research articles Journal of proteome research

Nanoliter Sample Handling Combined with Microspot MALDI−MS for Detection of Gel-Separated Phosphoproteins

Jacek Stupak,[†] Huaizhi Liu,[†] Zhengping Wang,[†] Brenda J. Brix,[‡] Larry Fliegel,[‡] and Liang Li*,[†]

Departments of Chemistry and Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Received November 17, 2004

We describe a microspot matrix-assisted laser desorption ionization (MALDI) mass spectrometric approach to analyze gel-separated phosphoproteins. This method involves in-gel digestion of phosphoproteins after gel separation, followed by open tubular capillary (OTC) immobilized metal-ion affinity chromatography (IMAC) to capture the phosphopeptides with markedly reduced interferences from nonphosphorylated peptides. Nanoliter-volume of ammonium phosphate is used to elute the phosphopeptides captured on the capillary tube. After mixing with a small volume of matrix solution in the capillary, the effluent is deposited in a microspot on a sample plate for MALDI−MS analysis. It is also shown that, with peptide esterification after in-gel digestion of a phosphoprotein, negative ion detection in MALDI gives a distinct advantage over the positive ion mode of operation for phosphopeptide analysis, even without IMAC enrichment. However, the OTC−IMAC technique is demonstrated to be superior to the approach of negative ion detection of esterified in-gel digests without IMAC. OTC−IMAC is found to be sufficiently selective to capture phosphopeptides from in-gel digest of a gel band containing predominately one protein and the combination of peptide esterification and IMAC enrichment does not provide any real advantage. Using a standard phosphoprotein α-casein as a model system, we demonstrate that this OTC−IMAC method can detect a number of phosphopeptides after in-gel digestion with mid-fmol protein sample loading. An example of real world applications of this method is illustrated in the characterization of a fusion protein, His182, expressed in *E. coli*.

Keywords: phosphoprotein • open tubular column • IMAC • MALDI

Introduction

Phosphorylation of proteins on Ser, Thr, and Tyr residues is one of the most commonly encountered post-translational modifications, and has been implicated in a number of cellular regulatory mechanisms and transduction pathways.[1−5] Perturbations in the workings of cellular phosphorylation machinery have been implicated in a number of pathological states, including cancer.[6,7] To fully understand the molecular basis of these cellular mechanisms, it is necessary to detect the phosphoproteins and identify the sites of phosphorylation.

Two-dimensional gel electrophoresis (2-DE) is currently one of the most widely used methods for displaying the proteome of a group of cells. Its unsurpassed resolution for proteins, protein isoforms, and modified proteins, sample loading capacity, ability to quantitate stained protein spots, and its relatively wide dynamic range, combined with well-established mass spectrometric methods[8−12] for protein identification, have made it a staple of many proteomic efforts.[13,14] With the growing number of protein sequence databases, it is now possible to identify most of the spots visualized on a 2-DE gel. The next step in the challenging venture of proteomics is to characterize post-translational modifications directly from spots observed on a gel. It has been suggested that up to one-quarter of such spots may be post-translationally modified.[14] The problem associated with direct MS analysis of phosphopeptides obtained from the corresponding phosphoproteins observed in gels is the low stoichiometry of phosphorylation, and low ionization efficiency of multiply phosphorylated peptides. For real samples, peptides bearing the electronegative phosphoryl group are often detected with very low efficiency or not at all, and the detection of multiply phosphorylated peptides is most difficult of all. In addition, the fact that phosphorylated peptides generally make up only a very small portion of a typical protein digest leads to a more general suppression effect of low concentration ions by much more abundant ones.

To overcome the inherent problem associated with detection of phosphopeptides, immobilized metal-ion affinity chromatography (IMAC)[15,16] has commonly been used as an enrichment technique. Immobilized Fe^{3+}, and several other metal ions have been shown to have the ability to bind the phosphate moiety of phosphorylated peptides, thereby selectively retaining these while removing more abundant nonphosphorylated peptides from the mixture. One disadvantage of this approach is the co-enrichment of peptides bearing multiple carboxylic acid groups, which also bind the chelated metal in IMAC. To overcome this problem, Ficarro et. al. developed a method for selective esterification of carboxylate groups of phosphopeptides prior to IMAC, followed by HPLC−MS analysis, with very prom-

* To whom correspondence should be addressed. E-mail: Liang.Li@ualberta.ca.
[†] Department of Chemistry.
[‡] Department of Biochemistry.

10.1021/pr049789t CCC: $30.25

Published on Web 03/04/2005

ising results.[17] It has been reported that nickel loaded nitrilotriacetic acid beads which use a tetradentate ligand to bind iron—as opposed to the more commonly used bidentate iminodiacetic acid—retain phosphopeptides more selectively,[18] although this improvement has been challenged by Posewitz et al.[19] Phosphoproteins from electrophoretic gels have also been studied at the picomole and subpicomole level by Fe^{3+}—IMAC columns in combination with reversed-phase (RP) LC ESI—MS and MALDI—MS,[20] LC—MS/MS,[21−23] CE—ESI—MS^n,[24] with precursor ion scanning in negative ion mode on a triple-quadrupole mass spectrometer,[25] by enzymatic dephosphorylation followed by MS,[26] and most recently using microfluidic compact disk technology.[27] Determination of phosphorylation degree in cysteine and/or methionine containing residues has also been performed by way of LC inductively coupled plasma (ICP) MS.[28] Classical methods for the analysis of protein phosphorylation, such as Edman sequencing and ^{32}P labeling, although well established, are currently out-favored by mass spectrometry protocols due to the ease and sensitivity of the latter, and the hazards associated with handling of radioactive samples.

In this study, we report an extension of our recently published open tubular IMAC (OT-IMAC) technique for phosphopeptide enrichment: a nanoliter sample handling open tubular IMAC for effective enrichment of small amounts of phosphopeptides from in-gel digest of phosphoproteins. Microspot MALDI is used to detect phosphopeptides from the enriched sample. The technique involves the use of open tubular *capillary* IMAC (OTC—IMAC) columns using iminodiacetic acid as the Fe^{3+}-binding ligand, to enrich phosphopeptides into ~500 μm diameter micro-spots for MALDI—MS analysis. We also examine the potential of esterifying peptides from the in-gel digest to further improve the selectivity of OTC—IMAC. We discover that a combination of peptide esterification and negative ion MALDI analysis can enhance the detection of phosphopeptide even without IMAC. However, peptide esterification does not lead to any improvement in the performance of OTC—IMAC. We show that OTC—IMAC is superior to the non-IMAC peptide esterification/negative ion detection method, particularly in dealing with in-gel digests of femtomole amounts of proteins. Finally, we report the application of this technique for detection of low-level phosphopeptides from Na^+/H^+ exchanger fusion protein His182, following its separation by SDS-PAGE.

