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Affinity-Tagged Phosphorylation Assay by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (ATPA-MALDI): Application to Calcium/Calmodulin-Dependent Protein Kinase

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A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)–based kinase assay using a peptide substrate tagged with a biotinyl group has been developed. The peptide moiety was designed to serve as an efficient substrate for calcium/calmodulin-dependent protein kinase II, based on the *in vivo* phosphorylation site of phosrestin I, a *Drosophila* homolog of arrestin. In the assay, the quantitative relationship was determined from the ratio of the peak areas between the two peaks respectively representing the unphosphorylated and the phosphorylated substrate. Attempts to assay phosphorylated peptides directly from the reaction mixture, gave inaccurate results because of the high noise level caused by the presence of salts and detergents. In contrast, after purifying the substrate peptides with the biotin affinity tag using streptavidin-coated magnetic beads, peak areas accurately represented the ratio between the unphosphorylated and phosphorylated peptide. By changing the substrate peptide to a peptide sequence that serves as a kinase substrate, it is expected that an efficient non-radioactive protein kinase assay using MALDI-TOF MS can be developed for any type of protein kinase. We call this technique "Affinity-Tagged Phosphorylation Assay by MALDI-TOF MS (ATPA-MALDI)." ATPA-MALDI should serve as a quick and efficient non-radioactive protein kinase assay by MALDI-TOF MS.

Key words: avidin, biotin, kinase, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, phosphorylation.

Abbreviations: AIP, autocamtide-2-related inhibitory peptide; CaMK II, calcium/calmodulin-dependent protein kinase II; ESI, electrospray ionization; Fmoc, 9-fluorenylmethyloxycarbonyl; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PMSF, phenylmethanesulfonyl fluoride; PRI, phosrestin I; TFA, trifluoroacetic acid.

During the past several decades, protein phosphorylation cascades have been revealed as major pathways of signal transduction underlying numerous cellular events (*1*). To characterize these phosphorylation cascades, which are mediated by kinases and phosphatases, it is necessary to determine phosphorylation stoichiometry and identify phosphorylation sites. Recent developments in mass spectrometry make it possible to obtain structural information, such as the phosphorylation site. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in particular provides a simple and an easy-to-use technique for structural study of phosphoproteins and phosphopeptides (*2–4*).

It has been reported that MALDI-TOF MS can be used for the quantification of proteins and other bioactive molecules by evaluating the ratio of the peak heights between the analyte and an internal standard (*5*, *6*). In this manner, by using highly purified calcium/calmodulin-dependent protein kinase II (CaMK II) and substrate peptide for CaMK II, we developed a quantification method for phosphopeptide (*7*). However, we could not easily apply this method to an assay system using a crude kinase extract, which is usually used as an enzyme source in place of purified kinase. We had to solve two problems. The first problem was that phosphorylation adds one negative charge to the substrate peptide and influences the ionization efficiency of the phosphopeptide (*8*). To solve this problem, we designed a substrate peptide that was a portion of phosrestin I (PRI) containing one phosphorylation site and many basic amino acid residues. PRI is a *Drosophila* homolog of the arrestin family, and is phosphorylated by CaMK II (*9*). Using HPLC-electrospray ionization (ESI) mass spectrometry, the phosphorylation site was determined to be exclusively Ser-366 (*10*). In our previous report, we used a portion of PRI [W-PRI (349–377), WAPGTIEKKRS-NAMKKMKS*IEQHRNVKGYY] containing one phosphorylation site, eight basic and two acidic residues as the CaMK II substrate peptide. The Ser marked with the asterisk corresponds to Ser-366. Upon phosphorylation by CaMK II, there was no significant difference in net charge

