

Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide Microcolumns*

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Reversible phosphorylation of proteins regulates the majority of all cellular processes, e.g. proliferation, differentiation, and apoptosis. A fundamental understanding of these biological processes at the molecular level requires characterization of the phosphorylated proteins. Phosphorylation is often substoichiometric, and an enrichment procedure of phosphorylated peptides derived from phosphorylated proteins is a necessary prerequisite for the characterization of such peptides by modern mass spectrometric methods. We report a highly selective enrichment procedure for phosphorylated peptides based on TiO₂ microcolumns and peptide loading in 2,5-dihydroxybenzoic acid (DHB). The effect of DHB was a very efficient reduction in the binding of nonphosphorylated peptides to TiO₂ while retaining its high binding affinity for phosphorylated peptides. Thus, inclusion of DHB dramatically increased the selectivity of the enrichment of phosphorylated peptides by TiO₂. We demonstrated that this new procedure was more selective for binding phosphorylated peptides than IMAC using MALDI mass spectrometry. In addition, we showed that LC-ESI-MSMS was biased toward monophosphorylated peptides, whereas MALDI MS was not. Other substituted aromatic carboxylic acids were also capable of specifically reducing binding of nonphosphorylated peptides, whereas phosphoric acid reduced binding of both phosphorylated and nonphosphorylated peptides. A putative mechanism for this intriguing effect is presented. *Molecular & Cellular Proteomics* 4: 873–886, 2005.

Phosphorylation is among the most widespread post-translational modifications in nature, and it has been estimated that more than 30% of the proteins in a given mammalian cell at some point during their expression are phosphorylated (1). Phosphorylation and dephosphorylation of proteins regulates a large number of biological processes such as signal transduction (2), molecular recognition and interaction, and other cellular events. A fundamental understanding of these biological processes at the molecular level thus requires a charac-

terization of the phosphorylated sites in the proteins. It is therefore essential to develop sensitive and selective methods for this task.

A wide variety of methods are known for characterization of phosphorylated proteins. The most widely used have been peptide sequencing using Edman degradation combined with ³²P labeling. This method is well established and very robust but has several limitations. For example, in Edman degradation the peptides have to be separated before the analysis using liquid chromatography. This decreases the overall sensitivity and increases analysis time, and it is therefore not well suited for analysis of complex samples.

Recently a number of MS-based strategies have been developed that are relatively sensitive and in many cases easier to perform than Edman degradation with respect to handling complex mixtures (e.g. Ref. 3). The increased sensitivity is especially needed for low stoichiometric phosphorylation. However, presently none of these MS-based methods can individually provide a complete characterization of a phosphorylated protein. For the MS-based strategies, it is common that the phosphorylated protein is enzymatically degraded to peptides, which are subsequently analyzed by MS to detect a mass increment of 80 Da per phosphate group. Because sulfonation gives the same mass shift, this strategy is often combined with phosphatase treatment to specifically cleave off the phosphate group from the peptide. This mass shift can be monitored by MS as a loss of 80 Da. This differential peptide mass mapping can be combined with purification of peptides using microcolumns packed with material of increasing hydrophobicity (4). In MALDI-TOF MS operating in reflector ion mode, the loss of phosphoric acid in the gas phase is often detected from phosphorylated peptides as a poorly resolved peak originating from metastable fragmentation (5). The exact site of phosphorylation can often be localized using tandem MS; however, the loss of phosphoric acid upon CID is frequently observed as the major fragmentation pathway, and this may interfere with the interpretation due to inadequate fragmentation of the peptide backbone.

The phosphate group is believed to have an effect on the ionization of phosphorylated peptides in MS, resulting in decreased signal intensity for phosphorylated peptides in the

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presence of non-phosphorylated peptides (*i.e.* an ion suppression phenomenon). Matrix additives like ammonium citrate (6) or phosphoric acid (7) have been shown to enhance the relative abundance of phosphorylated peptides in the presence of non-phosphorylated peptides in MALDI MS.

To reduce the suppression of phosphorylated peptides caused by the presence of non-phosphorylated peptides, it is advantageous to prepurify the phosphorylated peptides, especially from complex peptide mixtures. Enrichment of phosphorylated peptides from peptide mixtures using IMAC is widely used (8–14). With this approach the negatively charged phosphorylated peptides are purified by their affinity to metal ions like Fe^{3+} or Ga^{3+} . However, frequently non-phosphorylated peptides, including those containing multiple acidic residues, are also enriched by this method (15). Blocking the acidic residues by *O*-methyl esterification has been shown to enhance the specificity of the phosphopeptide binding (15). Nonetheless in our experience the yield of this derivatization is below 100%, which compromises the sensitivity of this procedure and increases the complexity of the sample. In addition, *O*-methyl esterification often causes a partial deamidation and subsequent methylation of Asn and Gln residues, and these byproducts complicate the MS analysis and data interpretation further (16). Furthermore this method requires evaporation of the aqueous solvent from the peptide sample prior to the addition of the esterification reagent, and this step is known to cause adsorptive losses resulting in decreased sensitivity (17, 18).

Chemical modification by β -elimination and concurrent Michael addition has also been widely used for affinity purification and quantitation of phosphorylated peptides (*e.g.* Ref. 19). However, this strategy suffers from several shortcomings including lack of reproducibility and sensitivity and introduction of unwanted side reactions (20).

Recently a promising strategy was introduced by Pinkse *et al.* (21) where titanium dioxide (TiO_2) was used as an alternative to IMAC for the selective enrichment of phosphorylated peptides prior to ESI liquid chromatography tandem MS. They used an on-line TiO_2 precolumn coupled directly to a reversed-phase capillary column, and with this setup successful analysis of various phosphorylated peptides was achieved. However, the selectivity of this method was somewhat compromised by the detection of several acidic non-phosphorylated peptides that were also retained by their TiO_2 column.

Here we present a new and improved procedure for using TiO_2 microcolumns that significantly enhanced the binding selectivity of TiO_2 toward phosphorylated peptides, thereby enabling phosphorylated peptide characterization from low femtomole level phosphorylated proteins. Phosphorylated peptides obtained from model proteins were used to optimize and test the procedure. In addition, the method was compared with the IMAC method.

EXPERIMENTAL PROCEDURES

Materials

Modified trypsin was from Promega (Madison, WI). Poros R2 and Poros Oligo R3 reversed-phase material were from PerSeptive Biosystems (Framingham, MA). GELoader tips were from Eppendorf (Hamburg, Germany). 2,5-Dihydroxybenzoic acid (DHB)¹ was from Fluka (St. Louis, MO). The 3M Empore™ C₈ disk was from 3M Bioanalytical Technologies (St. Paul, MN). Syringes for HPLC loading (P/N 038030, N25/500, 7C PKT 2) were from Scientific Glass Engineering (Victoria, Australia). The water was from a Milli-Q system (Millipore, Bedford, MA). Titanium dioxide beads were obtained from a disassembled TiO_2 cartridge (4.0-mm inner diameter, catalog number 5020–08520-5u- TiO_2) purchased from GL Sciences Inc. (Tokyo, Japan). All other chemicals and reagents were of the highest grade commercially available.

Model Proteins and Peptide Mixtures

Serum albumin (bovine), β -lactoglobulin (bovine), carbonic anhydrase (bovine), β -casein (bovine), α -casein (bovine), and ovalbumin (chicken) were from Sigma. Each protein was dissolved in 50 mM ammonium bicarbonate, pH 7.8 and treated with trypsin (1–2%, w/w) at 37 °C for 12 h.

Peptide Mixture 1—Peptide mixture 1 contained peptides originating from a tryptic digestion of 0.5 pmol of commercial α -casein.

Peptide Mixture 2—Peptide mixture 2 contained peptides originating from tryptic digestions of serum albumin, β -lactoglobulin, carbonic anhydrase, β -casein, α -casein, and ovalbumin. Peptide mixture 2 (ratios 1:1, 1:10, and 1:50) refers to a mixture of peptides originating from a tryptic digestion of 0.5 pmol of the phosphorylated proteins (β -casein, α -casein, and ovalbumin) and 0.5, 5, and 25 pmol of the non-phosphorylated peptides (serum albumin, β -lactoglobulin, and carbonic anhydrase), respectively.

Purification of Phosphorylated Peptides Using TiO_2 Microcolumns

TiO_2 microcolumns with a length of ~3 mm were packed in GELoader tips. A small plug of C₈ material was stamped out of a 3M Empore C₈ extraction disk using an HPLC syringe needle and placed at the constricted end of the GELoader tip. The C₈ disk serves only as a frit to retain the titanium dioxide beads within the GELoader tip. Note that the solvent used for either washing or loading the sample onto the TiO_2 microcolumn contains organic solvent (50–80% CH_3CN), which abrogates adsorption of peptides to the C₈ material. The TiO_2 beads were suspended in 80% acetonitrile, 0.1% TFA, and an aliquot of this suspension (depending on the size of the column) was loaded onto the GELoader tip. Gentle air pressure created by a plastic syringe was used to pack the column as described previously (22, 23).