Experimental Section

Chemicals and Reagents. α-Casein and bovine trypsin were obtained from Sigma Aldrich Canada (Oakville, ON). 4-hydroxycinnamic acid (HCCA), acetyl chloride, 2-[*N*-Morpholino]-ethanesulfonic acid, iron(III) chloride, ammonium dihydrogenphosphate, ammonium bicarbonate, hydrochloric acid, trifluoroacetic acid, glacial acetic acid, 3-glycidoxypropyltrimethoxysilane, iminodiacetic acid, sodium hydroxide, and all solvents used were of the highest available purity, and were also obtained from Sigma Aldrich Canada. HCCA was purified by recrystallization from ethanol prior to use. Deionized water was from NANOpure water system (Barnstead/Thermolyne).

Protein Digests and Electrophoresis. Solution tryptic digestion of α-casein was carried out at a concentration of 1 μg/uL with an enzyme-to-substrate ratio of 1:20 for 30 h at 37 °C. In-gel tryptic digestion was performed using the method of Shevchenko et al.[29] Briefly, gel pieces were dehydrated with acetonitrile, dried, and incubated with 4.5 mM DTT in 50 mM NH_4HCO_3 for 30 min., at 56 °C, cooled to room temperature, and resuspended in 50 mM NH_4HCO_3 containing 9 mM iodoacetamide for 30 min. in the dark. The gel slices were washed twice with water, dehydrated with acetonitrile, dried under vacuum, and swelled in 10 ng/μL bovine trypsin in 50 mM ammonium bicarbonate; excess trypsin solution was then removed, the gel piece was placed in approximately 200 μL of 50 mM ammonium bicarbonate buffer pH 8, and digestion was allow to proceed overnight at 37 °C. Peptides were extracted three times with 50% acetonitrile/water/0.2% trifluoroacetic acid, Speedvac dried, cleaned by way of commercially available reverse phase C_{18} Zip-Tip^R cartridges (Millipore, Bedford, MA), redissolved in deionized water and stored at −20 °C. For IMAC experiments, samples were Speedvac dried and reconstituted in 3 μL 0.1 M acetic acid, and loaded directly onto OTC—IMAC. SDS-PAGE was carried out using standard 12% mini-gels stained with Coomasie Blue dye (BioRad, Hercules, CA).

Expression and Purification of Fusion Proteins. The carboxyl-terminal 182 acids of the human Na^+/H^+ exchanger (NHE1) was expressed as a fusion protein with a C-terminal hexahistidine tag (His182) using the plasmid pDest 14 and the Gateway Cloning System as described earlier.[30] *E. coli* strain BL21-SI was induced with 0.3 M NaCl for 3 h. His182 protein was harvested using standard conditions, and the protein was purified from the supernatant of *E. coli* via Ni—NTA affinity chromatography according to manufacturer's directions (Qiagen). Residues 2—183 of this protein correspond to amino acids 634—815 of the NHE1 isoform of the human Na^+/H^+ exchanger.[31] An initiator methionine residue was necessary at the N-terminus, and a hexahistidine tag at the C-terminus was necessary for protein purification.

In Vitro Phosphorylation of the Na^+/H^+ Exchanger Fusion Proteins. The standard reaction conditions for phosphorylation of His182 contained 10.0 μg of His182, 0.03 μg Erk2 (Biomol, Plymouth Meeting, PA), 12.5 mM 3-(4-morpholino)propanesulfonic acid (MOPS) pH 7.2, 0.5 mM EGTA, 2 mM DTT, 8.5 mM magnesium chloride, 6 μM okadaic acid, 0.24 mM sodium fluoride, 500 μM ATP, and 1 μL of 10 μCi/μL of [γ-^{32}P]ATP in a final volume of 24 μL. Samples were incubated at 30 °C for 90 min., and the reaction was terminated by the addition of SDS-PAGE loading buffer. Samples were run on a 12% SDS-PAGE gel, dried, and exposed for autoradiography.

Open Tubular Capillary IMAC Construction and Phosphopeptide Purification. The method used to attach iminodiacetic acid to the silyl groups on the surface of a fused silica capillary was a modified version of that used to make Sepharose-bead IMAC.[32−34] 4.2 g of NaOH pellets were mixed with 2.8 g of iminodiacetic acid (IDA) in 100 mL of deionized water in a round-bottom flask kept on ice; to this solution, 10 mL of 3-glycidoxypropyltrimethoxysilane (GLYMO) were added slowly with stirring. The resulting GLYMO—IDA solution was then stirred at 65 °C overnight. 1 m of a 50 μm i.d., 365 o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ) was activated by flushing it with: ammonia/hydrogen peroxide/water (1:1:5) overnight followed by several column volumes of water; with 6 M HCl overnight; and again with several column volumes of water until the pH of the effluent was neutral. The pH of the GLYMO—IDA solution was adjusted to 3.5 with 6 M HCl, and the conditioned capillary was flushed with it overnight at 95 °C in a thermostated oven under nitrogen gas. The capillary was then flushed with a large volume of deionized water, and baked under nitrogen gas at 150 °C.

The IMAC device, shown in Figure 1, was constructed by cutting 10 mm of the surface modified capillary, and joining it to a cutoff pipet tip through 2 cm of 0.3 mm i.d. Teflon tubing

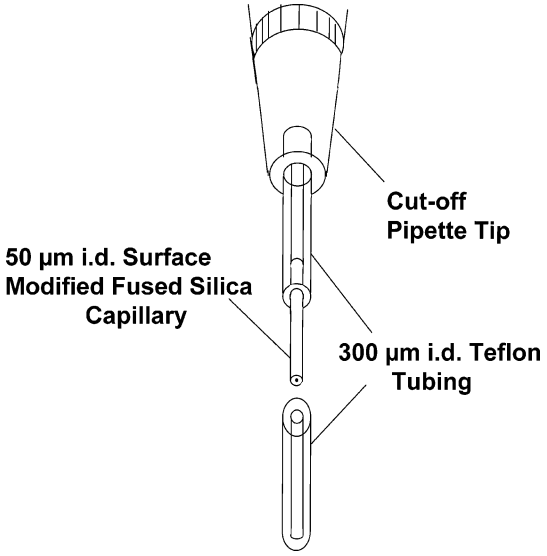

Figure 1. Schematic representation of the open tubular capillary IMAC (OTC−IMAC) device described in this work.