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and ionization efficiency between unphosphorylated and phosphorylated W-PRI (349–377) (*7*). In this study, we chose a shorter PRI peptide, PRI (346–371) (LQPAPGTIEKKRSNAMKKMKS*IEQHR) instead of W-PRI (349–377) because of its easier synthesis. As the two peptides have almost identical sequences, PRI (346–371) was expected to show no significant difference in ionization efficiency between unphosphorylated and phosphorylated forms. The second problem was that contaminants originating from the enzyme source could suppress ionization of peptides. To overcome this problem in this study, we used a one-step, selective purification technique using the interaction between biotinylated substrate peptide and streptavidin-coated magnetic beads after phosphorylation. Using N-terminally biotinylated substrate, biotin-LQPAPGTIEKKRSNAMKKMKS*IEQHR [biotin-PRI (346–371)], we developed a quantitative kinase assay using MALDI-TOF MS. This method makes it possible to easily remove contaminants and to detect sharper peaks corresponding to unphosphorylated and phosphorylated substrate with low background in the MALDI-TOF mass spectrum. We show here details of the combination technique using biotin-avidin interaction and subsequent MALDI-TOF MS-based quantification. We call this technique "Affinity-Tagged Phosphorylation Assay by MALDI-TOF MS (ATPA-MALDI)."

MATERIALS AND METHODS

The CaMK II substrate peptide PRI (346–371) and CaMK II inhibitory peptide, [Lys^3,Phe^{10},Tyr^{13}]-autocamtide-2–related inhibitory peptide (AIP) were synthesized according to Fmoc chemistry. The N-terminal of PRI (346–371) was biotinylated using biotin *N*-hydroxysuccinimide ester before the deblocking reaction. The peptides were purified by preparative HPLC. Streptavidin-coated magnetic beads (Dynabeads M-280) were purchased from Dynal AS (Oslo, Norway). The beads are superparamagnetic polystyrene beads with streptavidin covalently attached to their surface. The beads are supplied as a suspension containing 6.7 $\times$ 10^8 beads/ml (10 mg/ml) in phosphate-buffered saline. Recombinant CaMK II was purchased from New England BioLabs (MA, USA). The RC DC protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Calmodulin, biotin *N*-hydroxysuccinimide ester and other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

A typical CaMK II reaction mixture (50 μl) consisted of 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 5 mM β-mercaptoethanol, 200 μM ATP, 2 mM $CaCl_2$, 1 μg of bovine brain calmodulin, 50 μM substrate peptide and 10 μl of enzyme source. Phosphorylation reactions were typically performed at 30°C for 20 min, and started by the addition of enzyme source. Five microliters of the reaction mixture was taken from the reaction pool, and 0.1% TFA (50 μl) and 500 mM Tris-HCl (pH 6.8) (50 μl) were added to quench and neutralize the reaction, respectively. A suspension of streptavidin-coated magnetic beads (5 μl) was then added. After the beads had been immobilized with the magnetic concentrator, the supernatant was carefully removed. The substrate-bead complex was washed three times with 100 μl of H_2O, then suspended in 20 μl of 0.1% TFA. The suspension (0.5 μl) and 0.5 μl of matrix solution (10 mg/ml of α-cyano-4-hydroxycinnamic acid in TFA/acetonitrile/H_2O, 0.1:70:30, v/v/v) were mixed and applied to a MALDI sample plate.

The mass spectrometric analysis was performed using Voyager Elite MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) in the linear positive ion mode with delayed extraction. To avoid loss of detector function, the low mass gate was set at m/z = 2,000. The signals from 128 laser shots were averaged, and the peak area was integrated using GRAMS/386 software (Galactic Industries Corp., Salem, NH, USA). Statistical analysis was performed using Microsoft Excel. For kinase assays using frog brain homogenate, dissected frog (*Rana catesbeiana*) brain was homogenized on ice with a homogenization buffer containing 0.32 M sucrose, 50 mM HEPES-NaOH (pH 7.5), 5 mM DTT, 1 mM $MgSO_4$, 1 mM EGTA, 1 mM EDTA, 0.1 mM Na_3VO_4, 10 mM KF, 10 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 50 μg/ml aprotinin and 100 μg/ml phenylmethanesulfonyl fluoride (PMSF). The homogenates were centrifuged at 100,000 $\times$ *g* for 30 min at 4°C, and the supernatant was carefully collected and subjected to RC DC protein assay. The protein solution (1 mg/ml) prepared in homogenization buffer was stored at –80°C before use.