The efficacy of four different procedures for selective binding of phosphorylated peptides was investigated. The first procedure (A) was adopted from the method published previously (21). In the following optimization (procedures A–D), peptide mixture 1 was used. In procedure A, peptides were loaded onto TiO_2 columns in 0.1 M acetic acid. The columns were washed with 20 μl of 80% acetonitrile, 0.1 M acetic acid, and the bound peptides were eluted with 3 μl of 250 mM ammonium bicarbonate, pH 9.0. An aliquot of the eluate (0.7 μl) was mixed with 0.3 μl of 2% TFA and 0.5 μl of DHB/PA matrix solution (DHB (20 g/liter) in 50% acetonitrile, 1% phosphoric acid) directly on the MALDI target. Procedure B was the same as procedure A with the

¹ The abbreviations used are: DHB, 2,5-dihydroxybenzoic acid; PA, phosphoric acid.

TABLE I
Overview of observed phosphorylated peptides derived by tryptic digestion of ovalbumin (Ov),
 α -casein S1 (α -S1) and S2 (α -S2), and β -casein (β -C)

The phosphorylation sites are underlined, and the amino acid position numbers are given in parentheses.

Peptide sequence	Number of phosphate groups	(M + H) ⁺
		Da
EVVGS <u>AEAG</u> VDAA (Ov-(340–352)) ^a	1	1254.52
EQL <u>ST</u> SEENSK (α -S2-(141–151))	1	1331.53
EQL <u>ST</u> SEENSK (α -S2-(141–151))	2	1411.50
TVDME <u>ST</u> EVFTK (α -S2-(153–164))	1	1466.61
TVDME <u>ST</u> EVFTKK (α -S2-(153–165))	1	1594.70
VPQLEIVPNSAEER (α -S1-(121–134))	1	1660.79
YLGEYLIVPNSAEER (α -S1) ^b	1	1832.83
DIG <u>S</u> ESTEDQAMEDIK (α -S1-(58–73))	1	1847.69
DIG <u>S</u> ESTEDQAMEDIK (α -S1-(58–73))	2	1927.69
YKVPQLEIVPNSAEER (α -S1-(119–134))	1	1951.95
FQSEEQQTEDQLDK (β -C-(33–48))	1	2061.83
EVVGS <u>AEAG</u> VDAASVSEEFr (Ov-(340–359))	1	2088.89
NVPGEIV <u>ES</u> LSSESISITR (β -C-(7–25)) ^c	4	2352.85
NTMEHV <u>SS</u> SEESISQETIK (α -S2-(17–36))	4	2619.04
VNELSKDIG <u>S</u> ESTEDQAMEDIK (α -S1-(52–73))	3	2678.01
QMEAES <u>IS</u> SSSEIVPNSVEAQK (α -S1-(74–94))	5	2720.91
NTMEHV <u>SS</u> SEESISQETIKQ (α -S2-(17–37)) ^d	4	2747.10
EKVNELSKDIG <u>S</u> ESTEDQAMEDIK (α -S1-(50–73))	3	2935.15
ELEELNVPGEIV <u>ES</u> LSSESISITR (β -C-(17–40))	4	2966.16
NANEEEEY <u>S</u> IGSSSEESAEVATEEVK (α -S2-(61–85))	4	3008.01
NANEEEEY <u>S</u> IGSSSEESAEVATEEVK (α -S2-(61–85))	5	3087.99
RELEELNVPGEIV <u>ES</u> LSSESISITR (β -C-(16–40))	4	3122.27
KNTMEHV <u>SS</u> SEESISQETIKQEK (α -S2-(16–39)) ^c	4	3132.20

^a Unusual cleavage (sequence was verified by MALDI tandem MS).

^b The peptide signal at m/z 1832.8 represents a new sequence variant of the α -S1 casein in the region 104–119 corresponding to the sequence YLGEYLIVPNSAEER (where pS is phosphoserine) in contrast to the published sequence ¹⁰⁴YKVPQLEIVPNSAEER¹¹⁹. This sequence could originate from an alternative splicing. The sequence was verified by MALDI tandem MS.

^c Chymotryptic cleavage (sequence was verified by MALDI tandem MS).

^d Sequence partly verified by MALDI tandem MS. Trypsin indicates Lys instead of Gln in the C terminus.

exception that the peptides were eluted with NH₄OH, pH 10.5. In procedure C, the peptides were loaded onto the TiO₂ columns in 0.1% TFA, and the columns were washed first with 10 μ l of the DHB solution (DHB (20 mg/ml) in 50% acetonitrile) and then with 10 μ l of 80% acetonitrile, 0.1% TFA before the bound peptides were eluted using 3 μ l of NH₄OH, pH 10.5. In procedure D, the peptides were loaded onto the TiO₂ column in DHB solutions of different concentrations (1–350 mg/ml in 80% acetonitrile, 0.1% TFA). The columns were washed with 10 μ l of the DHB solution and 20 μ l of 80% acetonitrile, 0.1% TFA. The bound peptides were eluted using 3 μ l of NH₄OH, pH 10.5.

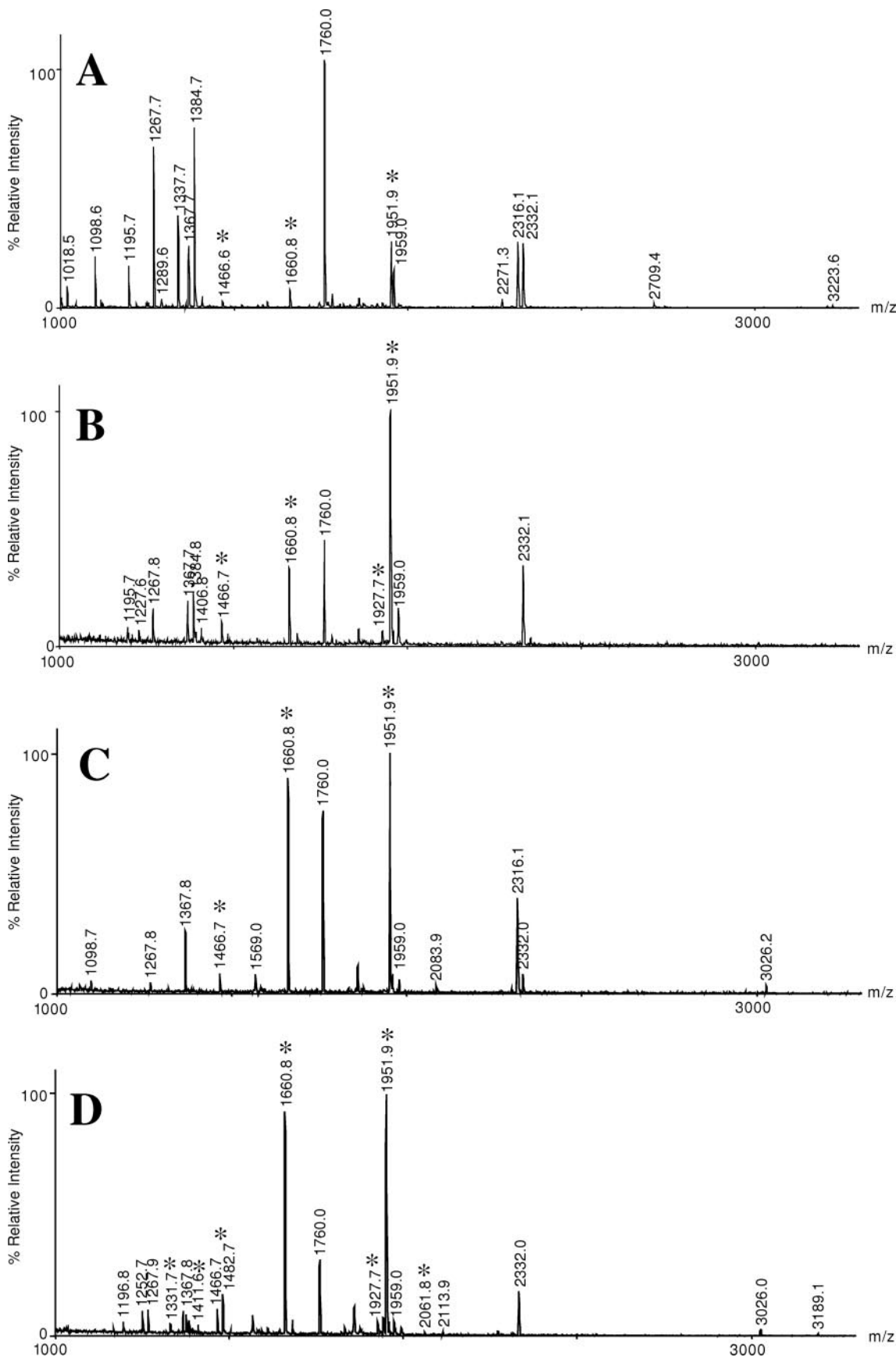
To investigate the selective adsorption of phosphorylated peptides relative to non-phosphorylated peptides a series of organic acid solutions were tested as loading/washing solvents. TiO₂ microcolumns were loaded with peptide mixture 2 (ratio, 1:1). The peptides were loaded onto the TiO₂ microcolumns in a 0.13 M solution of one of the following acids in 1:1 (v/v) H₂O/CH₃CN with 0.1% TFA: phosphoric acid, benzoic acid, cyclohexane-carboxylic acid, phthalic acid, salicylic acid, and 2,5-dihydroxybenzoic acid. In the case of acetic acid, TFA was omitted from the solution. After loading the peptides onto the TiO₂ columns, the columns were washed with 10 μ l of 50% acetonitrile, 0.1% TFA. Each column was eluted with 3 μ l of NH₄OH, pH 10.5. Each eluate was purified on a Poros Oligo R3 microcolumn (see below) prior to MALDI MS analysis. For LC-ESI-MSMS analysis the eluted peptides were purified by Poros Oligo R3 reversed-phase material and eluted by 50% acetonitrile, partly dried, and diluted to 13 μ l in 0.5% acetic acid.