(Supelco, Bellefonte, PA). For IMAC experiments, OTC−IMAC columns were wetted with 50% acetonitrile/0.1% acetic acid, charged with Fe^{3+} by pipetting a solution of 200 mM $FeCl_3$ in 50 mM acetic acid through the capillary; excess iron was washed away with water, and the capillary was then equilibrated with 0.1 M acetic acid (binding buffer).

Phosphopeptides dissolved in 100 mM acetic acid buffer were loaded by repeated pipetting back and forth through the capillary (no less than twenty times), which was then washed five times with approximately 300 nL of binding buffer and again five times with 15% acetonitrile/0.1% acetic acid to further remove nonspecifically bound acidic peptides. For derivatized samples, the capillary was washed five times with binding buffer and twice with a solution of 100 mM NaCl containing 25% acetonitrile and 1% acetic acid, in an attempt to remove all derivatized peptides bound nonspecifically through hydrophobic interactions.

Phosphopeptides were eluted with a solution of 200 mM ammonium dihydrogen phosphate (pH 4.5) by pipetting a small plug of approximately 40 nL through the capillary. To allow for movement of this solution back and forth through the capillary, and thus improve elution efficiency, a 1 cm long, 300 μm i.d. Teflon tubing sleeve was attached to the other end of the capillary to prevent loss of analyte. An equal volume of 10 mg/mL HCCA matrix containing 1% HCl was mixed with the eluting solution inside the capillary and connecting Teflon tubing. The resulting solution was then spotted onto a thin first layer of HCCA matrix according to a modified version of the two-layer method.[35] One percent HCl was originally used in order to acidify pH 9 ammonium phosphate to prevent dissolution of the first layer of HCCA matrix, and was observed to have no detrimental effect on signal intensities. It was fortuitous to discover later that including this additive with the pH 4.5 ammonium phosphate made for much easier spotting from the capillary IMAC, perhaps due to the high viscosity of HCl. The spots resulting from this methodology were then washed with deionized water and analyzed by mass spectrometry. Alternatively, an equal volume of 60 mg/mL DHB matrix was mixed with the eluate inside the capillary and connective tubing, the Teflon sleeve shown in Figure 1 was connected to prevent loss of analyte and allow for mixing of matrix and eluate by pipetting; the solution was then spotted on a MALDI plate.

For experiments involving derivatization of carboxylate groups without modifying the phosphoryl residue, the method of Ficarro et al. was used. Briefly, 1 M methanolic HCl was prepared by addition of 200 μL acetyl chloride, with mixing, to 2.5 mL of absolute methanol through a septum with a gastight syringe to keep all conditions anhydrous. Two microliters of this solution was added to previously Speedvac-dried phosphopeptide mixtures and the derivatization was allowed to proceed for 1 h at room temperature. Samples were then Speedvac dried once more, reconstituted in 10 μL 0.1% trifluoroacetic acid and purified by reverse phase C_{18} Zip-Tip^R cartridges, dried, and redissolved in 1 μL HCCA or DHB matrix for direct analysis, or 3 μL binding buffer (0.1 M acetic acid) for loading onto OTC−IMAC columns.

Elution from OTC−IMAC was achieved using 10 μL 200 mM ammonium phosphate solution, and 0.3 M NH_4OH for the Zip-Tip according to manufacturer's protocol. Spotting was done by the two-layer method for HCCA and the dried droplet method with DHB.

Mass Spectrometry. MALDI was performed on a Voyager Elite reflectron time-of-flight mass spectrometer (Framingham, MA) equipped with a nitrogen laser ($\lambda = 337$ nm) by collecting signals averaged from 400 laser shots. Spectra were calibrated externally using monoisotopic ions of a pepcal standard, or internally using trypsin autolysis peaks. MALDI spectra were analyzed using the Igor Pro Software package (WaveMetrics, Lake Oswego, OR).

Results and Discussion

Optimization of Derivatization Conditions. Ficarro et al. developed a method for selectively converting carboxylate groups of peptides to methyl esters, thereby eliminating nonselective binding of phosphopeptides to IMAC columns.[17] 100 μL of 2 M methanolic HCl was used by the authors to esterify carboxylate groups of peptides obtained from a complex mixture of several proteins. Through our initial experiments, we were able to derivatize only bulk quantities of the model phosphoprotein α-casein using this procedure. The volumes of reagents had to be scaled down in order to work on small amounts such as would be present in gel samples. The digest from 50 ng (2 pmol) α-casein derivatized in 2 M MeOH/HCl clearly showed the trend of signal improvement as a function of decreasing the derivatizing reagent volume (data not shown). All reagents were of the highest purity available, so it is not exactly clear why this should be the case. 2 μL of 2 M MeOH/HCl was chosen as the reagent composition for the subsequent derivatization experiments.

During these experiments, it was also observed that certain α-casein peptides gave rise to peaks with a mass shift of 15 Da from the fully derivatized peptide (i.e., with all its glutamic and aspartic acid residues methylated). Since the formation of methyl esters gives rise to increases of 14 Da for each carboxylate group present, the plus-15 Da products suggest another amino acid besides glutamic and aspartic acid may be reacting under the conditions used. It has been suggested that under the harsh condition used for esterification the expected deamidation reaction may take place;[36] however, to our knowledge, no evidence has been provided to show that it does indeed occur. This evidence is now shown in Supplementary Figure S1 in Supporting Information for a couple of α-casein peptides where modification on asparagines residue was observed. We note that recognition of these minor modifications which can occur using the esterification chemistry is important for peak assignment in the MALDI mass spectrum of the derivatized digest.

