-MS analysis 0.3 μL of sample solution was applied onto a MALDI target plate. Hence, the sampling amounts of α-casein were 30 fmol/well and 3 fmol/well, respectively. Five phosphorylated peptides (CP1, CP2, CP3, CP4 and CP5; see Table 1) were generated after tryptic digestion along with non-phosphopeptides up to *m/z* 3000. Figure 5 presents six panels of mass spectra in three sample preparation conditions, as in Fig. 4, with addition of no additive (a, d), PA (b, e), and MDPNA (c, f) for the two model solutions. At 30 fmol/well of α-casein, all of five phosphopeptides were observed when using MDPNA as an additive (c); however, CP1 was not detected when using PA (b), and CP1 and CP3 were missing

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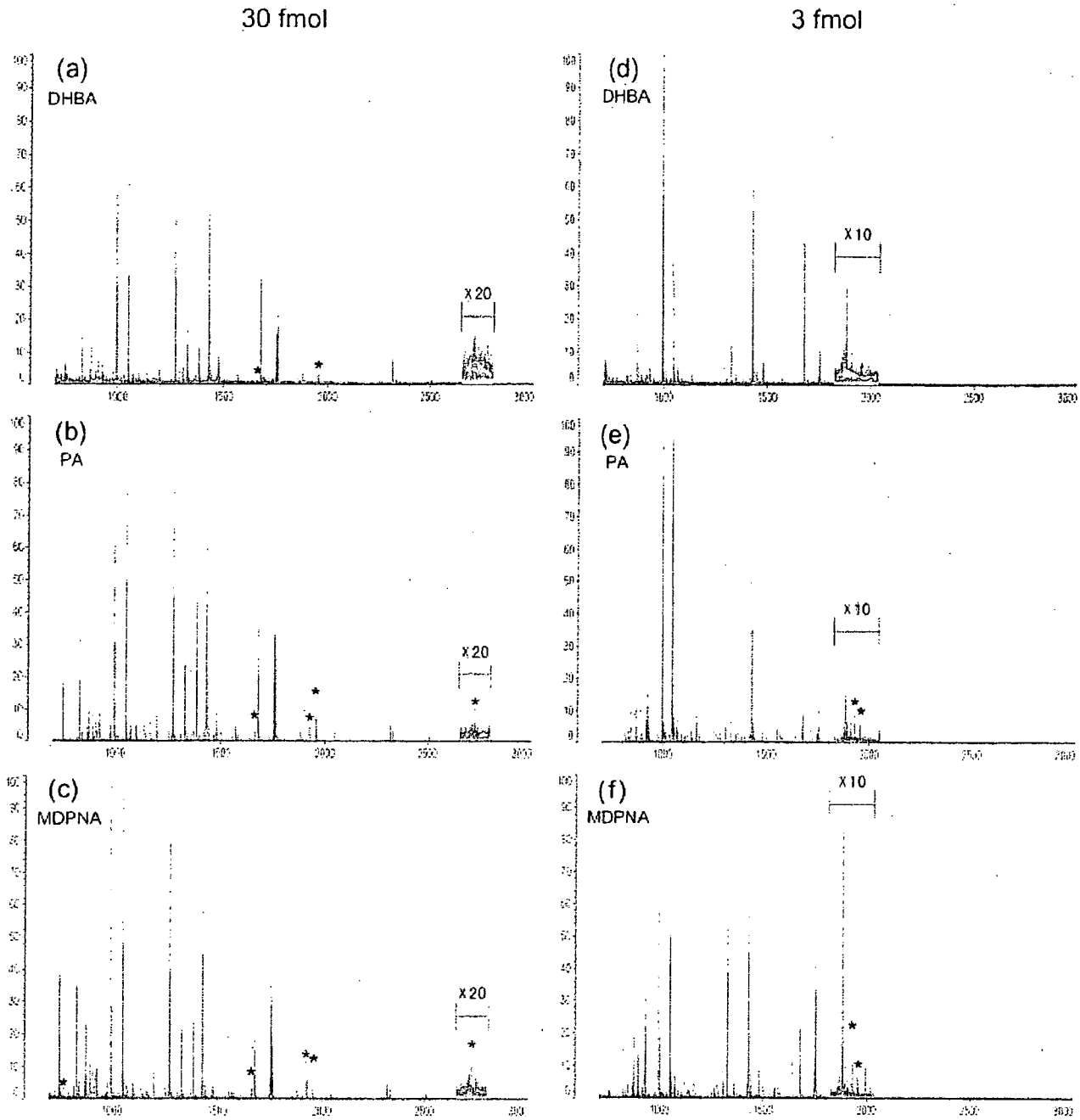