For the determination of phosphorylation site, biotin-PRI (346–371) was phosphorylated under the same conditions as mentioned above using frog brain homogenate for 1 h. The biotinylated peptide was collected with streptavidin-coated magnetic beads. The peptide-bead complex was washed twice with 100 μl H_2O and suspended in 0.1% TFA (100 μl). The suspension was immobilized with magnetic concentrator, and the supernatant was collected and reduced in volume to 20 μl with a SpeedVac concentrator (ThermoElectron, Bellefonte, PA, USA). Addition of 50 mM NH_4HCO_3 (pH 8.5, 20 μl) to neutralize the solution was followed by 10 μl of immobilized TPCK trypsin (Pierce Biotechnology Inc, Rockford, IL, USA), and the resulting solution (pH 8.5) was incubated for 17 h at 4°C. After the centrifugation at 15,000 rpm for 5 min, the supernatant was concentrated by SpeedVac to 5 μl. The resulting peptide mixture (0.5 μl) was applied to MALDI-TOF MS measurement in positive linear mode using α-cyano-4-hydroxycinnamic acid as matrix.

RESULTS

To evaluate the reliability of the Affinity-Tagged Phosphorylation Assay by MALDI-TOF MS (ATPA-MALDI), we first aimed to establish the purification protocol using biotinylated substrate and streptavidin-coated magnetic beads. Figure 1a shows the MALDI-TOF mass spectrum of a crude assay mixture using frog brain homogenate as the enzyme source. Although both S and SP peaks were recognizable, it was hard to quantify the peak areas because the peaks were broad and the background was high. In contrast, after selective purification using streptavidin-coated magnetic beads, both S and SP peaks observed in the MALDI-TOF mass spectrum became sharper (Fig. 1b). The average molecular weights of unphosphorylated and phosphorylated biotin-PRI (346–371) were calculated as 3233.88 and 3313.84, respectively. Here, S and SP were observed by MALDI-TOF MS as protonated ions at

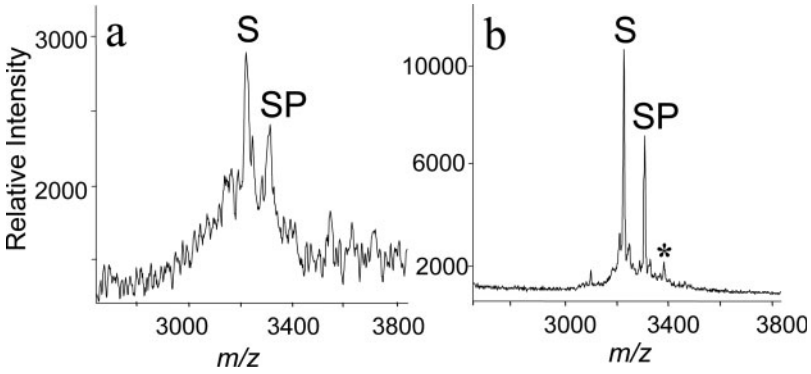

Fig. 1. **(a) Phosphorylation of biotin-PRI (346–371), monitored by MALDI-TOF MS in the crude assay mixture. (b) Phosphorylation of biotin-PRI (346–371), monitored by MALDI-TOF MS in the assay mixture purified using streptavidin-coated magnetic beads.** Crude frog brain homogenate was used as the enzyme source. S and SP indicate unphosphorylated and phosphorylated biotin-PRI (346–371), respectively. The peak indicated with the asterisk did not correspond to double phosphorylated biotin-PRI (346–371) because the mass difference between SP and the indicated peak was 74 Da.