Desalting and Concentration of Eluted Peptides

Custom made chromatographic reversed-phase microcolumns used for desalting and concentration of peptides were prepared using GELoader tips as described in detail earlier (22, 23). The eluates from the TiO₂ microcolumns were diluted in formic acid to a final concentration of 5% and applied onto Poros Oligo R3 microcolumns using gentle air pressure. The columns were washed with 20 μ l of 0.1% TFA. The retained peptides were eluted using 0.5 μ l of DHB/PA matrix solution directly onto the MALDI target.

IMAC

IMAC purification of phosphorylated peptides was performed according to Cole *et al.* (24) with minor changes. Briefly 40 μ l of iron-coated PHOS-selectTM metal chelate beads (Sigma) were washed two times in 100 μ l of washing/loading solution (0.25 M acetic acid, 30% acetonitrile) and resuspended in 40 μ l of washing/loading solution. An aliquot of this solution (20 μ l) was incubated with the peptide solution in a total volume of 40 μ l of washing/loading solution for 30 min with constant rotating. After incubation, the solution was loaded onto a constricted GELoader tip, and a gentle air pressure was used to pack the beads. Subsequently the beads were washed extensively with the washing/loading solution. The bound peptides were eluted using 3 μ l of NH₄OH, pH 10.5, and desalted using Poros R3 microcolumns prior to MALDI MS analysis (as describe above).



MALDI-TOF MS

MALDI MS was performed using a Voyager STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delayed extraction or a MALDI Q-TOF mass spectrometer (Micromass, Manchester, UK). All spectra were obtained in positive reflector mode. Mass spectrometric data analysis was performed using either the MoverZ software (www.proteometrics.com) or the software MassLynx 3.5. Sequence analysis and peptide assignment were accomplished using the GPMW software (welcome.to/gpmw). For analysis of phosphorylated peptides, DHB (20g/liter) in 50% acetonitrile, 1% phosphoric acid was used as the matrix. Note that inclusion of 1% phosphoric acid in the MALDI matrix solution increases the relative abundance of multiphosphorylated peptides (7). Because some of the test proteins used in this study (α - and β -casein) include several multiphosphorylated peptides the analyses were all performed using 1% phosphoric acid in the matrix solution (DHB/PA).

Nanoflow LC-ESI-MSMS

LC-ESI-MSMS analysis was performed using a Q-TOF Ultima mass spectrometer (Waters/Micromass UK Ltd., Manchester, UK) utilizing automated data-dependent acquisition. A nanoflow HPLC system (Ultimate, Switchos2, Famos, LC Packings, Amsterdam, The Netherlands) was used for chromatographic separation of the peptide mixture prior to MS detection. The peptides were concentrated and desalted on a precolumn (75- μ m inner diameter, 360- μ m outer diameter, Zorbax® SB-C18 3.5 μ m (Agilent, Wilmington, DE)) and eluted at 200 nl/min by an increasing concentration of acetonitrile (2%/min gradient) onto an analytical column (50- μ m inner diameter, 360- μ m outer diameter, Zorbax SB-C18 3.5 μ m (Agilent)). A MS-TOF survey spectrum was recorded for 1 s. The three most abundant ions present in the survey spectrum were automatically mass-selected and fragmented by collision-induced dissociation (4 s per MSMS spectrum). The MSMS data were converted to a pkl file format using the MassLynx 3.5 ProteinLynx software, and the resulting pkl file was searched against the NCBI protein sequence data bases using an in-house Mascot server (version 1.8) (Matrix Sciences, London, UK).

RESULTS AND DISCUSSION

Optimization of the Purification Procedure Using TiO₂ Microcolumns—Initial optimization of the procedure was performed using tryptic peptides originating from α -casein (*i.e.* peptide mixture 1, see “Experimental Procedures”). Commercial α -casein consists of α -casein-S1 and α -casein-S2, and the preparations are usually contaminated with traces of β -casein and statherin. A list of the theoretical tryptic phosphorylated peptides derived from α - and β -caseins and their molecular masses is shown in Table I. Evaluation of the phosphorylated peptide binding selectivity of the TiO₂ microcolumns and optimization of the washing/elution conditions was performed by comparing the relative intensities of the non-phosphorylated tryptic peptides with those of the phosphorylated peptides.

A direct analysis of a tryptic digestion of 0.5 pmol of commercial α -casein by MALDI MS using a dried droplet sample

preparation in which the peptide mixture is mixed with 0.1% TFA and DHB matrix solution (including 1% phosphoric acid) resulted in detection of a few of the theoretical phosphorylated peptides (Fig. 1A, marked with *asterisks*).

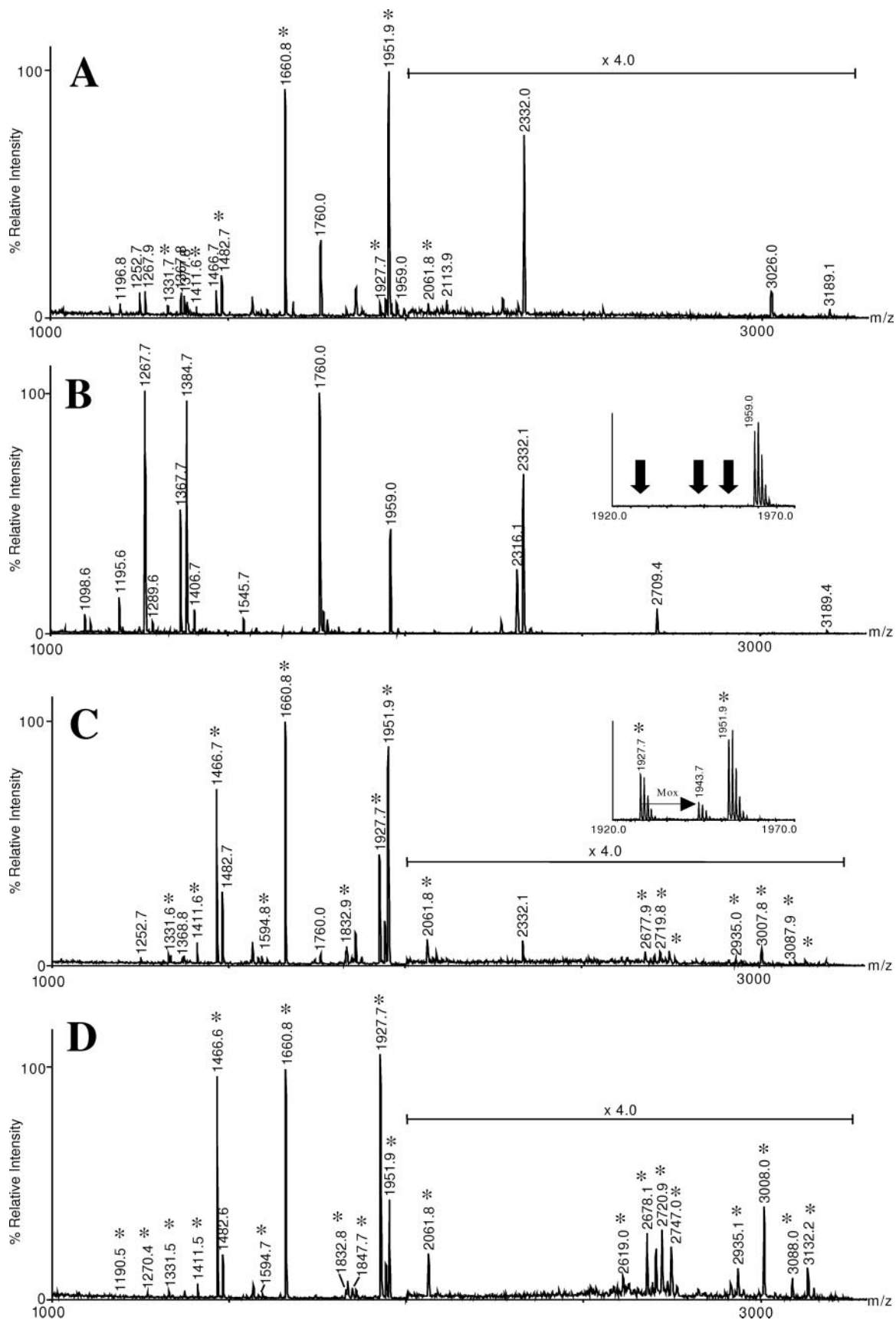
The MALDI MS spectrum obtained from the TiO₂ enrichment of phosphorylated peptides from peptide mixture 1 using the purification conditions as described by Pinkse *et al.* (21) (elution with 250 mM ammonium bicarbonate, pH 9.0) is shown in Fig. 1B. A significant number of non-phosphorylated peptides were observed together with four phosphorylated peptides (marked with *asterisks*). No signals were observed from the multiphosphorylated peptides.