Figure 5. MALDI mass spectra of tryptic digest of the four-protein mixture. Two model mixtures were prepared that contained α-casein as a component at 90 fmol/μL and 9 fmol/μL, from which a portion (0.3 μL) was applied to a target plate with or without an additive: no additive (a, d), PA as an additive (b, e), MDPNA as an additive (c, f). Signals with an asterisk represent phosphopeptides of CP1 ($[M+H]^+$: 769.4), CP2 ($[M+H]^+$: 1660.8), CP3 ($[M+H]^+$: 1927.7), CP4 ($[M+H]^+$: 1952.0) and CP5 ($[M+H]^+$: 2720.9).

without an additive (a). At 3 fmol/well of α-casein, no signals of these five phosphorylated peptides were detected when using DHBA only (d). CP1, CP2 and CP5 were not observed even with PA or MDPNA addition. Though CP3 and CP4 were observed in both (e) and (f), the S/N ratios of the two signals were better with MDPNA addition (f). In this experiment using a four-protein mixture, the enhancement efficiency of MDPNA was clearly demonstrated in comparison with no additive (DHBA only).

These results indicated that adding MDPNA to DHBA matrix significantly enhances detection of ovalbumin and α-casein phosphopeptides by MALDI-MS.

When DHBA is used as a matrix, an intrinsic problem is pronounced hot-spot formation due to inhomogeneous sample preparation, which causes prolonged measurement times and is unfavorable for automated data acquisition. The use of MDPNA and other compounds incorporating multiple phosphonic acid moieties improves sample

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homogeneity after crystallization, which significantly alleviates hot-spot formation. This increases measurement quality and applicability to automated data acquisition.

In this study, we did not use a pre-treatment process such as Ziptip (Millipore) desalting for MALDI-MS measurement because an analyte with rather high hydrophilicity (e.g., phosphopeptide) may be lost in the desalting step, which leads to a failure to detect the analyte. Therefore, we used a tryptic digest of sample proteins directly for MALDI-MS analysis. A sample solution processed from in-gel digestion can also be used without pre-treatment for MALDI-MS analysis (data not shown).

MDPNA addition, as seen from Fig. 4, seems to have enhancing effect on signals of both phosphopeptides and non-phosphopeptides. It has been discussed[29] from several viewpoints of solubilization, crystallization, protonation and so on that the increase in sensitivity is not limited to the peptides of interest when using ammonium salts as additives. However, the role of the additive in improving the signal is not understood yet. The detailed investigation of how MDPNA serves as a 'good' matrix additive for sensitive detection of phosphopeptides by MALDI-MS is outside the scope of this paper and is not addressed here. It can only be suggested to date from the data in this report that relative intensities of phosphopeptide signals increase upon addition of the additive.

The results obtained in this study indicate the robustness and versatility of MDPNA as an additive that is suitable for practical use.

CONCLUSIONS

Adding MDPNA to the DHBA matrix enhanced ion formation and improved the S/N ratio in both positive and negative mode MALDI analysis of phosphopeptides. In addition, signals of alkali-metal ion adducts $[M+Na]^+$ and $[M+K]^+$ were eliminated. Furthermore, the phenomenon of producing fewer peptide ions, in particular in the low-mass region, was not observed with MDPNA added to the DHBA matrix, which leads to improved detectability of phosphopeptides as well as non-phosphorylated peptides. Although the mechanism(s) for the enhancement of phosphopeptide signals by MALDI-MS with MDPNA addition remains to be investigated, the use of MDPNA as an additive to the DHBA matrix is useful for MALDI analysis of phosphopeptides in both positive and negative modes.