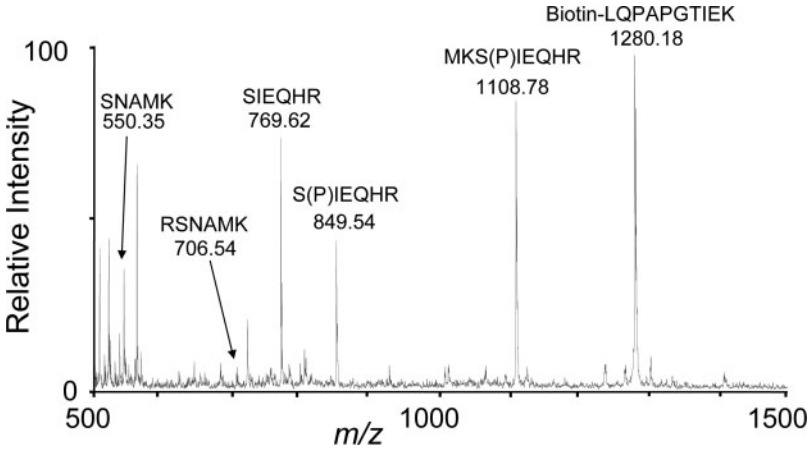

Fig. 2. **Mass spectrum of tryptic digest of phosphorylated biotin-PRI (346–371).** Biotin-PRI (346–371) (biotin-LQPAPGTIEK-KRSNAMKKMKSIEQHR) was phosphorylated with crude frog brain homogenate. The peptide was digested with immobilized trypsin after the purification using streptavidin-coated magnetic beads. The resulting peptide mixture was measured by MALDI-TOF MS. The observed m/z values and the peak assignments were indicated. The calculated monoisotopic masses of protonated ions were SNAMK (550.27), RSNAMK (706.37), SIEQHR (769.40), S(P)IEQHR (849.40), MKS(P)IEQHR (1,108.53), and Biotin-LQPAPGTIEK (1,280.59). Peaks corresponding to phosphorylated peptides, S(P)NAMK (630.27), RS(P)NAMK (786.37), and biotin-LQPAPGT(P)IEK (1,360.59) were not detected.

m/z = 3,235.06 and 3,315.27, respectively. This shows that S and SP represent the unphosphorylated and phosphorylated forms of biotin-PRI (346–371), respectively. The small peak in Fig. 1b indicated by the asterisk did not correspond to the double phosphorylated peptide, because the mass difference between SP and the indicated peak was 74, not 80: single phosphorylation of a peptide adds 80 Da to the molecular mass. The identity of this peak is currently unclear.

On the other hand, using the reaction mixture containing frog brain homogenate in the absence of Ca^{2+} and calmodulin, a signal corresponding to the phosphorylated biotin-PRI (346–371) has not been observed in the MALDI-TOF MS spectrum (data not shown). This result shows that the kinase activity of crude brain homogenate in this system is Ca^{2+}/calmodulin-dependent. In the phosphorylation reaction using frog brain homogenate in the presence of Ca^{2+} and calmodulin, only the mono-phosphorylated biotin-PRI (346–371) was observed. This kinase activity was almost completely inhibited by CaMK II specific inhibitory peptide, [Lys^3,Phe^{10},Tyr^{13}]-AIP (KKKLRRQEAFDAY) (*11*) (data not shown). Although there are three potential phosphorylation sites, one threonine and two serine residues in the substrate peptide, we previously showed that only the serine residue near the C-terminal was phosphorylated by *Drosophila* CaMK II (*9*, *10*) and rat CaMK II (*12*). We determined the phosphorylated site of biotin-PRI (346–371) in this assay system using frog brain homogenate. Figure 2 shows the MALDI-TOF MS spectrum of tryptic digest of the phosphorylated biotin-PRI (346–371). In this spectrum, S(P)IEQHR and MKS(P)IEQHR were observed as phosphorylated peptides, while the phosphorylated forms of SNAMK, RSNAMK, and Biotin-LQPAPGTIEK were not detected. This result clearly demonstrates that the serine residue near the C-terminal was exclusively phosphorylated under this condition. The above results, taken together, demonstrate that in this assay system with frog brain homogenate, the phosphorylation of biotin-PRI (346–371) is catalyzed by a CaMK II, and the C-terminal half of serine is the phosphorylation site.

