



## BRIEF COMMUNICATIONS

# Identification of WEHI-231 Phosphoproteins and Phosphorylation Sites Using IMAC and LC-MS/MS\*

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*Abstract: A goal of the Alliance for Cellular Signaling (AfCS) Protein Chemistry Laboratory is the identification of phosphoproteins in mouse B lymphocytes (B cells). In order to identify phosphoproteins on a proteome-wide basis, WEHI-231 cells were treated with calyculin A, a serine/threonine phosphatase inhibitor, or left untreated. Proteins were extracted from whole cell lysates and treated with trypsin to generate peptides. Phosphorylated peptides were enriched using immobilized metal affinity chromatography (IMAC) and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 107 proteins were identified using these methods. Forty-two of these proteins were previously reported to be phosphorylated, and 17 of them are on the AfCS protein list. The list also includes 11 proteins that appear to be completely novel. A total of 193 phosphorylation sites were identified within these proteins.*

\*A version of this data has been published in *Molecular and Cellular Proteomics*, January 17, 2004 (Epub ahead of print). [\[PubMed\]](#)

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## Introduction

An important goal of the AfCS Protein Chemistry Laboratory is the global analysis of ligand-induced changes in protein phosphorylation. Important steps in this process are the identification of phosphoproteins present in the AfCS model cell systems and the determination of their sites of phosphorylation. This information will be used to generate phospho-specific antibodies that will provide quantitative information on the effects of ligands on phosphorylation of specific sites. Recent improvements in phosphopeptide enrichment by immobilized metal affinity chromatography (IMAC) and in mass spectrometry make it possible to identify phosphoproteins on a proteome-wide basis (1). We used this approach to identify phosphorylation sites in whole cell lysates from the WEHI-231 B cell line. In this communication, we present the identification of phosphoproteins and their phosphorylation sites in control and calyculin A-treated WEHI cells.

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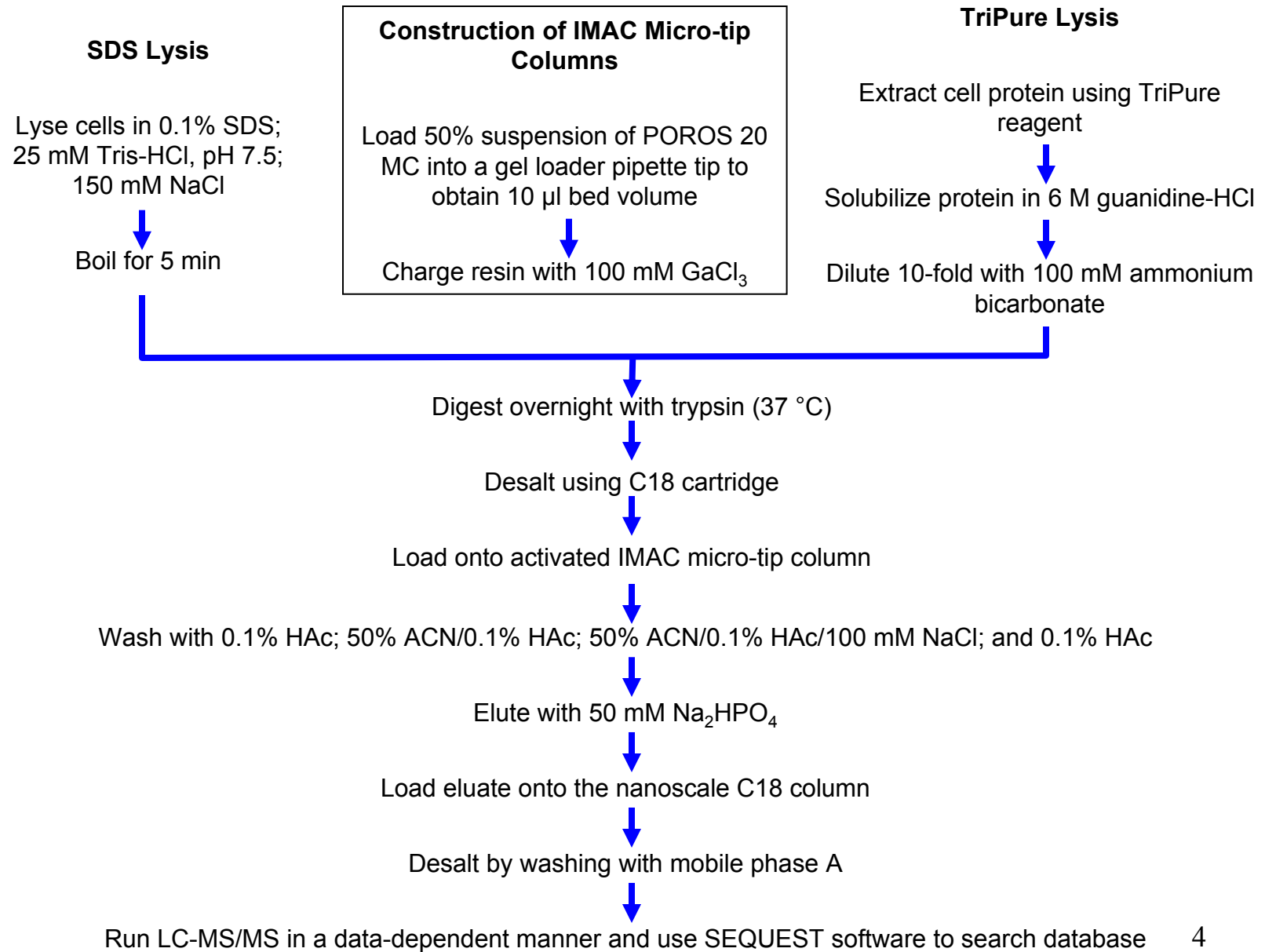