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Direct on-membrane peptide mass fingerprinting with MALDI–MS of tyrosine-phosphorylated proteins detected by immunostaining☆

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Abstract

We have identified tyrosine-phosphorylated proteins on membrane from A-431 human epidermoid carcinoma cells by using detection with anti-phosphotyrosine antibody followed by PMF analysis. In there, on-membrane digestion for these protein spots was carried out on microscale region using chemical inkjet technology and the resulting tryptic digests were directly analyzed by MALDI–TOF MS. Proteins identified by a database search included phosphoproteins that are known to be markedly phosphorylated on tyrosine sites after the cells are treated with epidermal growth factor (EGF). This procedure is a rapid and easily handled approach that enables both detection and identification of phosphoproteins on a single blot membrane.
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Keywords: Phosphorylation; Immunodetection; On-membrane digestion; Protein identification; MALDI–TOF MS

1. Introduction

Post-translational modifications are known to play a significant role in many biological processes, such as signal transduction-mediated cellular events. A number of proteins in all cells become post-translationally modified with a variety of functional groups, which include phosphate, glycan, lipid, sulfate, nitric oxide and ubiquitin, etc. In particular, protein phosphorylation, one of the post-translational modifications, plays a crucial role in eukaryotic signal transduction, DNA transcription, protein synthesis, cell cycle progression and cell metabolism [1,2]. Since the discovery that reversible phosphorylation regulates the activity of glycogen phosphorylase, a wide variety of phosphorylation cascades regulated by protein kinases and phosphatases have been characterized in which specific serine, threonine and tyrosine residues in proteins become phosphorylated or dephosphorylated [3]. The approach to rapidly detect and identify phosphoproteins has important implications because of the crucial role of protein phosphorylation in biological processes. Currently, there are several approaches for detecting phosphoproteins, such as traditional metabolic labeling using the radioactive isotope ^{32}P, chemical modification of phosphate groups followed by MS analysis, and immunostaining or immunoprecipitation using specific phosphoserine, phosphothreonine or phosphotyrosine antibodies [4]. In current proteomics, mass spectrometry is widely used for identification of detected phosphoproteins [5]. Several previous reports have described the use of mass spectrometric analysis to identify proteins detected by Western blotting. These approaches require dual separations of the same sample on gels, or a step in which proteolytic peptides are extracted from the membrane used for immunodetection before MS analysis [6,7].

In this study we carry out direct identification of phosphoproteins from A-431 human epidermoid carcinoma cells, detecting them using an anti-phosphotyrosine antibody and carrying out on-membrane digestion using piezoelectric chemical inkjet printing in combination with MALDI–TOF MS. In general, in order to identify protein by mass spectrometry after detecting them by Western blotting, it is necessary to carry out a

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time-consuming operation such as extraction of digested peptides from the blot membrane, or preparation of an alternative gel to separate the proteins by 2-dimensional electrophoresis (2-DE) for in-gel digestion. However, this approach utilizes piezoelectric technology, and direct on-membrane analysis enables us to omit the above time-consuming operations and to rapidly identify the proteins visualized with Direct Blue 71 after immunostaining [8]. Furthermore, the microdispensing function of reagents using piezoelectric inkjet technology could be used to improve on-membrane PMF analysis in the microscale region of the protein spots without cross-contamination between proximate proteins [9]. Reagents at sub-nanoliter volume levels can be microdispensed, allowing digestion of only a tiny region within a protein spot. As a result, we could identify proteins from A-431 human epidermoid carcinoma cells by this method. The proteins identified in this manner included proteins, known to be increasingly phosphorylated on specific tyrosine sites after EGF treatment of A-431 cells. Thus, this procedure has the potential to become a powerful tool for direct identification of phosphoproteins on membrane.