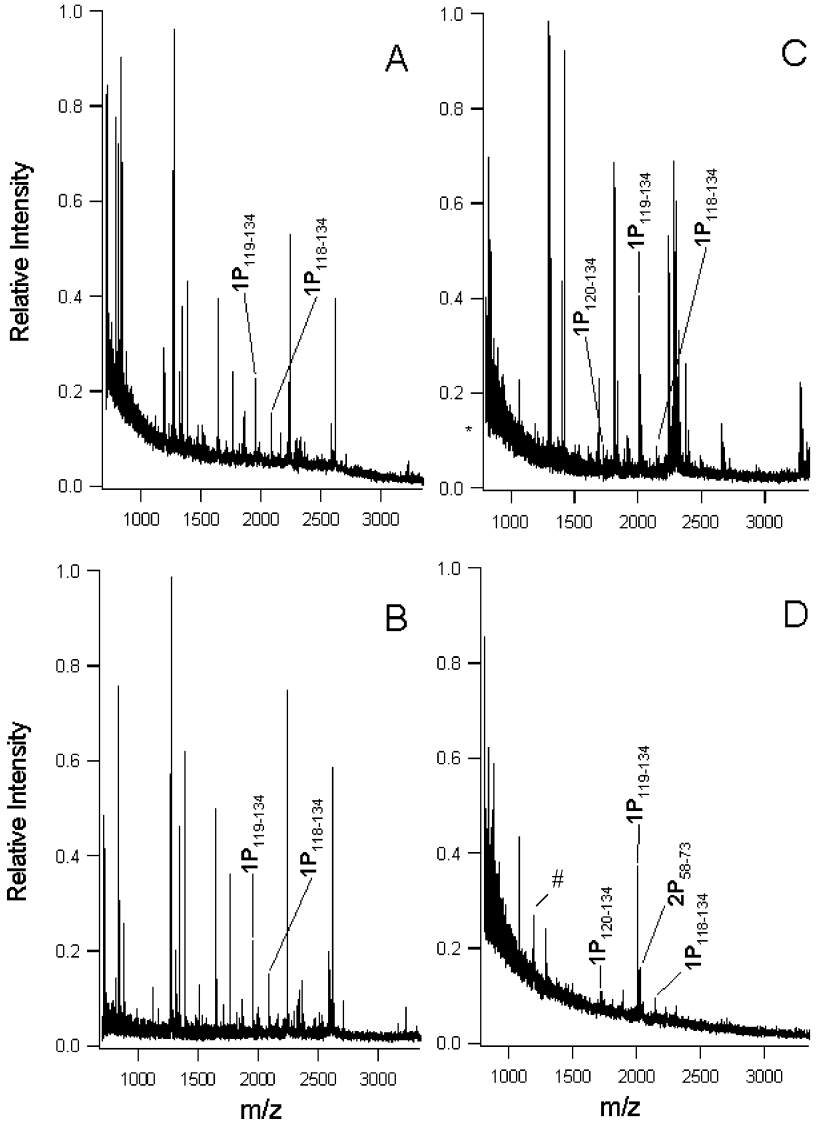

Figure 2. MALDI mass spectra of tryptic α-casein digests following methylation of carboxylate groups. (A) 4 pmol in-gel-digested α-casein unesterified control; positive ion mode. (B) 4 pmol in-gel-digest α-casein unesterified control; negative ion mode. (C) 4 pmol in-gel digested esterified α-casein in the positive ion mode; (B) 4 pmol in-gel digested esterified α-casein in the negative ion mode. Peak labels: S2 α-casein $1P_{153-164}$ − TVDMESTEVFTK at *m/z* 1522.6; S1 α-casein $1P_{121-134}$ − VPQLEIVPNSAEER at *m/z* 1716.8; S1 $2P_{58-73}$ − DIGSESTEDQAMEDIK at *m/z* 2025.7; S1 $1P_{119-134}$ − YKVPQLEIVPNSAEER at *m/z* 2008.0; S1 $1P_{118-134}$ − KYKVPQLEIVPNSAEER at *m/z* 2136.0; # − background peak observed with DHB in the negative-ion mode.

Positive/Negative Ion Detection and Esterification. It has been suggested by several authors that comparison of relative intensities of phosphopeptide peaks in positive and negative ion MALDI−MS could be used as a criterion for identification of such phosphopeptides, since their ionization is thought to be much more efficient in the negative ion mode.[37−40] We have observed the foregoing to be a very unreliable criterion and likely to be protein dependent. Since relative peak intensities from a real sample digest vary significantly from repeat experiments in MALDI, observing minor relative peak intensity changes as an identification strategy in MALDI−MS would not be very useful in general. This is especially true where spotting techniques such as the dried droplet are used, since this method gives heterogeneous crystals with 'hot-spots', a number of which must be averaged in order to obtain a representative spectrum.

To determine whether the ion detection mode (positive or negative ion), esterification, or a combination of the two, has any influence on the detectability of the phosphorylated peptides, 4 pmol (100 ng) of α-casein were separated by SDS-PAGE and digested with trypsin in-gel, and esterified. The positive and negative ion MALDI mass spectra of the underivatized digest and the esterified digest were obtained. Figure 2 shows the results obtained where the spectra shown in panels A and B are from the underivatized digest and panels C and D are from the esterified digest. Only phosphopeptides are labeled in the mass spectra. From the spectral comparison of panels A and B, we can conclude that application of the negative ion mode of detection does little to affect relative peak intensities of the underivatized peptides. However, in comparing the esterified digests analyzed in positive and negative ionization modes (panels C and D), it is evident that nonphosphorylated peptides which have had their anionic carboxylate groups methylated are no longer able to ionize very efficiently in the negative ion mode, for obvious reasons: there are no acidic groups left on these peptides. Certain nonphosphorylated