## Methods

- WEHI-231 cells were either left untreated or treated with 100 nM calyculin A for 45 min according to AfCS protocol [PS00000537](#).
- Cells were lysed and the protein was extracted using TriPure reagent according to protocol [PP00000162](#), or the cells were lysed in a solution containing 0.1% sodium dodecyl sulfate (SDS; see [Supplemental Methods](#)).
- Immobilized metal affinity chromatography (IMAC) columns were prepared according to protocol [PP00000163](#), or as described in [Supplemental Methods](#), to compare different resins.
- Proteins were digested with trypsin and the phosphopeptides were enriched by IMAC according to protocol [PP00000164](#).
- Proteins were identified by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using a nanoscale C18 column coupled in-line with an ion trap mass spectrometer according to protocol [PP00000164](#).
- The mass spectra and MS/MS data were used to search the nonredundant NCBI mouse protein database using SEQUEST software. Software parameters were set to detect a modification of 80 Da on Ser, Thr, or Tyr.
- The assignments of phosphopeptide sequences were then manually confirmed by comparing the acquired MS/MS spectra to the theoretical fragmentation pattern.

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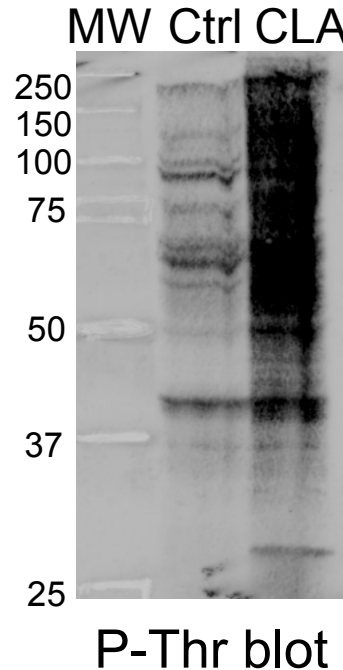
# *Flow chart of IMAC procedure for the enrichment of phosphorylated proteins from whole cell lysates*



## Results

### *Generation of phosphorylated proteins by calyculin A treatment*

To maximize detection and identification of phosphoproteins, cells were treated with the serine/threonine phosphatase inhibitor calyculin A (CLA). A Western blot with an anti-P-Thr antibody shows the increase in threonine phosphorylation upon CLA treatment (Fig. 1).



**Fig. 1. Generation of phosphorylated proteins by calyculin A treatment.** WEHI-231 cells were either left untreated (Ctrl) or treated with 20 nM CLA for 45 min (CLA) and lysed in SDS sample buffer. The proteins were resolved on a 10% SDS gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-phosphothreonine polyclonal antibody P-Thr-polyclonal (Cell Signaling Technologies). The mass of each molecular weight marker (MW) is shown at the left.

## ***Comparison of IMAC resins for the enrichment of phosphopeptides***

To optimize enrichment of phosphopeptides, different metal chelating resins were charged with either iron or gallium and tested with a known mixture of phosphorylated and non-phosphorylated peptides. Of the resins tested, Ga<sup>3+</sup>-charged POROS 20 MC and BRX-IDA resins resulted in the lowest background and highest recovery of each of the phosphopeptides (see Fig. 2). When compared to Fe<sup>3+</sup>, the resins charged with Ga<sup>3+</sup> resulted in a higher signal-to-noise ratio with our test sample.