2. Experimental

2.1. Materials

Phospho-enriched whole cell lysates, A-431/PE and A-431 + EGF/PE were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylpyrrolidone (PVP-40), Direct Blue 71 and 2,5-dihydroxy benzoic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Trypsin was obtained from Promega (Madison, WI, USA) and the Immobilon-FL PVDF membrane was purchased from Millipore (Bedford, MA, USA). Phospho-tyrosine mouse mAB (P-Tyr-100) was obtained from Cell Signaling Technology (Danvers, MA, USA) and rabbit anti-phosphoserine was obtained from Zymed Laboratories Inc. (S. San Francisco, CA, USA). Alexa Fluor 633 goat anti-mouse IgG (H + L) and Texus Red goat anti-rabbit IgG (H + L) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Instruments

Direct analyses on the PVDF membrane was performed using a MALDI–TOF MS instrument, AXIMA-CFR plus (Shimadzu Corporation, Kyoto, Japan and Kratos Analytical, Manchester, UK), that was operated in a positive ion mode by using an internal calibration method with trypsin autodigests (m/z = 842.51, 2211.10). For on-membrane digestion, the chemical inkjet printer (Shimadzu Corporation, Kyoto, Japan) was used for microdispensing the reagents onto blotted protein spots as we have previously reported [10].

2.3. Preparation of 2-DE blotted membranes using A-431 human epidermoid carcinoma cells lysates

Phospho-enriched whole cell lysates, A-431/PE and A-431 + EGF/PE (200 μg) were purchased from Santa Cruz Biotechnology. The proteins from the cell lysates were recovered by TCA precipitation and then dissolved in 200 μL Protein Extraction Reagent Type 3 solution (from ProteoPrep Sample Extraction Kit, Sigma). Proteins from the cell lysates of A-431 and A-431 + EGF were prepared according to the ProteoPrep sample extraction kit protocol. Solubilized proteins were reduced with 5 mM tributylphosphine for 60 min at room temperature and then alkylated with 15 mM iodoacetamide for 90 min at room temperature. Pharmalyte (pH 3–10) was added to a final concentration of 0.2% and a trace of bromophenol blue (BPB) was also added. The protein solution was centrifuged at 15,000 × g for 20 min at 20 °C and the supernatant was used for rehydration of IPG strips. Amersham IPG strips (pH 3–10, 13 cm) were rehydrated for 8 h with the prepared sample solution (200 μL) and focused on a Protean IEF Cell apparatus (Bio-Rad, Hercules, CA, USA) for 100 kV/h at a maximum of 8 kV. The focused IPG strips were equilibrated for 10 min with equilibration buffer, and SDS-PAGE (10–20%) was then performed for these strips. The proteins separated by 2-dimensional electrophoresis (2-DE) were blotted onto the Immobilon-FL membrane by the semi-dry electroblotting method. Blotting was performed at constant 200 mA for 40 min using three type of blotting buffer (A: 0.3 M Tris, 20% methanol, 0.02% SDS, B: 25 mM Tris, 20% methanol, 0.02% SDS, C: 25 mM Tris, 40 mM ε-amino-n-hexanoic acid, 20% methanol, 0.02% SDS) [11]. The membrane was air-dried after rinsing it with water.

2.4. Immunostaining with an anti-phosphotyrosine and an anti-phosphoserine antibodies

Immunostaining with anti-phosphotyrosine and anti-phosphoserine antibodies was performed according to rapid immunodetection, which has an advantage in that blocking is not required, thus saving time [12]. The blot must be thoroughly dry before beginning rapid immunodetection immunostaining and the following step can be carried out. Dried PVDF membranes were rewetted by dipping into 100% methanol for 10 s and then were air-dried on a filter paper for 15 min. Subsequently, the blot was dried in a vacuum chamber for 30 min. The blot was incubated at 37 °C for 30 min and then was air-dried for 2 h. Primary and secondary antibodies were diluted 1:2000 and 1:5000 for phosphoserine, 1:5000 and 1:5000 for phosphotyrosine, respectively, with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.25% PVP-40. The blot was incubated with each antibody at a ratio of 0.09 ml/cm^2 of membrane surface area for 30 min. The blot was then washed in plastic containers using TBS at a ratio of 0.9 ml/cm^2 of membrane surface area for 5 min twice. After rinsing with water, a fluorescent image of the blot was acquired using the FLA-5000 analyzer (Fujifilm, Tokyo, Japan).