To investigate whether the purification system using biotinylated substrate and streptavidin-coated magnetic beads can be applied to quantitative measurement based on MALDI-TOF MS, we measured the extent of phosphorylation of the biotinylated substrate peptide by integrating the S and SP peak areas in the MALDI-TOF mass spectrum after the purification with streptavidin-coated magnetic beads. The extent of phosphorylation was calculated as [SP]/([S]+[SP]). Here, [S] and [SP] represent the peak areas in the MALDI-TOF mass spectrum of S and SP, respectively. Figure 3 shows the peak area ratio, [SP]/([S]+[SP]) at each time point, with recombinant CaMK II (0.25 μl of original solution) used as the enzyme source. These results show that the biotin-tagged peptide, biotin-PRI (346–371) exhibited a linear correlation for the peak area ratio versus reaction time (slope = 0.0230, r^2 = 0.996, n = 5).

We next confirmed whether the observed peak area ratio values, [SP]/([S]+[SP]), from the mass spectra were

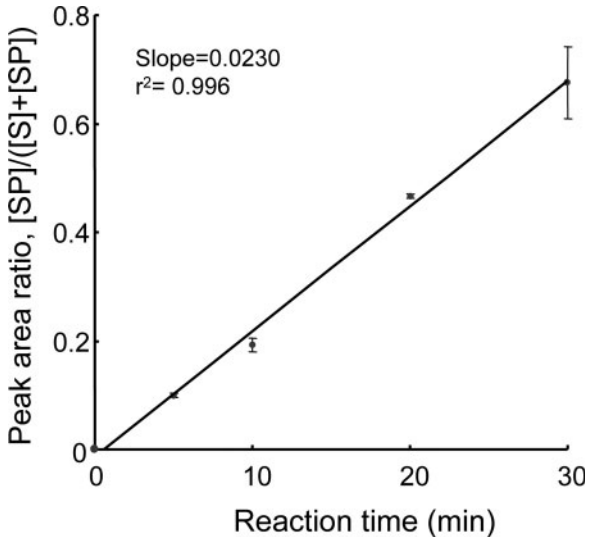

Fig. 3. **Time-course of phosphorylation of biotin-PRI (346–371) monitored by MALDI-TOF MS using a purified CaMK II.** The amounts of substrate [S] and phosphorylated peptide [SP] were integrated from the ion counts against the m/z domain on the MALDI-TOF spectrum. Error bars represent standard deviation (n = 5).