After elution with 250 mM ammonium bicarbonate, pH 9.0, the same microcolumn was subsequently eluted with 3 μ l of NH₄OH, pH 10.5, and the MALDI MS analysis of 0.7 μ l of this solution yielded very abundant phosphorylated peptides (Fig. 1C), thereby demonstrating that elution with pH 9 only releases a small fraction of the adsorbed phosphorylated peptides, whereas pH 10.5 elutes most of the bound phosphorylated peptides. Subsequent elution using higher pH values did not result in further improvement in the recovery of phosphorylated peptides from the TiO₂ microcolumns.

In previous purification procedures for enrichment of phosphorylated peptides using both TiO₂ and IMAC 0.1–0.25 M acetic acid (pH 2.7–2.9) has been used as the loading buffer. The reason for choosing this pH value in the loading is to ensure that the acidic residues in the peptides are neutral, whereas the pK_{a1} value of phosphoric acid is 1.8, and therefore the phosphate group will still have a negative charge at pH 2.9. However, a significant amount of non-phosphorylated acidic peptides binds to either IMAC or TiO₂ under those conditions (15, 21). The substitution of an alkyl group onto an acidic phosphate oxygen atom increases the acidity, *e.g.* the pK_{a1} value of phosphoric acid decreases to 1.1 upon methylation (*i.e.* CH₃OPO(OH)₂) (25). Thus, we anticipate that the pK_{a1} value of the phosphate group also decreases when it is linked to a peptide. Therefore 0.1% TFA, which has a pH value of 1.9, was used in the following buffers for the purification of phosphorylated peptides using TiO₂.

A TiO₂ microcolumn was loaded with peptide mixture 1 in 0.1% TFA. After washing with 80% acetonitrile, 0.1% TFA, the phosphorylated peptides were eluted from the TiO₂ with 3 μ l of NH₄OH, pH 10.5, and the MALDI MS analysis of 0.7 μ l of this solution resulted in the MALDI spectrum shown in Fig. 1D. Here the intensity of the phosphorylated peptides increased relative to the non-phosphorylated peptides, indicating a more selective enrichment of phosphorylated peptides when 0.1% TFA was used as loading buffer. However, still a signif-

FIG. 1. **Optimization of the procedure for enrichment of phosphorylated peptides using TiO₂ microcolumns.** Shown are MALDI mass spectra obtained from peptide mixture 1 without TiO₂ enrichment (A), enriched by TiO₂ using acetic acid as loading buffer and NH₄HCO₃, pH 9.0 as elution buffer (B) and subsequent elution with NH₄OH, pH 10.5 (C), and enriched by TiO₂ using 0.1% TFA as loading buffer and NH₄OH, pH 10.5 as elution buffer (D). The phosphorylated peptides are marked with *asterisks*.



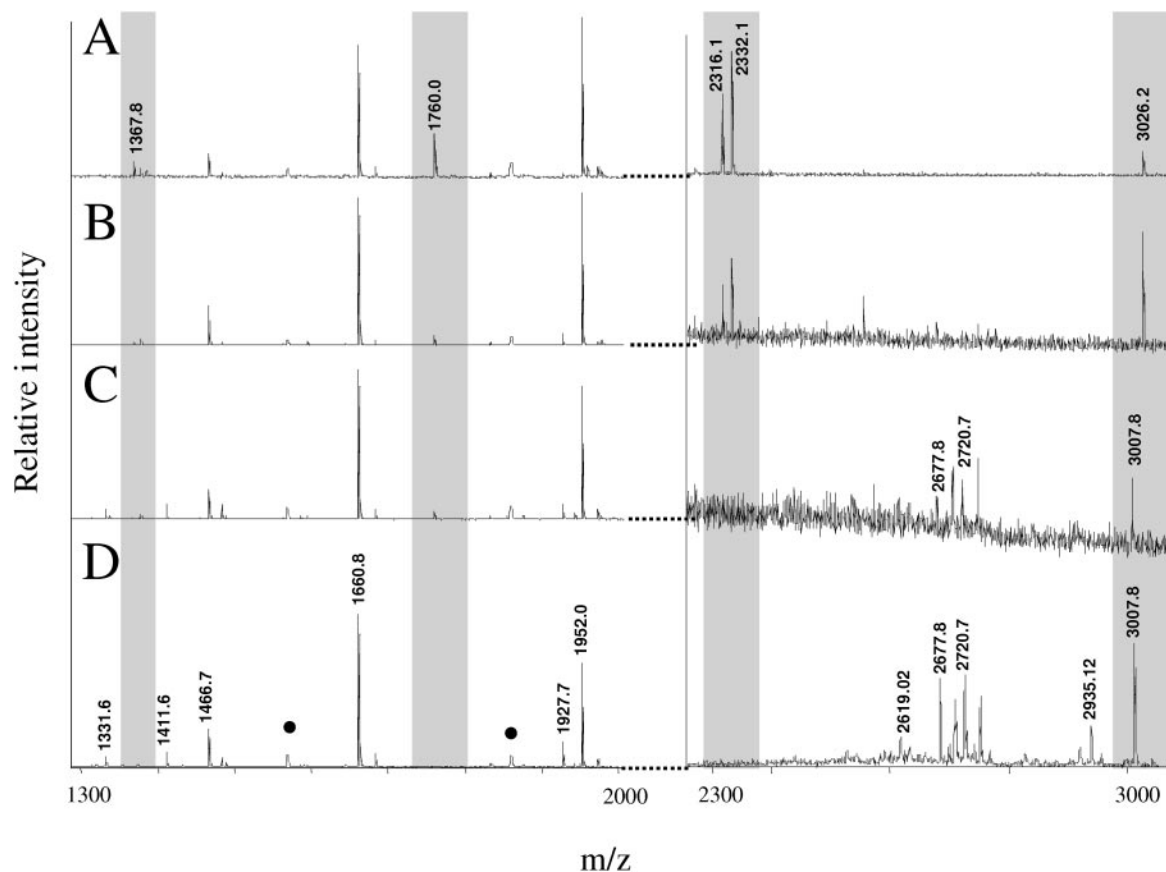


FIG. 3. The effect of the DHB concentration in the loading buffer on the selective enrichment of phosphorylated tryptic α -casein peptides using TiO_2 microcolumns. The MALDI mass spectra of TiO_2 -enriched peptides from peptide mixture 1 using 0, 1, 10, and 20 mg/ml DHB (in 80% acetonitrile, 0.1% TFA) are shown in A, B, C, and D, respectively. The circles mark the loss of phosphoric acid by metastable fragmentation, and the m/z ranges containing non-phosphorylated peptides are marked with gray boxes.