2.5. Direct identification of proteins detected with an anti-phosphotyrosine antibody on membrane

Antibodies on the membrane were removed by washing it in 0.2 M glycine–HCl (pH 2.0) after acquiring the fluorescent image. After washing for 15 min three times, the blot membrane was rinsed with water and then dried at room temperature. The

membrane was stained with 0.008% (w/v) Direct Blue 71 in 40% ethanol, 10% acetic acid for 7 min and washed with 40% ethanol, 10% acetic acid for 5 min twice [8]. After rinsing with water, the blot membrane was subjected to microscale on-membrane digestion using the chemical inkjet printer. Firstly, the blot membrane was adhered to the stainless steel plates for MS analysis using 3 M™ electrically conductive tape 9713 (St. Paul, MN). A visualized image of the adhered blot was acquired with a scanner of the chemical inkjet printer, and the target protein spots were selected on the basis of the scanned images. Subsequently, the reagents for on-membrane digestion were printed onto microscale region of the protein spots [9]. Ten nanoliters of 0.1% (w/v) PVP solution in 60% MeOH was printed to pre-wet the membrane and then 50 nl of trypsin at 40 μg/ml in 10 mM NH_4HCO_3 containing 10% (v/v) 2-propanol was microdispensed to each target position. On-membrane digestion was performed for 16 h at 30 °C in a humidified chamber. After digestion, 100 nl of 5 mg/ml 2,5-DHB in 0.1% trifluoroacetic acid (TFA) containing 25% (v/v) acetonitrile was printed onto each position on the membrane. Then the blot was subjected to the AXIMA-CFR plus instrument for on-membrane MS analysis. Positional information for the printed region was transferred to the mass spectrometer as an output file from the chemical inkjet printer, and MS analysis was performed for the digested region on the basis of this information. On the basis of the obtained MS spectrum, a database search was conducted using the Sprot database with the aid of Mascot software (Matrix Science, MA), which was set at a tolerance of 0.3 Da for the MS analysis, and at one missed cleavage site as fixed parameters.

3. Results and discussion

3.1. Immunostaining of A-431 cell lysates with an anti-phosphotyrosine antibody

Enriched phosphoproteins from A-431 lysates incubated in the presence and absence of EGF were separated with 2-DE and blotted onto a PVDF membrane. The blot membrane was analyzed using a modified rapid immunodetection approach with an anti-phosphotyrosine antibody [10]. Fig. 1(b) shows the result of immunodetection for proteins containing phosphotyrosines from A-431 cell lysates. After removing the antibodies by washing with 0.2 M glycine–HCl (pH 2.0), the blot membrane was visualized with Direct Blue 71. The stained image is shown in Fig. 1(a). The results, which are shown in Fig. 1(b) indicate the presence of phosphotyrosine on a number of proteins and also suggest that phosphorylation of a number of proteins on tyrosine residues is remarkably induced in A-431 cells treated with EGF. In general, it is known that the binding of EGF to EGF receptors results in receptor dimerization, autophosphorylation and activation of various downstream phosphorylation cascades, especially tyrosine kinase activation [13]. Furthermore, the human epidermoid carcinoma cell line A-431 has already been shown to contain an extraordinarily high concentration of membrane receptors for EGF [14]. The results shown in Fig. 1(b) indicate that a number of specific tyrosine sites in A-431 cells are phosphorylated by EGF-treatment.