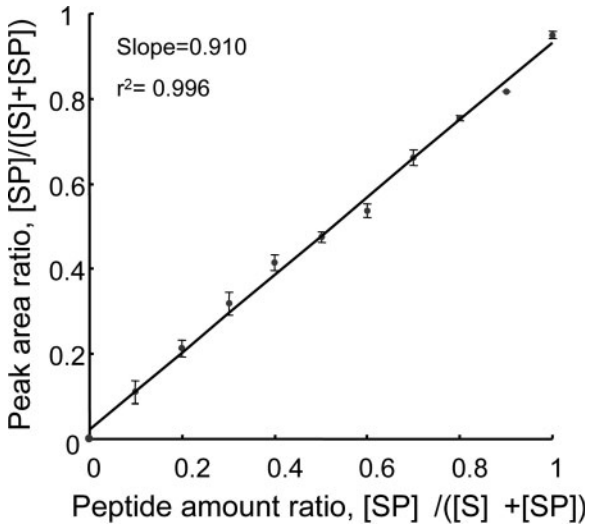

Fig. 4. **Correlation between the peak area ratios, [SP]/([S]+[SP]), on the MALDI-TOF mass spectra and the ratios of the actual peptide amounts, [SP]′/([S]′+[SP]′).** The [SP]/([S]+[SP]) values were obtained by measuring the MALDI-TOF mass spectra of corresponding [SP]/([S]+[SP]) ratios. [S]′ and [SP]′ represent the amounts of unphosphorylated and phosphorylated biotin-PRI (346–371) peptides, respectively. Phosphorylated biotin-PRI (346–371) was prepared by complete phosphorylation using CaMK II for 2 h and confirmed by MALDI-TOF MS. Error bars represent standard deviation (n = 5).

linearly correlated with the ratios of peptide amounts, [SP]′/([S]′+[SP]′). [S]′ and [SP]′ represent the peptide amounts, as designated by the peptide concentration. To obtain a standard set of samples for the calculation of [SP]′/([S]′+[SP]′), 0.5 mM biotin-PRI (346–371) solution was divided into two equal aliquots (100 μl each). To prepare species S, one aliquot was mixed into the assay mixture (final volume in 1 ml) without an enzyme source. To prepare species SP, the other aliquot was completely phosphorylated using purified CaMK II (2 μl) for 2 h, then 1 μl of TFA was added to quench the kinase reaction (final volume 1 ml). The completion of phosphorylation was confirmed by the complete disappearance of the S peak and the appearance of the SP peak (data not shown). Therefore, each of the two reaction mixtures contained S or SP at equivalent concentrations. We then mixed these solutions such that the ratios of S and SP were 0:10, 1:9, 2:8, 3:7, ... 9:1 and 10:0 (final volume 50 μl). From each mixture, 5 μl was subjected to purification with streptavidin-coated magnetic beads. Figure 4 shows the ratio of the observed peak areas of [SP]/([S]+[SP]) on the MALDI-TOF mass spectrum plotted against the corresponding ratio of [SP]′/([S]′+[SP]′) in actual peptide amounts. A linear correlation was observed over the entire observed range of [SP]′/([S]′+[SP]′) (slope = 0.910, r^2 = 0.996, n = 5). We previously observed that the slope was 0.917 when the correlation was examined by plotting the phosphorylation ratio determined from the peak areas on the MALDI-TOF mass spectra *versus* the peak areas on HPLC chromatograms (7). Taken together, these results showed that the biotin-tagged peptide and the purification step using biotin-avidin interaction did not affect the result of the quantification in the kinase assay using MALDI-TOF MS. We finally examined the reliability of ATPA-MALDI for true kinase assays using crude tissue homogenates as the enzyme source in place of purified CaMK II enzyme. As

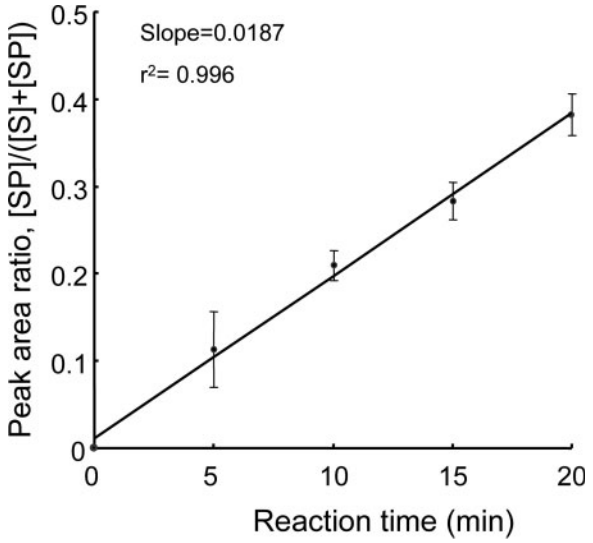

Fig. 5. **Time-course of phosphorylation of biotin-PRI (346–371) monitored by MALDI-TOF MS using crude frog brain homogenate as the enzyme source.** The amounts of substrate [S] and phosphorylated [SP] peptide were integrated in the ion counts against the m/z domain on the MALDI-TOF mass spectra. Error bars represent standard deviation (n = 5).