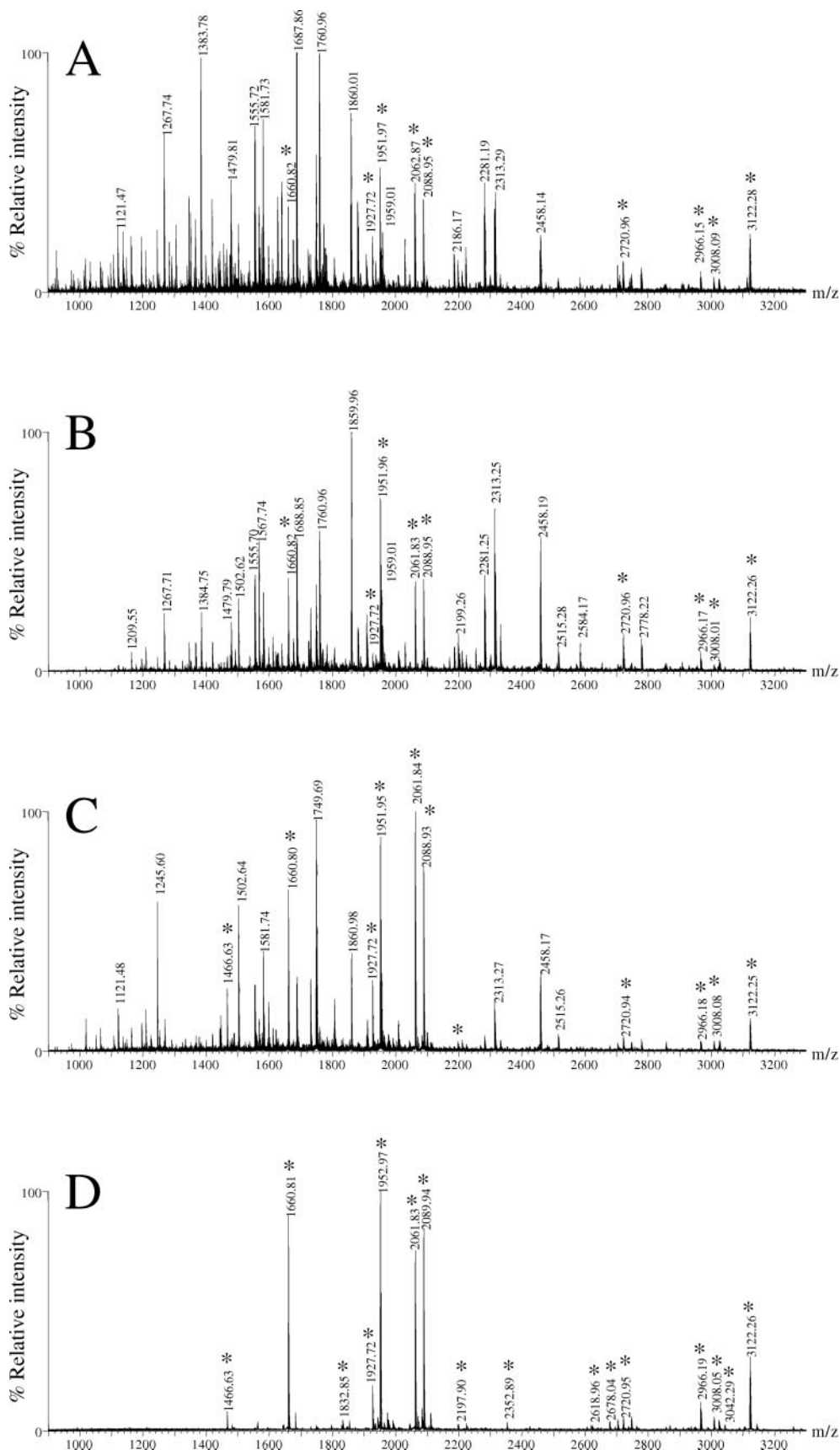
icant number of non-phosphorylated peptides were observed using this procedure.

Elution of phosphorylated peptides from IMAC material using the DHB matrix solution has previously been shown to increase the recovery of some phosphorylated peptides from this chromatographic material (26). Because the binding of phosphorylated peptides to TiO_2 is attributed to its ion exchange properties (21) and is similar to the binding observed in IMAC experiments, we attempted to elute the phosphorylated peptides with the DHB matrix solution.

The enrichment of phosphorylated peptides from peptide mixture 1 using TiO_2 loaded in 0.1% TFA followed by washing in 80% acetonitrile, 0.1% TFA and elution of the phosphorylated peptides with pH 10.5 is shown in Fig. 2A. Peptides from peptide mixture 1 were loaded onto a new TiO_2 microcolumn as above; however, after washing with 80% acetonitrile, 0.1%

TFA, the peptides were eluted directly onto the MALDI target using DHB matrix solution (20 mg/ml in 50% acetonitrile, 0.1% TFA). Phosphoric acid (1%) was added after the elution, and the eluate was subsequently analyzed by MALDI MS (Fig. 2B). Only non-phosphorylated peptides were detected as illustrated by the insets in the figure where arrows indicate the expected masses of some of the phosphorylated peptides. Thus, all phosphorylated peptides were retained by the TiO_2 resin after elution with the DHB matrix solution. Subsequent elution from the same column with NH_4OH , pH 10.5, recovered a larger number of phosphorylated peptides (Fig. 2C, marked with asterisks) compared with the procedure where the column was only washed with 80% acetonitrile, 0.1% TFA (Fig. 2A). In addition, a surprisingly low abundance and number of non-phosphorylated peptides were observed (four in total). In IMAC, DHB is sufficient to displace the bound phos-

FIG. 2. Optimization of the procedure for enrichment of phosphorylated peptides using TiO_2 microcolumns. Shown are MALDI mass spectra obtained from peptide mixture 1 enriched by TiO_2 using 0.1% TFA as loading buffer and NH_4OH , pH 10.5 as elution buffer (A), enriched by TiO_2 using 0.1% TFA as loading buffer and acidic DHB solution as elution buffer (B) and subsequent elution with NH_4OH , pH 10.5 (C), and enriched by TiO_2 using acidic DHB solution as loading buffer and NH_4OH , pH 10.5 as elution buffer (D). The insets show the m/z range 1920–1975. The phosphorylated peptides are marked with asterisks.



phorylated peptides, whereas the interaction between TiO_2 and the phosphate group appears to be much stronger and cannot be dissociated by DHB. However, acidic non-phosphorylated peptides can be displaced from TiO_2 by competitive binding of DHB thereby increasing the selective binding of phosphorylated peptides.

Because DHB is capable of displacing nonspecifically bound acidic peptides from the TiO_2 microcolumn, another experiment was performed in which peptide mixture 1 was applied onto a TiO_2 microcolumn in 20 μl of a DHB matrix solution (20 mg/ml in 80% acetonitrile, 0.1% TFA). The column was subsequently washed with 10 μl of the DHB solution and 20 μl of 80% acetonitrile, 0.1% TFA, respectively. The bound peptides were eluted with 3 μl of NH_4OH , pH 10.5, and 0.7 μl of this solution was mixed with 0.3 μl of 2% TFA and 0.5 μl of DHB/PA matrix solution on the MALDI target. The resulting MALDI peptide mass map is shown in Fig. 2D. Here a total of 20 signals were detected of which all represent phosphorylated peptides, and no significant signals were detected from non-phosphorylated peptides. Note the absence of the signal at m/z 1760. The flow-through from the loading was collected directly on the MALDI target and analyzed for the presence of phosphorylated peptides. Here only non-phosphorylated peptides could be detected (data not shown). The two very low abundant ions at m/z 1190.5 and m/z 1270.4 carrying one and two phosphate groups, respectively, originate from the contaminating protein statherin as verified by MALDI tandem MS (data not shown).

The Effect of the DHB Concentration on Phosphopeptide Isolation from Simple Mixtures—The concentration of DHB in the loading buffer was found to have a large effect on the adsorption of non-phosphorylated peptides onto TiO_2 . A series of experiments were performed using peptide mixture 1 and different concentrations of DHB in the loading buffer. Peptides from peptide mixture 1 were loaded onto equal length TiO_2 microcolumns in 0, 1, 10, and 20 mg/ml DHB (in 80% acetonitrile, 0.1% TFA), respectively. The MALDI mass spectra covering the mass range 1000–2000 and 2300–3100 Da obtained from the elution with NH_4OH , pH 10.5, are shown in Fig. 3, A–D. This figure shows that the number of non-phosphorylated peptides decreases with the increasing concentration of DHB. In the experiment where the peptides were loaded in 80% acetonitrile, 0.1% TFA alone, five signals from non-phosphorylated peptides were observed (Fig. 3A, shaded areas). Inclusion of as little as 1 mg/ml DHB significantly decreased the abundance of these peptides. A further increase in the concentration of DHB to 20 mg/ml completely abrogated the adsorption of these non-phosphorylated peptides to TiO_2 as is evident by their absence in the mass

spectra. Interestingly the multiphosphorylated peptides in the m/z 2300–3100 ranges are only observed at DHB concentrations above 10 mg/ml. This indicates a pronounced suppression of the ionization of multiphosphorylated peptides in the presence of non-phosphorylated peptides.