3.2. Immunostaining of A-431 cell lysates with an anti-phosphoserine antibody

Phosphoproteins from A-431 cells were subsequently detected by Western blotting with an anti-phosphoserine antibody. Fig. 2(b) shows the result of immunostaining. The blot membrane stained with Direct Blue 71 is shown in Fig. 2(a). Protein spots, especially some proteins having a molecular weight of approximately 35–90 kDa, that were observed with an intense signal in Fig. 1(b) could not be detected with anti-phosphoserine antibody. This result suggests that these proteins of molecular weight 35–90 kDa become phosphorylated on specific tyrosine residues upon EGF treatment. On the other hand, most of the signals detected in immunostaining with the anti-phosphoserine antibody are observed as weak signals, as compared with the anti-phosphotyrosine antibody. This may result from low affinity or specificity of the anti-phosphoserine antibody for its antigen in comparison with the anti-phosphotyrosine antibody. These results suggest that the immunodetection using a highly specific antibody is of importance for further detection with MS analysis. We tried direct on-membrane PMF analysis for some protein spots having an intense signal, which had slight differences in the phosphorylation level on serine residues when comparing the spots in the presence and absence of EGF, but most of these proteins could not be identified (data not shown). Most of the proteins detected with immunostaining using the anti-phosphoserine antibody appeared as faint spots on the membrane stained with Direct Blue 71. Therefore, the microscale on-membrane identification for small amounts of proteins might be unsuccessful in some cases because of decreased practical sensitivity when only a tiny part of a protein spot is digested [9].

3.3. On-membrane direct identification of proteins detected with an anti-phosphotyrosine antibody

We carried out microscale on-membrane digestion using the microdispensing function based on piezoelectric inkjet technology for some proteins immunodetected with a highly intense signal using anti-phosphotyrosine (Fig. 3). The resulting tryptic digests were analyzed directly on the membrane by MALDI–TOF MS, and a database search was performed to find a match to the MS spectrum. The results of the database search are shown in Table 1, which lists proteins that were identified with a reliable score. With regard to proteins 2–8, sequentially arranged proteins, which may have multiple isoforms or modifications such as phosphorylation on different amino acids within a single protein, were analyzed in the microscale region of a single protein spot using piezoelectric inkjet printing technology. These proteins (proteins 2–8) that were slightly shifted according to pI were identified as the same protein, and differences derived from isoforms or modifications could not be observed on the MS spectrum (data not shown). Such differences may depend on ion suppression from the negative charge of a phosphate group or incomplete sequence coverage. When we performed direct on-membrane MS analysis in this experiment, decreasing resolution was observed, compared with analysis carried out on the stainless steel plate [9]. Although roughness of