## ***Enrichment of phosphorylated peptides by IMAC***

In order to test the IMAC procedure under more rigorous conditions, a test sample containing 1 pmol of a synthetic P-tyr phosphopeptide, 2 pmol alpha-casein, and 400 pmol of four non-phosphorylated proteins was used. Samples from each step of the procedure were monitored using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Fig. 3). The phosphopeptides were not detected in the total digest due to the abundance of non-phosphorylated peptides present. The total digest was loaded onto a Ga<sup>3+</sup>-charged BRX-IDA column. None of the phosphopeptides were detected in either of the wash fractions. Analysis of the material eluted with 200 mM Na<sub>2</sub>HPO<sub>4</sub> showed that all four phosphopeptides were recovered with very little contamination by non-phosphorylated peptides.

## ***Identification of proteins enriched by IMAC***

Proteins were identified using LC-MS/MS. The mass spectra and MS/MS data (Fig. 4) were used to search the nonredundant NCBI mouse protein database using SEQUEST software. Software parameters were set to detect a modification of 80 Da on Ser, Thr, or Tyr. The assignments of phosphopeptide sequences were then manually confirmed by comparing the acquired MS/MS spectra to the theoretical fragmentation pattern.

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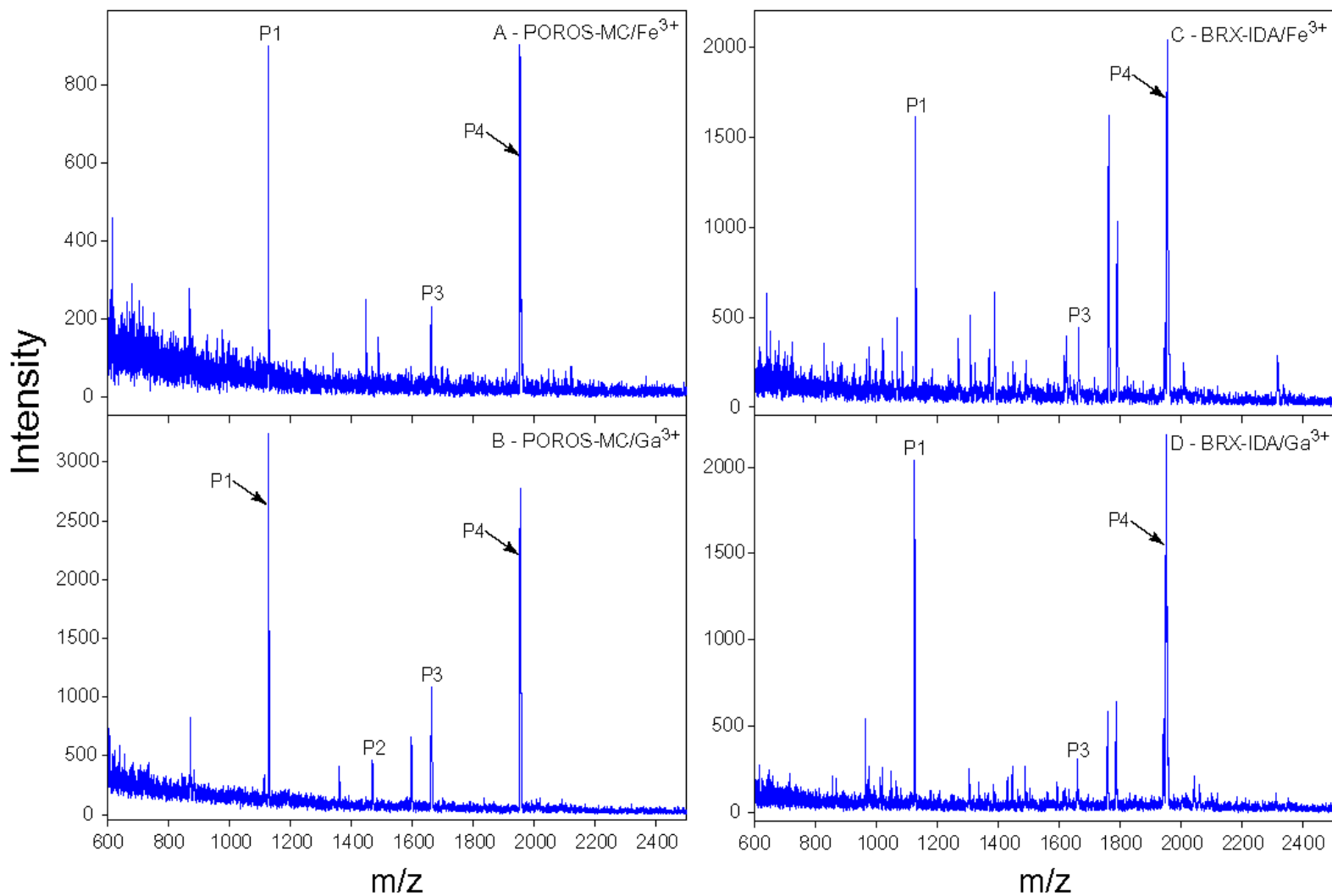
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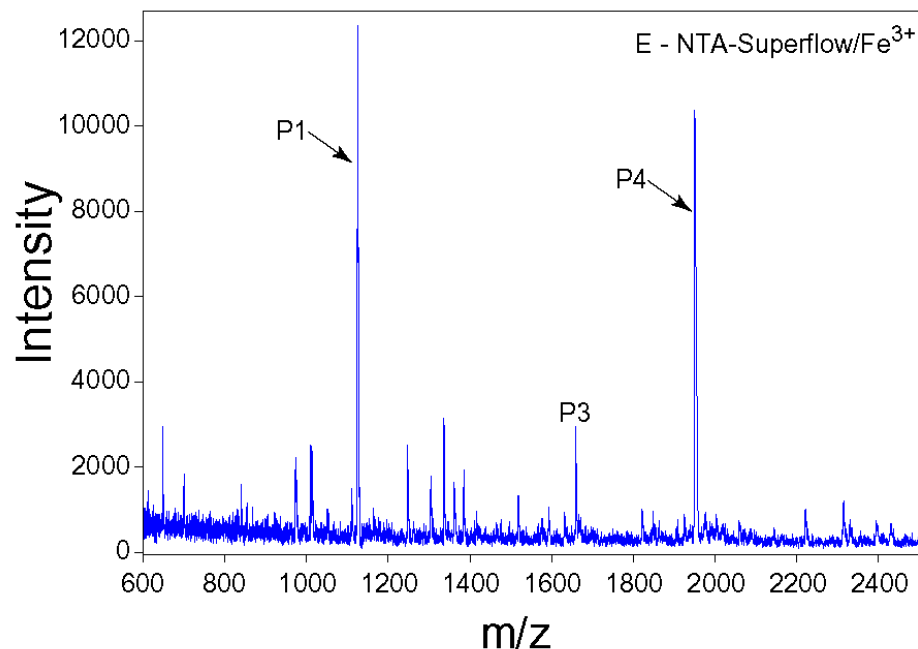
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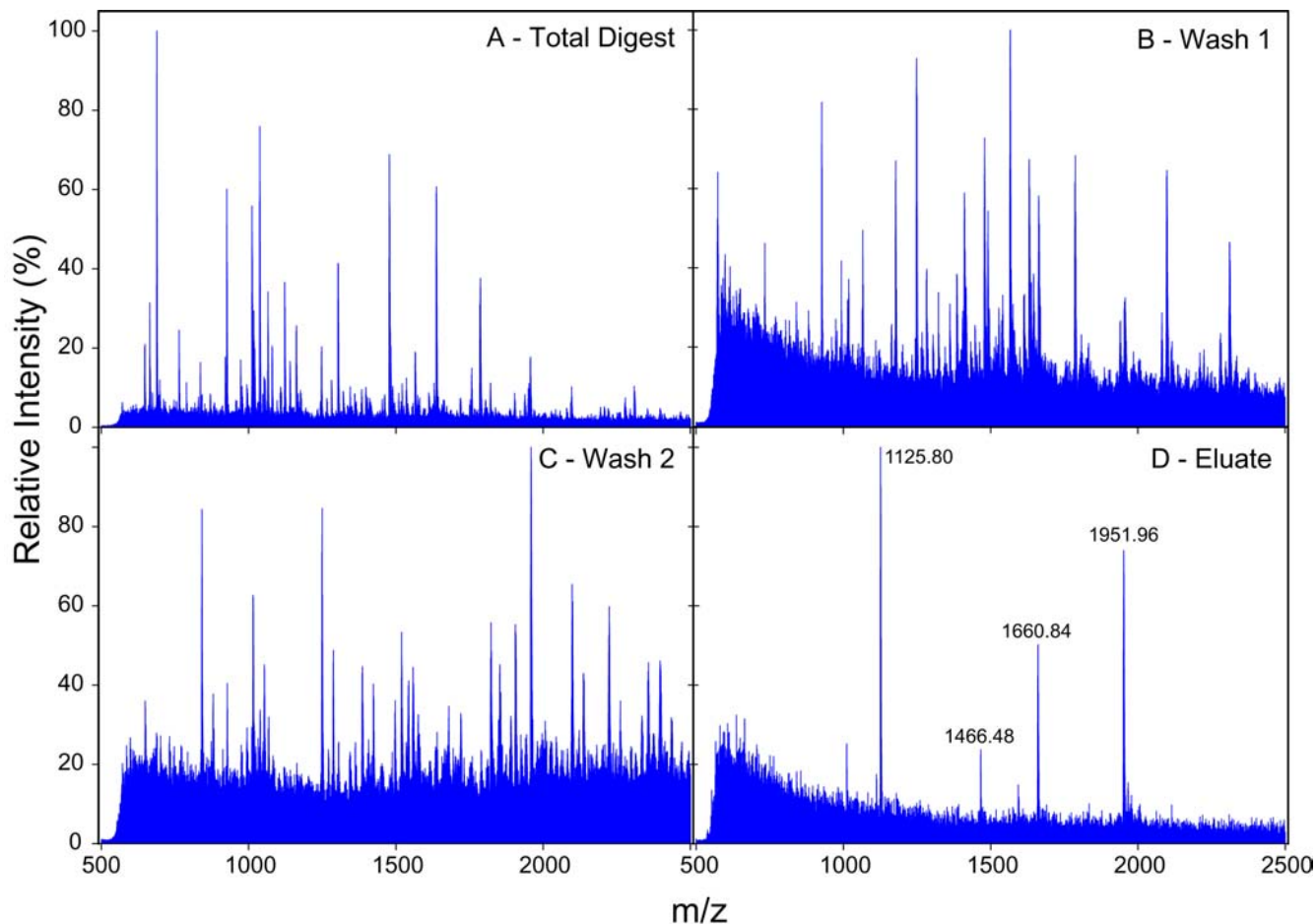