Purification of Phosphorylated Peptides from Semicomplex Peptide Mixtures Using TiO_2 Microcolumns—The previous analyses using TiO_2 microcolumns were performed with peptides derived from a single protein (and low amounts of contaminating proteins). Here a semicomplex peptide mixture (peptide mixture 2 (ratio, 1:1), see “Experimental Procedures”) was analyzed by TiO_2 microcolumns using the optimized procedure. In this mixture a total of a minimum 18 phosphorylated peptides (listed in Table I) are present. The theoretical number of peptides derived from the six proteins by tryptic digestion is 296, allowing for one missed cleavage and a mass range of 700–3500 Da. Peptides from peptide mixture 2 (ratio, 1:1) were analyzed by MALDI MS using the normal dried droplet method (Fig. 4A). Here only nine phosphorylated peptides could be detected (marked with asterisks) probably due to the ion suppression effect caused by the non-phosphorylated peptides. A similar amount of peptides from peptide mixture 2 (ratio, 1:1) was applied onto a TiO_2 microcolumn using the procedure described by Pinkse *et al.* (21). The peptides were eluted off the column using 3 μl of NH_4OH , pH 10.5, and the eluted peptides were subsequently purified using a Poros Oligo R3 microcolumn from which the peptides were eluted directly onto the MALDI target using the DHB/PA matrix solution. The resulting MALDI MS peptide mass map is shown in Fig. 4B. Here the same nine phosphorylated peptides could be detected; however, a significant amount of non-phosphorylated peptides was observed in the eluate. The experiment was repeated using 0.1% TFA instead of 0.1 M acetic acid in the loading procedure. The resulting MALDI MS peptide mass map is shown in Fig. 4C. Here a significant amount of non-phosphorylated peptides was still observed in the eluate, but the relative signal intensity of the phosphorylated peptides was markedly increased compared with loading in acetic acid. In addition, two extra phosphorylated peptides could be detected.

Loading peptide mixture 2 (ratio, 1:1) in DHB (300 mg/ml in 80% acetonitrile, 0.1% TFA) resulted in the selective purification of phosphorylated peptides with hardly any “contamination” with non-phosphorylated peptides (Fig. 4D). Here a total of 16 phosphorylated peptides was detected. The purification of the eluate using Poros R3 resulted in the loss of at least two phosphorylated peptides (m/z 1331.5 and m/z 1411.5) because they do not bind to this reversed-phase material. However, these two phosphorylated peptides were detected by

FIG. 4. **Enrichment of phosphorylated peptides from peptide mixture 2 (ratio, 1:1).** Shown are MALDI mass spectra obtained without TiO_2 enrichment (A), enriched by TiO_2 using acetic acid as loading buffer (B), enriched by TiO_2 using 0.1% TFA as loading buffer (C), and enriched by TiO_2 using DHB (300 mg/ml) as loading buffer (D). The phosphorylated peptides are marked with asterisks.

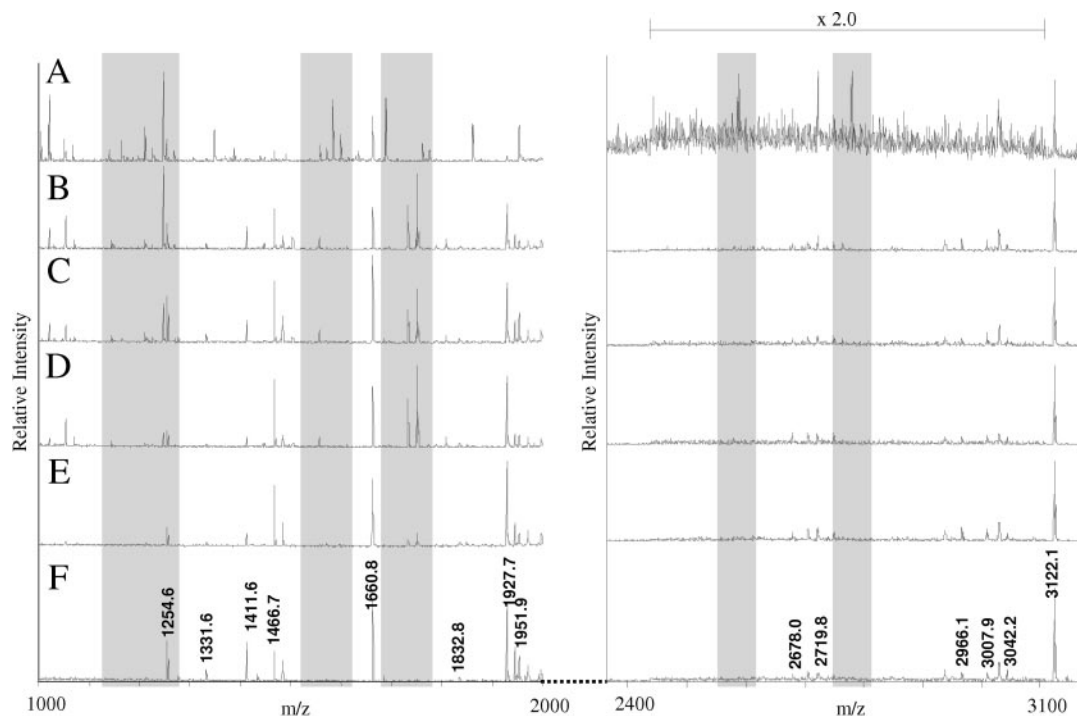


FIG. 5. The effect of the DHB concentration on the selective enrichment of phosphorylated peptides by TiO_2 obtained from peptide mixture 2 (ratio, 1:1). The MALDI mass spectra obtained from TiO_2 enrichments in a loading buffer containing 0, 10, 20, 50, 100, and 200 mg/ml DHB are shown in A, B, C, D, E, and F, respectively. The phosphorylated peptides are labeled, and the m/z ranges containing non-phosphorylated peptides are marked with gray boxes.

MALDI MS after purification of the flow-through from the R3 column by using a graphite microcolumn (4) (data not shown).

The absolute abundance of the phosphorylated peptides was markedly increased when using the DHB as loading buffer compared with either acetic acid or TFA despite the same amount of starting material. The same observation was made in all the other experiments performed in this study. This indicates a more efficient ionization for phosphorylated peptides in the absence of non-phosphorylated peptides.

The Effect of the DHB Concentration on Phosphopeptide Isolation from Semicomplex Mixtures—The effect of the inclusion of DHB in the loading and washing procedure for complex mixtures was investigated using peptide mixture 2 (ratio, 1:1). Peptides from peptide mixture 2 (ratio, 1:1) were applied onto TiO_2 microcolumns of the same length in 0, 10, 20, 50, 100, and 200 mg/ml DHB (in 80% acetonitrile, 0.1% TFA), respectively. The resulting MALDI peptide mass maps obtained from 0.7 μl of each of the elutions (performed with 3 μl of NH_4OH , pH 10.5) is shown in Fig. 5, A–F. The absence of DHB caused a high number of non-phosphorylated peptides to bind to the columns. The number of non-phosphorylated peptides decreased with the increasing concentration of DHB up to 200 mg/ml where no non-phosphorylated peptides were observed. In addition, the multiphosphorylated peptides were more clearly detected when a higher concentration of DHB was used, probably due to decreased ion suppression effect.

This clearly indicates that when the sample is more complex a higher concentration of DHB is needed to exclude the binding of non-phosphorylated peptides. For very complex samples a DHB concentration of 300–400 mg/ml (close to a saturated solution) is highly recommended.

Comparison of the Performance with IMAC for Semicomplex Samples—The selective enrichment of phosphorylated peptides using TiO_2 microcolumns was compared with IMAC. Peptides from peptide mixture 2 (ratios 1:1, 1:10, and 1:50) were purified using TiO_2 microcolumns and IMAC beads (PHOS-select (Sigma)), respectively. The resins were in both cases eluted with NH_4OH , pH 10.5, and the eluted peptides were further purified using Poros Oligo R3 microcolumns and eluted from this column directly onto the MALDI MS target using 0.7 μl of the DHB/PA matrix solution. Elution with DHB from IMAC beads has been shown to improve the recovery of phosphorylated peptides. However, in this study NH_4OH , pH 10.5 was used to directly compare the binding selectivity of phosphorylated between TiO_2 and IMAC. In addition, elution with DHB matrix solution limits the possibility for downstream applications like liquid chromatography coupled to MS. The resulting MALDI peptide mass maps obtained from the TiO_2 purifications are shown in Fig. 6, A–C, and the MALDI peptide mass maps obtained from the IMAC purifications are shown in Fig. 6, D–F. The signals corresponding to the detected phosphorylated peptides are indicated by dots in Fig. 6, A and D.