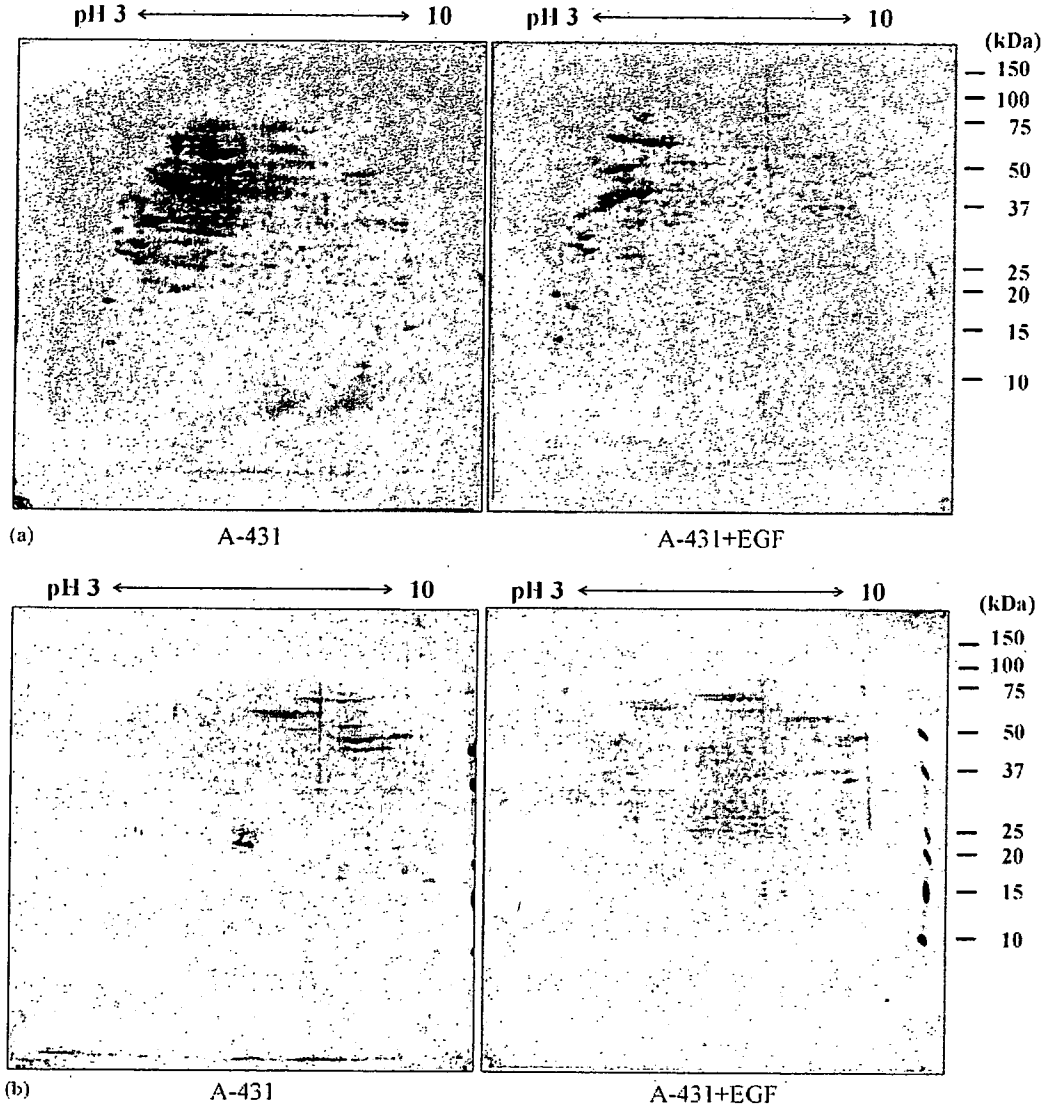

Fig. 1. 2-DE blot image of phospho-enriched whole cell lysates from A-431/A-431 + EGF cell lysates. (a) Image visualized with Direct Blue 71 staining; (b) fluorescent image detected with an anti-phosphotyrosine antibody.

Table 1
Phosphoproteins from EGF-stimulated A-431 cell lysates identified directly on membrane using a database search

	Accession no.	Protein name	M.W.	Score	Matched peptides
Protein 1	P13639	Elongation factor 2	96115	80	12
Protein 2	P26038	Moesin	67761	70	10
Protein 3	P15311	Ezrin (p81)	69339	63	10
Protein 4	P31948	Stress-induced-phosphoprotein 1 (STI1)	63227	75	11
Protein 5	P14618	Pyruvate kinase isozymes M1/M2	58339	118	11
Protein 6	P04406	Lyceraldehyde-3-phosphate dehydrogenase	36070	118	10
Protein 7	P08107	Heat shock 70 kDa protein 1	70294	181	18
Protein 8	P38646	Stress-70 protein, mitochondrial precursor	73920	164	16

The database search was performed using the Sprot database. Typical results of database search (proteins 6) are as follows: amino acid sequences (observed ion *m/z* /calculated ion *m/z*): VKVGVNGFGR (3–12), 1032.62/1032.60; VGVNGFGR (5–12), 805.43/805.43; AENGKLVINGNPITIFQER (61–79), 2113.13/2113.14; LVINGNPITIFQER (66–79), 1613.97/1613.90; LVINGNPITIFQERDPSK (66–83), 2041.18/2041.11; AGAHLQGGAKR (107–117), 1065.72/1065.59; DGRGALQNIIPASTGAAK (197–214), 1739.97/1739.94; GALQNIIPASTGAAK (200–214), 1411.70/1411.79, LTGMAFRVPTANVSVVDLTCR (227–247), 2323.10/2323.19; VPTANVSVVDLTCR (234–247), 1530.88/1530.80.