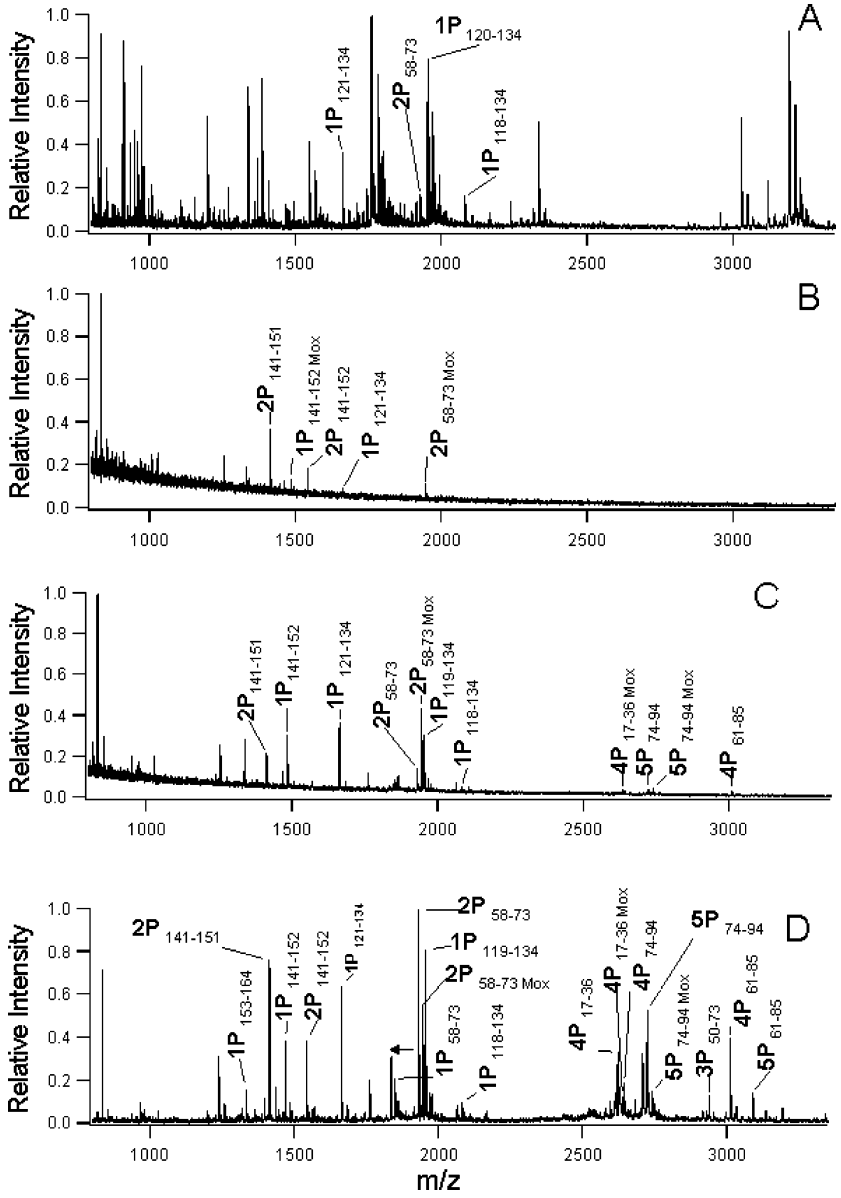

Figure 3. MALDI mass spectra of in-solution digest from 1 pmol α-casein using DHB matrix obtained (A) without the use of IMAC for phosphopeptide enrichment, (B) with the commercially available IMAC device from Millipore, (C) with OT-IMAC, and (C) with OTC−IMAC. Enrichment was performed without esterification of the digest. Peak labels: S2 $1P_{153-164}$ − TVDMESTEVFTK at *m/z* 1466.6; S2 $1P_{141-152}$ EQLSTSEENSKK at *m/z* 1539.6; S1 $1P_{121-134}$ – VPQLEIVPNSAEER at *m/z* 1660.8; S1 $2P_{58-73}$ − DIGSESTEDQAMEDIK at *m/z* 1927.7; S1 $1P_{119-134}$ − YKVPQLEIVPNSAEER at *m/z* 1952.0; S1 $1P_{118-134}$ − KYKVPQLEIVPNSAEER at *m/z* 2080.0; S2 $4P_{17-36}$ − NTMEHVSSSEESIISQETYK at *m/z* 2618.9; $5P_{74-95}$ − QMEAESISSSEEIVPNSVEQK at *m/z* 2720.9; S1 $3P_{50-73}$ − EKVNELSKDIGSESTEDQAMEDIK at *m/z* 2935.2; S2 $4P_{61-85}$ − NANEEEYSIGSSSEESAEVATEEVK at *m/z* 3008.0.

peptides are still detectable, especially in the low mass region; however, their intensity and number are significantly reduced compared to ionization in positive ion mode. As notable in the negative ion spectrum of the esterified digest (Figure 2D), a doubly phosphorylated peptide labeled $2P_{58-73}$ that is not detectable in the positive ion mode (Figure 2C), becomes detectable when this sample is derivatized. We also find that, when the protein amount loaded to the gel is below 4 pmol, signals corresponding to phosphopeptides are no longer observed for the in-gel digest even after derivatization. The rule of thumb is that if we can observe phosphopeptides in the positive ion mode following derivatization of carboxylate groups, application of the negative ion mode should then in large measure eliminate any nonphosphorylated peptides from the spectrum. This approach of carrying out esterification on peptides, followed by switching mode of ion detection in MALDI MS, appears to be an alternative way of detecting phosphopeptides without applying IMAC. However, it requires a relatively larger amount of sample (>4 pmol for α-casein). One potential advantage of this approach is that differential isotope labeling using esterification chemistry may be used to determine the stoichiometry of phosphopeptides which can be very important in studying regulatory functions of proteins or cells.

Next we examine whether peptide esterification combined with OTC−IMAC can further improve the selectivity of IMAC. For the single-protein digests used in this study (e.g., fusion protein His182 to be discussed below), as in most cases in analyzing gel-separated protein spots, we find that OTC−IMAC is already very selective and there is no real advantage to applying the esterification chemistry discussed above in order to

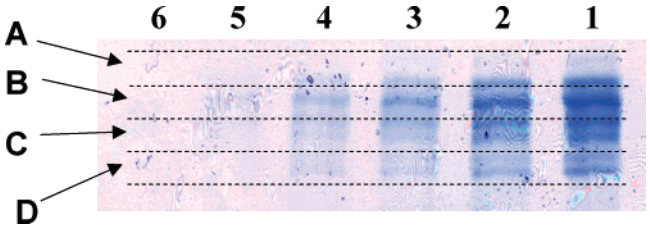

Figure 4. SDS-PAGE separation of serially diluted His182 fusion protein. Bands separated by degrees of phosphorylation were divided into 4 zones, labeled A−D, excised and analyzed using the herein described methods. Lanes: 1−1 μg protein; 2−500 ng protein; 3−400 ng protein; 4−300 ng protein; 5−200 ng protein; 6−100 ng protein.

eliminate nonphosphopeptide bindings. As it will be shown in the example of His182 analysis, derivatization combined with OTC−IMAC can actually reduce the overall detection sensitivity for phosphopeptide analysis. In addition, negative ion detection of OTC−IMAC enriched samples, with or with esterification, does not improve detection sensitivity over that of positive ion detection.

Detection Sensitivity. In our previous work, we have shown open tubular IMAC is generally more effective for enriching phosphopeptides from a protein digest compared to micropipet tips (from Millipore) packed with IMAC beads.[43]

OT-IMAC was initially developed for handling microliter volume of sample as all IMAC mini-columns do. However, OT-IMAC can be readily miniaturized to handle nanoliter samples by using a capillary tube as the capture media instead of an open glass tube. With the use of a capillary, samples can be deposited to a microspot for MALDI which can result in much improved detection sensitivity.[44−47] For the open tubular capillary IMAC device described in the Experimental Section, MALDI spots are on average 0.50 ± 0.04 mm in diameter. Considering a typical diameter of 2.5 mm for regular MALDI spots from a 1 μL droplet, capillary IMAC can potentially improve signals by a factor of approximately 25, assuming linearity of signal versus concentration. This is due to the fact that our analyte is concentrated into an area 1/25th the size of what it would be under normal conditions.