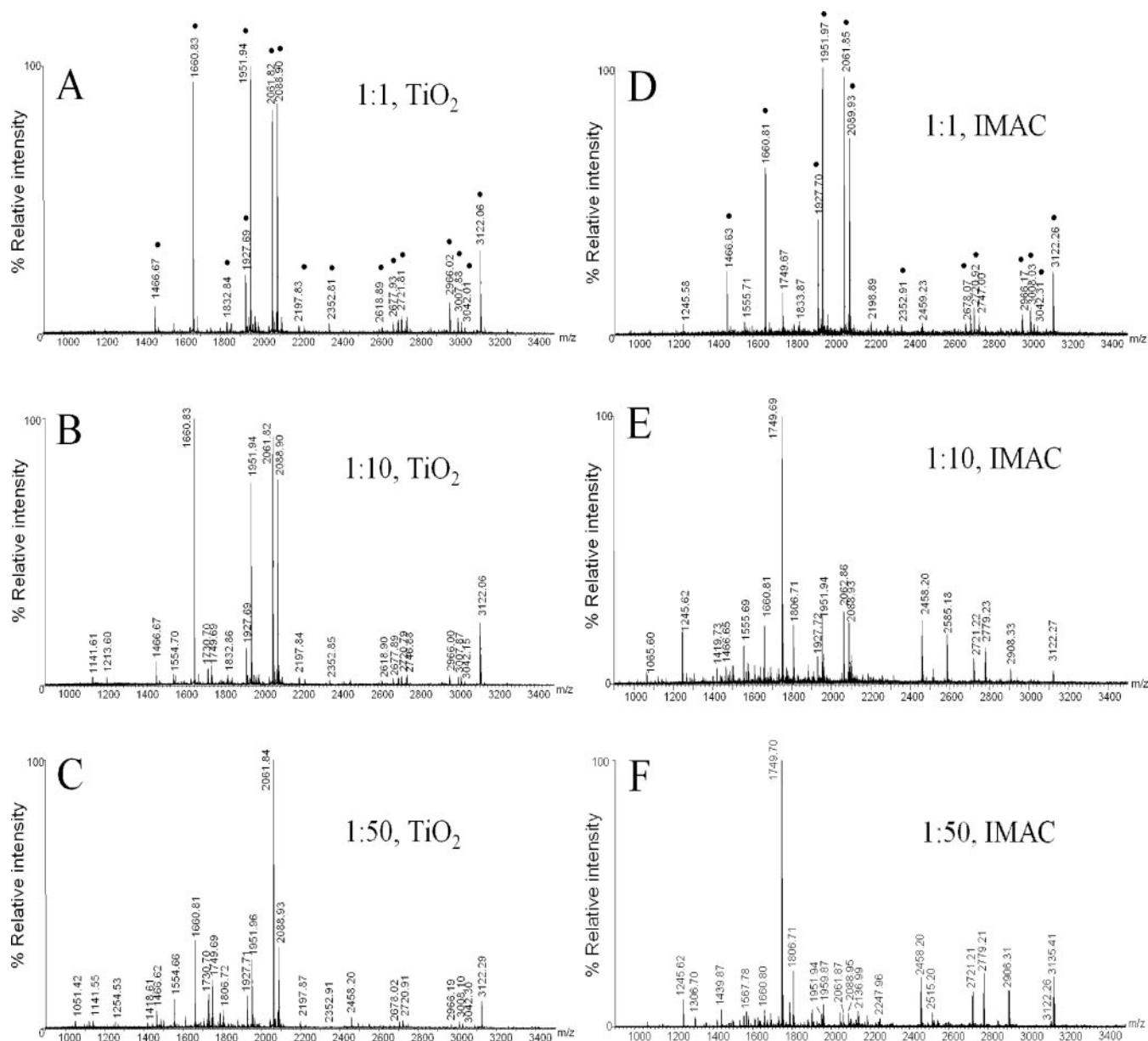


FIG. 6. Comparison of the performance of TiO₂ microcolumns and IMAC beads for the selective enrichment of phosphorylated peptides from complex mixtures. Phosphorylated peptides from peptide mixture 2 with the ratios 1:1, 1:10, and 1:50 were enriched by TiO₂ or IMAC (PHOS-select). MALDI mass spectra obtained from TiO₂ enrichments of the phosphorylated peptides in the three peptide mixtures are shown in A, B, and C, respectively. The MALDI mass spectra of similar enrichments using IMAC beads are shown in D, E, and F, respectively. The phosphorylated peptides detected here are marked with dots in A and D.

With peptide mixture 2 (ratio, 1:1) the two purification methods performed almost equally well with respect to number of detected phosphorylated peptides. However, a significantly higher number of non-phosphorylated peptides were observed in the IMAC experiment. With increasing ratios (1:10 and 1:50) the performance of the TiO₂ method significantly surpassed the IMAC method with respect to number of detected phosphorylated peptides and reduction of the number of non-phosphorylated peptides present in the eluate (e.g. Fig. 6, C and F). This indicates a much more selective binding

of the phosphorylated peptides on the TiO₂ microcolumn than on the IMAC resin. Optimization of the IMAC procedure, e.g. by loading in a more acidic condition, might improve the selectivity of the IMAC method.

Comparing the Performance of MALDI MS with LC-ESI-MS/MS for the Analysis of Phosphorylated Peptides Purified by TiO₂ Microcolumns—An aliquot of peptide mixture 2 (ratio, 1:1) (500 fmol) was loaded onto a TiO₂ microcolumn in DHB solution (350 mg/ml in 80% acetonitrile, 0.1% TFA), and the bound phosphorylated peptides were eluted by NH₄OH, pH

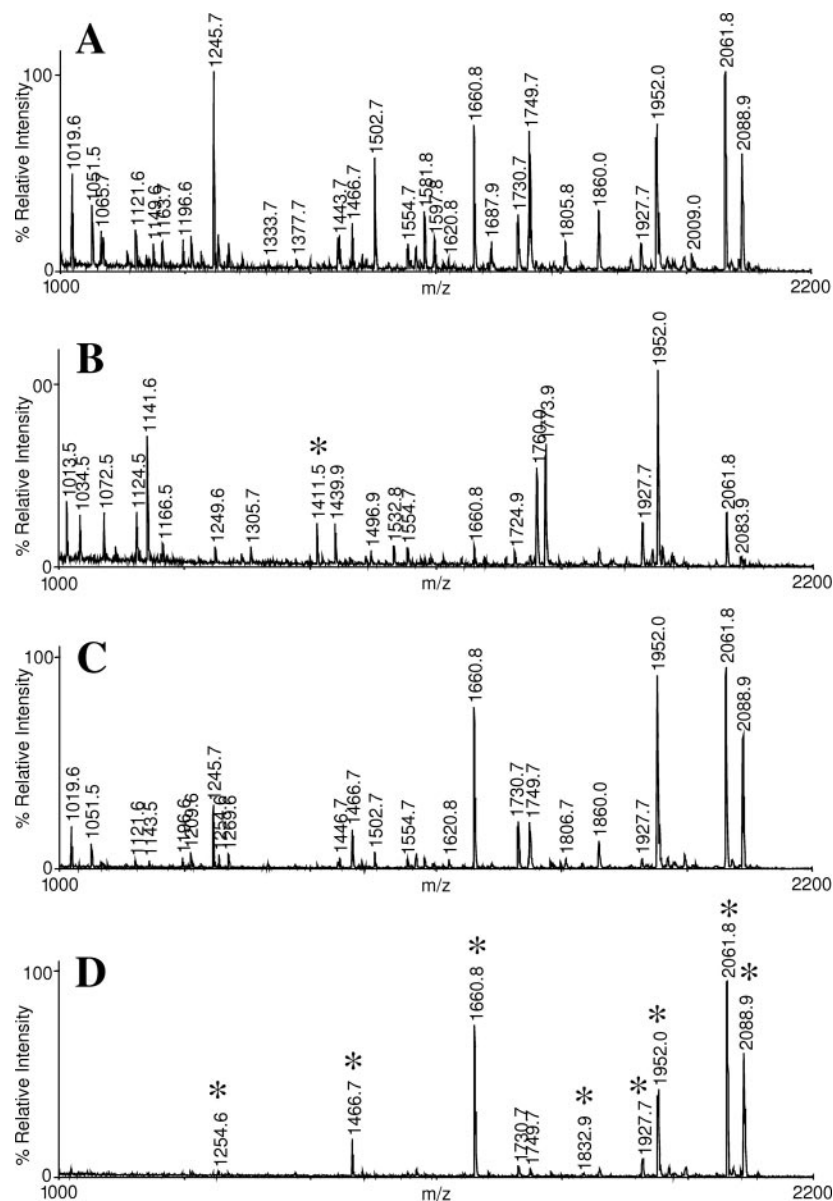


FIG. 7. The effect of various acids on the selective binding of phosphorylated peptides to TiO_2 . The peptides were obtained from peptide mixture 2 (ratio, 1:1). Shown are MALDI mass spectra obtained from TiO_2 enrichment using a loading buffer of 0.1% TFA (A), 0.13 M phosphoric acid (B), 0.13 M benzoic acid (C), and 0.13 M DHB (D). The asterisks in B and D mark phosphorylated peptides.