shown in Fig. 5, a linear correlation for [SP]/([S]+[SP]) against each time point was observed (slope = 0.0187, r^2 = 0.996, n = 5) after the use of streptavidin-coated magnetic beads. These data clearly demonstrate that a crude assay mixture containing tissue homogenate did not affect the linearity of the ATPA-MALDI.

DISCUSSION

We previously demonstrated that it is possible to quantify peptide phosphorylation using MALDI-TOF MS when the peptide exhibits only a slight change of isoelectric point following phosphorylation (*7*). Although MALDI possesses a high tolerance to salt and detergent, impurities that originate from the assay mixture cause a loss of quality of the mass spectra used for integrating the peak area. For this reason, a quick and efficient purification step is required before MALDI-TOF MS measurement.

Our method, ATPA-MALDI, uses the following two technical features. First is specific substrate purification using the interaction between biotin-tagged peptide substrate and streptavidin-coated magnetic beads. This can achieve sufficient purity to allow integration of peak areas on MALDI-TOF mass spectra. The biotin-tagged peptide was adequately observed by MALDI-TOF MS, although purified biotin-avidin complex was applied to MALDI-TOF MS measurement. This is attributed to dissociation of the biotin-avidin complex during sample preparation and/or ionization for MALDI-TOF MS. Second is the quantification based on MALDI-TOF MS using the peak area ratio, [SP]/([S]+[SP]), from the MALDI-TOF mass spectra, which represents the ratio of phosphorylation of substrate. Using this technique, we showed that crude brain homogenate could be directly applied to ATPA-MALDI.

We used approximately one four-hundredth of a 50 μl scale assay mixture containing 50 μM substrate peptide for the MALDI-TOF MS. The amount of substrate peptide used for MALDI-TOF MS analysis was calculated as 6 pmol. The amount of sample required for ATPA-MALDI is similar to that for kinase assays using [γ-^{32}P]ATP and phosphocellulose (*12*, *13*). Moreover, with ATPA-MALDI, the sensitivity on MALDI-TOF MS should not be a crucial issue because the detection limit for MALDI-TOF MS using biotinylated substance P analog and streptavidin-coated magnetic beads was reported to be 100 fmol (*14*). The abundant substrate is apt to attenuate shot-to-shot intensity variation on laser irradiation when obtaining MALDI-TOF mass spectra. Our data demonstrated that 128 laser shots from various target positions gave MALDI-TOF mass spectra of sufficient quality to permit integration of the peak areas to calculate [SP]/([S]+[SP]).

A method has been reported for incorporating the avidin-biotin interaction into MALDI-TOF MS using selective purification of peptides, proteins and oligosaccharides modified with commercially available biotinylation reagents (*15–17*). These biotinylation techniques can expand the application of ATPA-MALDI to a wide variety of substrates and assay systems. Recent developments in absolute quantification of protein phosphorylation and addressing phosphorylation stoichiometry *in vivo* are based on an isotope dilution method using HPLC-ESI mass spectrometry (*18*, *19*). In contrast, ATPA-MALDI is clearly useful as a simple and routine method for *in vitro* kinase assays in the biochemistry laboratory because of the easy and quick procedure. The features of ATPA-MALDI can be summarized as: (i) a non-radioactive kinase assay, (ii) availability for the structural information of substrate peptide, especially the phosphorylation (site) by subsequent MALDI-TOF MS analysis and/or tandem mass spectrometry, and (iii) potential for application to laboratory automation systems for high throughput kinase assays, *e.g.*, 96-well plate format assay (*20*) using avidin-coated microplates.

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