Figure 3 shows the results for comparing the sensitivity obtainable from in-gel tryptic digests of α-casein with open tubular and capillary IMAC versus that of packed pipet-tip IMAC from Millipore. The Millipore IMAC was used by 8/12 labs using IMAC in ABRF−PRG03[48]—and we found it to be useful down to a concentration of about 5 pmol using HCCA as matrix (data not shown).. Although a 'soft' matrix such as DHB is preferred for analysis of labile groups such as phosphate, HCCA can still be useful sometimes in that the metastable loss of phosphoric acid (−95 Da) can be used as a diagnostic marker for the presence of the phosphate group without the use of alkaline phosphatase. However, DHB is considerably more sensitive for phosphopeptide detection, and it was chosen for our purposes. With the un-derivitized in-gel digest of 1 pmol α-casein, Figure 3 shows a sensitivity comparison between the packed pipet-tip Millipore IMAC, open tubular IMAC, and capillary IMAC. We see that low intensity peaks corresponding to phosphopeptides can be observed with the commercial Millipore IMAC device (Figure 3B). The open-tubular device is shown to be more efficient than the latter (Figure 3C), and the inherent low-volume advantage of the OTC−IMAC is apparent by the total number of phosphopeptides observed—fifteen—and their high intensities (Figure 3D).

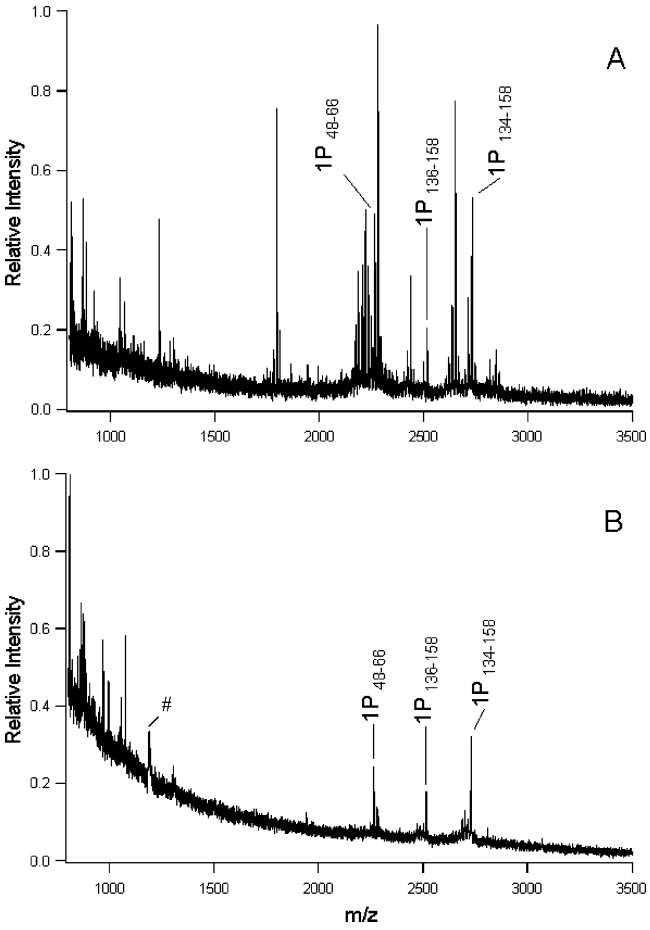

Figure 5. MALDI mass spectra of tryptically digested His182 following methylation of carboxylate groups. Band B from lane 2 in Figure 4 was used for this analysis; this corresponds to approximately 100−200 ng (5−10 pmol) of total protein analyzed. (A) Positive ion mode MALDI−MS; (B) Negative ion mode MALDI−MS.

All of the peaks detected at the 1 pmol level with OTC−IMAC are still detected at the 100 fmol level. At the 20 fmol level, several intense phosphopeptide peaks are observed, but naturally the total number of phosphopeptides detected decreases with decreasing concentration (data not shown).

Application to Histidine-Tagged Fusion Protein His182. To further explore the practical applications and relative analytical merits of the herein described methods, a histidine tagged phosphoprotein was separated by SDS-PAGE and subjected to enzymatic trypsin hydrolysis using the in-gel digestion method. We have previously used OT-IMAC to sequence the phosphorylation sites on His182, a Na^+/H^+ exchanger protein phosphorylated in vitro with the kinase Erk2. Ten micrograms of starting material per experiment was used for these purposes, with multiple experiments including dual-enzyme digestion finally giving a total of 5 phosphorylation sites identified.[43] We now wanted to see if we could apply OTC−IMAC for sensitive quality control for the production of the phosphorylated His182 for biological experiments, as it can be quite costly; and at once avoid the use of undersirable P^{32}. We ran the protein on a 12% SDS-PAGE gel, serially diluting it in adjacent lanes to gauge the sensitivity of the newly developed method for a real sample. An image of the gel obtained from dilutions of His182 is shown in Figure 4. It can be seen that this protein is visible with