**Fig. 2.** (See Fig. legend on next page.)



**Fig. 2. Comparison of IMAC resins for the enrichment of phosphopeptides.** The test sample was a standard mixture containing 1 pmol of a synthetic P-tyr phosphopeptide and a tryptic digest consisting of 1 pmol alpha-casein (containing three phosphopeptides) and 5 pmol each of four non-phosphorylated proteins (BSA, carbonic anhydrase, ubiquitin, and beta-lactoglobulin). The IMAC resins were (A) Fe<sup>3+</sup>-charged POROS MC; (B) Ga<sup>3+</sup>-charged POROS MC; (C) Fe<sup>3+</sup>-charged BRX-IDA; (D) Ga<sup>3+</sup>-charged BRX-IDA; and (E) Fe<sup>3+</sup>-charged NTA-superflow. Samples were loaded onto IMAC columns prepared in micro-tips, washed, and eluted. The eluate was analyzed by MALDI-TOF mass spectrometry. The labeled peaks represent phosphorylated peptides; (P1) synthetic P-tyr phosphopeptide (m/z 1127, DRVpYIHPF); (P2) alpha-casein (m/z 1467, TVDMEpSTEVFTK); (P3) alpha-casein (m/z 1662, VPQLEIVPNpSAERR); and (P4) alpha-casein (m/z 1953, YKVPQLEIVPNpSAERR).



**Fig. 3. Enrichment of phosphorylated peptides by IMAC.** Peptides present in each fraction from the IMAC procedure were monitored by MALDI-TOF mass spectrometry. A standard mixture containing 1 pmol of a synthetic P-tyr phosphopeptide and a tryptic digest of 2 pmol alpha-casein and 400 pmol each of four non-phosphorylated proteins (BSA, carbonic anhydrase, ubiquitin, and beta-lactoglobulin) was precleaned with a C18 cartridge. The total digest (A) was loaded onto an IMAC micro-tip containing  $\text{Ga}^{3+}$ -charged BRX-IDA resin, washed with 50% ACN/0.1% HAc (B) and 50% ACN/0.1% HAc/100 mM NaCl (C), and eluted with 200 mM  $\text{Na}_2\text{HPO}_4$  (D).