10.5. This peptide solution was diluted with 0.5% acetic acid and analyzed by LC-ESI-MS/MS. The resulting fragment ion spectra were searched by the Mascot data base search program, and a total of eight phosphorylated peptides were identified (the identified phosphorylated peptides according to Table I: α -S1 casein: m/z 1660.8, 1951.9, 2693.9 (2678 + oxidation); α -S2 casein: m/z 1331.5, 1411.5, 1466.6; β casein: m/z 2061.8; ovalbumin: m/z 2088.9) (data not shown). In addition to the phosphorylated peptides, five non-phosphorylated peptides were identified. Compared with the results obtained with MALDI MS (see e.g. Fig. 4D) where more than 16 phosphorylated peptides were observed, the LC-ESI-MS/MS clearly showed a bias toward monophosphorylated peptides as several multiphosphorylated peptides were not detected by the LC-ESI-MS/MS analysis. Their absence in the LC-ESI-MS/MS

analysis was manually validated. Data presented by Gruhler *et al.* (14) support this finding as they observed a significantly lower amount of multiphosphorylated peptides compared with singly phosphorylated peptides using LC-ESI-MS/MS. A similar effect has been observed in a number of ongoing studies by our group using both MALDI tandem MS and LC-ESI-MS/MS.² The reason for this bias is presently not known.

Investigating the Mechanism for the Selective Enrichment of Phosphorylated Peptides by TiO_2 —It is clear from the results presented above that the presence of DHB in the loading buffer dramatically enhances the selective retainment of phosphorylated peptides on TiO_2 . We attribute this effect to a

² M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff, and T. J. D. Jørgensen, unpublished results.

competition for binding sites on TiO_2 between non-phosphorylated peptides and DHB molecules. The large molar excess of DHB thus effectively competes with non-phosphorylated peptides for adsorption to the surface of TiO_2 , whereas phosphorylated peptide binding is virtually unaffected. To investigate the molecular determinants of DHB that are responsible for this intriguing effect we selected a number of benzoic acid derivatives as well as other acids and determined their effect on the selective adsorption of phosphorylated peptides from complex peptide mixtures. Peptides from peptide mixture 2 (ratio, 1:1) were applied onto TiO_2 microcolumns in 50% acetonitrile containing one of the following acids: trifluoroacetic acid, acetic acid, phosphoric acid, benzoic acid, salicylic acid, cyclohexane-carboxylic acid, phthalic acid, and DHB. The bound peptides were eluted using NH_4OH , pH 10.5 and desalted and concentrated on Poros Oligo R3 microcolumns prior to MALDI MS analysis. The phosphorylated peptide binding selectivity was evaluated by comparing the relative abundances of these peptides with those of non-phosphorylated peptides. Fig. 7 shows MALDI mass spectra obtained from TiO_2 enrichment of phosphorylated peptides using four different acids in the loading buffer (TFA, phosphoric acid, benzoic acid, and DHB). The spectra clearly show that DHB was the most efficient acid to prevent adsorption of nonphosphorylated peptides while retaining the ability of TiO_2 to bind phosphorylated peptides. In contrast, phosphoric acid was not as effective as DHB to reduce binding of nonphosphorylated peptides, and it appeared to inhibit the adsorption of some of the phosphorylated peptides. For example, the relative abundances of the phosphorylated peptides at m/z 2088.9, 1660.8, and 1466.7 were dramatically reduced when the loading buffer contained phosphoric acid, whereas the relative abundances of these peptides were rather similar in the case of the other acids. Using salicylic acid or phthalic acid in the loading buffer yielded spectra very similar to those obtained from DHB, whereas cyclohexane carboxylic acid gave results very close to that of benzoic acid (data not shown). The efficacy in inhibiting adsorption of nonphosphorylated peptides follows the order $\text{DHB} \sim \text{salicylic acid} \sim \text{phthalic acid} > \text{benzoic acid} \sim \text{cyclohexane carboxylic acid} > \text{phosphoric acid} > \text{TFA} > \text{acetic acid}$. Thus, the substituted aromatic carboxylic acids (*i.e.* DHB, salicylic acid, and phthalic acid) are markedly better competitors than the monofunctional carboxylic acid (*i.e.* TFA, acetic acid, cyclohexane-carboxylic acid, and benzoic acid) for preventing binding of non-phosphorylated peptides to TiO_2 . In line with this observation, infrared spectroscopic studies have shown that substituted aromatic carboxylic acids (including salicylic acid and phthalic acid) coordinate strongly to the surface of TiO_2 , whereas monofunctional carboxylic acids (including benzoic acid and acetic acid) only interact very weakly with TiO_2 (27). Interestingly phosphate binds to TiO_2 with affinity ($K_A = 4 \times 10^4 \text{ M}^{-1}$) similar to substituted aromatic carboxylic acids ($K_A = 10^4\text{--}10^5 \text{ M}^{-1}$) (28), but it appears to be signifi-

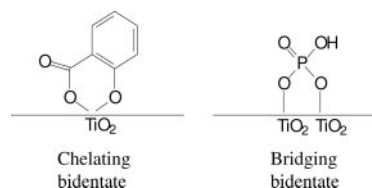


FIG. 8. Binding modes of phosphate and salicylate species adsorbed to TiO_2 (adapted from Refs. 27 and 28).

cantly less effective in preventing binding of nonphosphorylated peptides. Clearly a high TiO_2 binding affinity of the acidic loading buffer is not the decisive factor for the enhancement of phosphorylated peptide binding selectivity. In this context, it is important to note that the binding mode of phosphate to TiO_2 differs from that of substituted aromatic carboxylic acids. For example, the binding mode of salicylic acid to TiO_2 is a chelating bidentate salicylate species (27, 29), whereas the adsorption of phosphate anions to the surface of TiO_2 is a bridging bidentate surface complex (28) (see Fig. 8). The coordination geometry for an optimal phosphate binding site on TiO_2 is thus likely to differ from that of an optimal binding site for a substituted carboxylic acid. In this picture, phosphate will compete directly with phosphorylated peptides for binding sites on TiO_2 , whereas DHB targets other binding sites that appear to be similar to those preferred by non-phosphorylated peptides. Such nonequivalent binding sites on TiO_2 may result from a structural heterogeneity of the TiO_2 surface, but the adsorbate itself is also likely to generate nonequivalent binding sites on TiO_2 by the ability of TiO_2 to transmit inductive electronic effects over a few rows of atoms (30). Furthermore steric hindrance does also appear to play a role in preventing binding of non-phosphorylated peptides because bulky monofunctional carboxylic acids (*e.g.* cyclohexane-carboxylic acid) are more effective than less bulky acids (*e.g.* acetic acid). In conclusion, we attribute the selective enhancement of phosphorylated peptide binding by DHB to an effective competition predominantly with non-phosphorylated peptides for binding sites on TiO_2 . This effect is achieved by the existence of a heterogeneous array of adsorption sites on TiO_2 .

Conclusion—We used DHB to enhance the selective enrichment of phosphorylated peptides by TiO_2 adsorption. This novel methodology resulted in a remarkable increase in the selectivity of purification of phosphorylated peptides from complex mixtures of non-phosphorylated and phosphorylated peptides. In direct comparison with IMAC, our procedure proved superior in terms of selectivity and sensitivity of phosphorylated peptide binding. In addition, the TiO_2 purification was fast (typically less than 5 min per sample) and can be used in combination with high performance liquid chromatography coupled to either MALDI-MSMS or ESI-MSMS. However, the bias toward monophosphorylated peptides in LC-ESI-MSMS observed in this study clearly shows that both mass spectrometric methods should be applied in the analysis of purified phosphorylated peptides.

The mechanism of the selective enrichment of phosphorylated peptides by TiO_2 in combination with DHB was investigated by using other substituted aromatic acids as well as other acids. We attribute the enhancement of phosphorylated peptide binding selectivity to an effective competition between DHB and non-phosphorylated peptides for binding sites on TiO_2 .

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