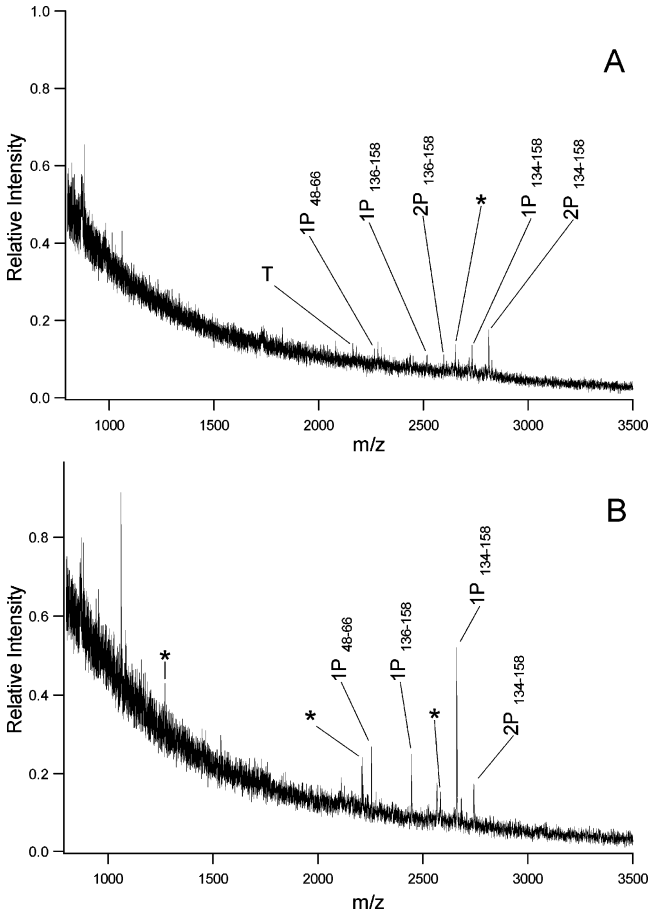

Figure 6. Positive ion MALDI mass spectra of in-gel digested ~25−50 ng (~1−3 pmol) His182. Band B from lane 6 in Figure 4 was used for this analysis. (A) With esterification, and OTC−IMAC enrichment; (B) Without esterification, with OTC−IMAC enrichment. Labels: $1P_{48-66}$ − INNYLTVPAHKLDSPTMSR $2P_{136-158}$ − ETSSPGTDDVFTPAPSDSPSSQR; $2P_{134-158}$ − SKETSSPGTDDVFTPAPSDSPSSQR; * − nonphosphorylated His182 peptides; T − trypsin autolysis.

Coomasie staining down to a level of approximately 200 ng total protein loaded (Figure 4, lane 5).

For the initial work, in-gel digest of selected gel bands was esterified so that both OTC−IMAC and non-IMAC negative ion detection methods could be applied to the same sample. Figure 5 shows the positive and negative ion MALDI−MS spectra obtained from the esterified in-gel digest of band B of lane 3 in Figure 4. No IMAC enrichment was used. This particular gel band corresponds to ~200 ng of His182. By applying the negative ion mode, we were able to ionize phosphopeptides at this level in a nearly selective manner, as evidenced by the disappearance of major nonphosphorylated peptides in the negative ion mode (Figure 5B), compared to those seen in the positive ion mode (Figure 5A). While this approach of negative ion analysis of the esterified digest is not perfect, as some nonphosphorylated peaks are still detected in the low-mass region below 1100 Da, it does enhance the phosphopeptide detection. However, when the amount of His182 decreased below 200 ng, it became increasingly difficult to detect the phosphopeptides. At the protein level of ~25−50 ng, no phosphopeptides could be detected with this approach.

On the other hand, with OTC−IMAC enrichment of the esterified His182 digests, we were able to detect phosphopeptides from this protein down to a level of about ~25−50 ng (~1−3 pmol), as shown in Figure 6A, where band B from lane 6 of the gel in Figure 4 was analyzed. This spectrum was obtained using the positive ion mode of detection and, in this case, no phosphopeptides were detected when negative ion detection was used.

From our experience in working with α-casein, we know that esterification of the in-gel digest does not improve the detection of phosphopeptide if OTC−IMAC is applied to the sample and, in fact, esterification can lead to the decrease of sensitivity when the protein amount used is near the detection limits of the OTC−IMAC technique. Thus, we repeated the experiment of OTC−IMAC on the in-gel digest of ~25−50 ng His182 except that no esterification was done on the digest. The positive ion MALDI spectrum of the OTC−IMAC enriched peptides from an underivatized in-gel digest is shown in Figure 6B. The signal-to-background ratios of all phosphopeptide peaks are increased compared to those shown in Figure 6A. This example demonstrates again that, when we deal with a smaller amount of proteins, OTC−IMAC provides superior performance to the non-IMAC esterification/negative ion detection method for analyzing phosphopeptides from an in-gel digest of a one-protein band.

Conclusions

We have developed a nanoliter-volume method for phosphopeptide enrichment by way of open tubular capillary IMAC, and have used the hand-held device so constructed to enrich phosphopeptides obtained from gel-separated proteins. Because of the small volume of the OTC−IMAC device, the resulting MALDI spots are on average 0.50 ± 0.04 mm in diameter, which allows us to detect phosphopeptides at levels at which conventional microliter-volume bead-packed IMAC pipettor tips show no signal; the sensitivity of the device is also an improvement on the previously developed open tubular IMAC.[43] Furthermore, we show that derivatization of carboxylate groups previously reported by Ficarro et al. can be scaled down to handle in-gel digests. We also demonstrate that application of the negative ion mode for MALDI−MS analysis shows much more of an advantage for phosphopeptide identification once the peptides in the sample have had their carboxylate groups methylated. However, we find little advantage to esterifying peptides from single-protein digests prior to IMAC enrichment. While the method of peptide esterification combined with negative ion detection can avoid the use of IMAC for enhanced phosphopeptide detection, it does not have the phosphopeptide enrichment capability offered by IMAC and thus the detection sensitivity of this method is not as good as the OTC−IMAC technique. We show that the OTC−IMAC technique is useful for detection of phosphopeptides from gel-separated phosphoproteins at the low pmol level for real world samples (sub-pmol for α-casein), and in the low fmol range for in-solution digested phosphoproteins.

Acknowledgment. This work was funded by the Protein Engineering Network of Centres of Excellence (PENCE) of Canada.

Supporting Information Available: Evidence to support the deamination, and subsequent esterification of glutamine (Q) and asparagines (N) residues (Supplementary Figure S1). These materials are available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rudolph, J.; Tolliday, N.; Schmitt, C.; Schuster, S. C.; Oesterhelt, D. *EMBO J*. **1995**, *14*, 4249−4257.
(2) Cantrell, D. *Annu. Rev. Immunol*. **1996**, *14*, 259−274.
(3) Cohen, P. *Trends Biochem. Sci*. **1992**, *17*, 408−413.
(4) Hunter, T. *Cell* **2000**, *100*, 113−127.
(5) MacKintosh, C. *Curr. Opin. Plant Biol*. **1998**, *1*, 224−229.
(6) Darnell, J. E., Jr. *Science* **1997**, *277*, 1630−1635.
(7) Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075−2080.
(8) Mann, M.; Wilm, M. *Anal. Chem*. **1994**, *66*, 4390−4399.
(9) Yates, J. R. III; Speicher, S.; Griffin, P. R.; Hunkapiller, T. *Anal. Biochem*. **1993**, *214*, 397−408.
(10) Henzel, W. J.; Billeci, T. M.; Stults, J. T.; Wong, S. C.; Grimley, C.; Watanabe, C. *Proc. Natl. Acad. Sci. U.S.A*. **1993**, *90*, 5011−5015.
(11) Yates, J. R. III; Eng, J. K.; McCormack, A. L. *Anal. Chem*. **1995**, *67*, 3202−3210.
(12) Shevchenko, A.; Jensen, O. N.; Podtelejnikov, A. V.; Sagliocco, F.; Wilm, M.; Vorm, O.; Mortensen, P.; Shevchenko, A.; Boucherie, H.; Mann, M. *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 14440−14445.
(13) Patterson, S. C.; Aebersold, R. H. *Nat. Genet. Suppl*. **2003**, *33*, 311−323.
(14) Blackstock, W. P.; Weir, M. P. *Tibtech*. **1999**, *17*, 121−127.
(15) Andersson, L.; Portath, J. *Anal. Biochem*. **1986**, *154*, 250−254.
(16) Muszynska, G.; Dobrowolska, G.; Medin, A.; Ekamn, P.; Porath, J. O. *J. Chromatogr*. **1992**, *604*, 19−28.
(17) Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. *Nat. Biotech*. **2002**, *20*, 301−305.
(18) Neville, D. C. A.; Rozanas, C. R.; Price, E. M.; Gruis, D. B.; Verkman, A. S.; Townsend, R. R. *Protein Sci*. **1997**, *6*, 2436−2445.
(19) Posewitz, M. C.; Tempst, P. *Anal. Chem*. **1999**, *71*, 2883−2892.
(20) Neville, D. C. A.; Rozanas, C. R.; Price, E. M.; Gruis, D. B.; Verkman, A. S.; Townsend, R. R. *Protein Sci*. **1997**, *6*, 2436−2445.
(21) Nuwaysir, L.; Stults, J. T. *J. Am. Soc. Mass Spectrom*. **1993**, *4*, 662−669.
(22) Watts, J. D.; Affolter, M.; Krebs, D. L.; Wange, R. L.; Samelson, L. E.; Aebersold, R., *J. Biol. Chem*. **1994**, *269*, 29520−29529.
(23) Figeys, D. G. S.; Zhang, Y.; Watts, J.; Gu, M.; Aebersold, R. *Electrophoresis* **1998**, *19*, 1811−1818.
(24) Cao P.; Stults, J. T. *Rapid Comm. Mass Spectrom*. **2000**, *14*, 1600−1606.
(25) Köcher, T.; Allmaier, G.; Wilm, M. *J. Mass Specrom*. **2003**, *38*, 131−137.
(26) Larsen, M. R.; Sorensen, G. L.; Fey, S. J.; Larsen, P. M.; Roepstorff, P. *Proteomics* **2001**, *1*, 223−238.
(27) Hirschberg, D.; Jagerbrink, T.; Samskog, J.; Gustafsson, M.; Stahlberg, M.; Alvelius, G.; Husman, B.; Carlquist, M.; Jornvall, H.; Bergman, T. *Anal. Chem*. **2004**, *76*, 5864−5871.
(28) Wind, M.; Wesch, H.; Lehmann, W. D. *Anal. Chem*. **2001**, *73*, 3006−3010.
(29) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem*. **1996**, *68*, 850−858.
(30) Li, X. J.; Liu, Y. S.; Kay, C. M.; Muller-Esterl, W.; Fliegel, L. *Biochemistry*. **2003**, *43*, 7448−7456.
(31) Fliegel, L.; Dyck, J. R. B.; Wang, H.; Fong, C.; Haworth, R. S. *Mol. Cell. Biochem*. **1993**, *125*, 137−143.
(32) Anspach, F. B. *J. Chromatogr*. A. **1994**, *672*, 35−49.
(33) Gimpel, M.; Unger, K. *Chromatographia* **1982**, *16*, 117−125.
(34) Vijayalakshmi, M. A. *Affinity Chromatogaphy and Biological Recognition*; Academic Press: New York, 1983.
(35) Dai, Y. Q.; Whittal, R. M.; Li, L. *J. Am. Soc. Mass. Spectrom*. **1999**, *10*, 512−520.
(36) Steen, H.; Pandey, A. *Tibtech*. **2002**, *20*, 361−364.
(37) Goodlett, D. R.; Keller, A.; Watts, J. D.; Newitt, R.; Yi, E. C.; Purvine, S.; Eng, J. K.; von Haller, P.; Aebersold, R.; Kolker, E. *Rapid Comm. Mass Spectrom*. **2001**, *15*, 1214−1221.
(38) Janek, K.; Wenschuh, H.; Bienert M.; Krause, E. *Rapid Comm. Mass Spectrom*. **2001**, *15*, 1593−1599.
(39) Ma, Y.; Lu, Y.; Zeng, H.; Ron, D.; Mo, W.; Neubert, T. *Rapid Comm. Mass Spectrom*. **2001**, *15*, 1693−1700.
(40) Bonewald, L. F.; Bibbs, L.; Kates, S. A.; Khatri, A.; McMurray, J. S.; Medzihradszky, K. F.; Weintraub, S. T. *J. Peptide Res*. **1999**, *53*, 53161−53169.
(41) Haydon, C. E.; Eyers, P. A.; Aveline-Wolf, L. D.; Resing, K. A.; Maller, J. L.; Ahn, N. G. *Mol. Cell Prot*. **2003,** *2*, 1055−1067.
(42) Wolfender, J.-L.; Chu, F.; Ball, H.; Wolfender F.; Fainzilber, M.; Baldwin, M. A.; Burlingame, A. L. *J. Mass Spectrom*. **1999**, *34*, 53447−53454.
(43) Liu, H.; Stupak J.; Zheng, J.; Keller, B.; Booth, B.; Li, L. *Anal. Chem*. **2004**, *76*, 4223−4232.
(44) Keller, B. O.; Wang, Z.; Li, L. *J. Chromatogr. B* **2002,** *25*, 317−329.
(45) Keller, B. O.; Li, L. *Anal. Chem*. **2001**, *73*, 2929−2936.
(46) Keller, B. O.; Li, L. *J. Am. Soc. Mass Spectrom*. **2000**, *11*, 88−93
(47) Whittal, R. M.; Keller, B. O.; Li, L. *Anal. Chem*. **1998**, *70*, 5344−5347.
(48) Arnott, D.; Gawinowicz, M. A.; Grant, R. A.; Neubert, T. A.; Packman, L. C.; Speicher, K. D.; Stone, K.; Trurck, C. W. J. *J. Biomol. Tech*. **2003**, *14*, 205−215.

PR049789T