

Application of Automated IMAC Enrichment for Bacterial Phosphopeptide Analysis

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Overview

Purpose: Demonstration of an automated Immobilized Metal Ion Affinity Chromatography (IMAC) enrichment system with utility for bacterial applications.

Method: Automated IMAC system with a large capacity IMAC column to enrich strong cation exchange-fractionated phosphopeptides from the soluble proteome of *Escherichia coli* MG1655.

Results: Identification of 75 unique peptides with phosphorylation on S/T/Y corresponding to 52 phosphorylated proteins.

Introduction

- While phosphoproteins play important roles in cell signaling and regulating protein activity, relatively little is known about protein phosphorylation in bacterial systems.
- Phosphoproteome enrichment is an essential step in the analysis of protein phosphorylation. While the IMAC strategy has proved valuable for enriching phosphopeptides in eukaryotic samples, it has not previously been demonstrated for bacterial cells.
- We applied an automated IMAC enrichment strategy for bacterial phosphopeptide analysis¹ and demonstrated:
 - Highly enriched phosphopeptides.
 - Enrichment factor (phosphorylated/total peptides) is 82.1%.
 - High throughput automated operation.
 - Without human intervention, nine samples were enriched in a day, with consistent performance.
 - Large-capacity IMAC column.
 - Facilitated unbiased recovery of phosphopeptides with a range of phosphorylation states.
 - Increased sample loading capacity (>20 times).
 - Reduced wash time (from 1-5 μ l/min to 20 μ l/min).
 - The number of injected solutions can readily be adjusted according to the purpose of the experiment.
 - Up to four buffers can be used in the conventional pump system.

Methods

Automated IMAC system

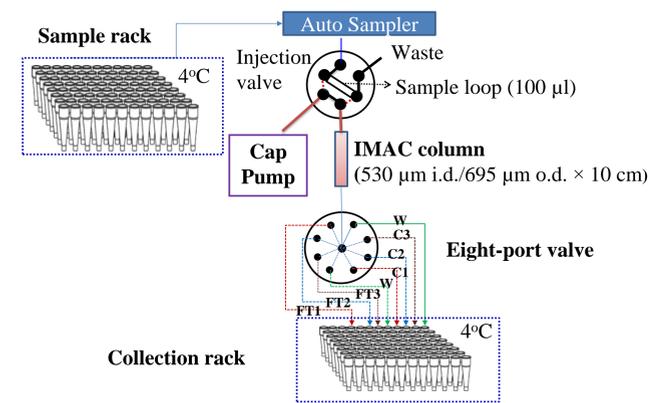


Fig. 1: Schematic representation of the automated IMAC enrichment setup. Fully controlled by the custom-designed software, the sample is loaded by the autosampler PAL onto the sample loop. The content of the sample loop is pushed to the Fe³⁺-bound IMAC column to trap the phosphopeptides. The flow-through (FT) (mainly non-specifically bound peptides) is collected through the valve position (FT1, FT2, or FT3). Lastly, the trapped phosphopeptides are eluted with phosphate buffer and collected through the valve position (C1, C2, or C3).

Experimental workflow

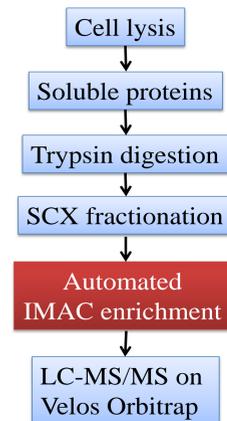


Fig. 2: Overview of phosphoproteome analysis for *E. coli*.

- 1) Tryptic digestion of the soluble proteins from *E. coli* MG1655 grown in M9 minimal medium.
- 2) Fractionation of the resulting peptides using strong cation exchange (SCX).
- 3) Enrichment of phosphopeptides using automated IMAC system.
- 4) Analysis of the enriched peptides using LC-MS/MS.
- 5) Data analysis.

Results

Identification of *E. coli* phosphopeptides

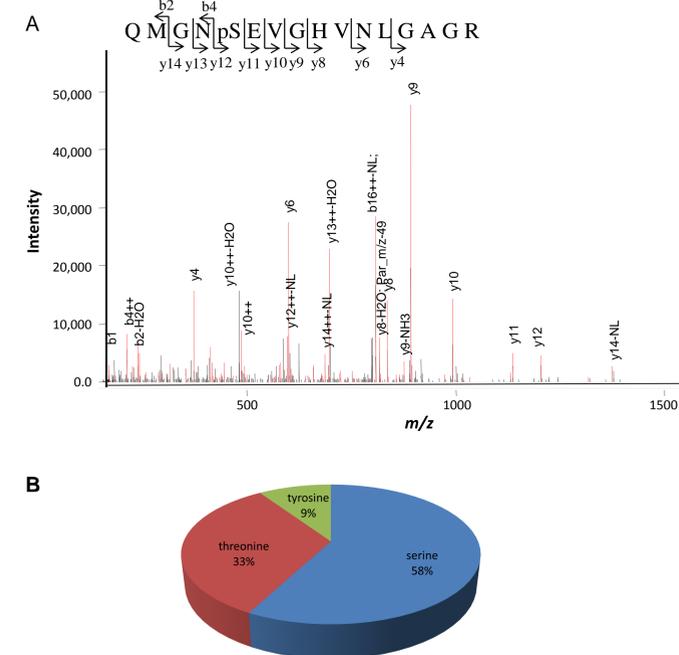


Fig. 3A: Representative MS/MS spectrum of phosphoglyceromutase III (gpmI) phosphopeptide QMGNpSEVGHVNLGAGR. 3B: Distribution of phosphorylation sites.

- The phosphorylation site localization was evaluated based on an A-score², and the identified phosphorylated peptides (FDR <5%) were manually confirmed (Fig. 3A as an example).
- Results were consistent with previous reports that phosphorylation occurs mainly on serine/threonine.
- Phosphorylation on histidine or aspartic acid was not observed, which could be caused by the instability of phosphorylation on histidine or aspartic acid under the acid conditions required for the IMAC enrichment strategy.

Deeper understanding of the *E. coli* phosphoproteome

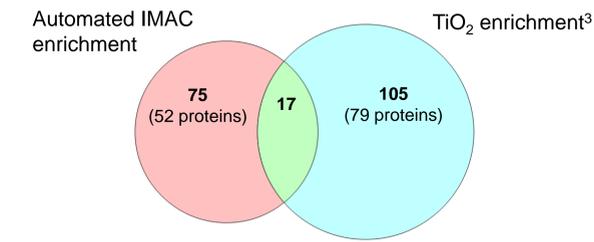


Fig. 4: Identification of *E. coli* phosphopeptides.

- Phosphorylation events revealed by the IMAC enrichment strategy included 75 unique phosphopeptides that cover 52 phosphorylated proteins.
- 58 of the 75 phosphopeptides were novel identifications, highlighting the complementary nature of the IMAC and TiO₂ enrichment strategies for bacterial phosphoproteomics.
- Noted differences between the two enrichment strategies:
 - Growth conditions (i.e., M9 minimal medium in our study vs. LB rich medium in Macek et al.³).
 - Starting material (i.e., soluble proteins in our study vs. global proteins in Macek et al.³).

Biological implications

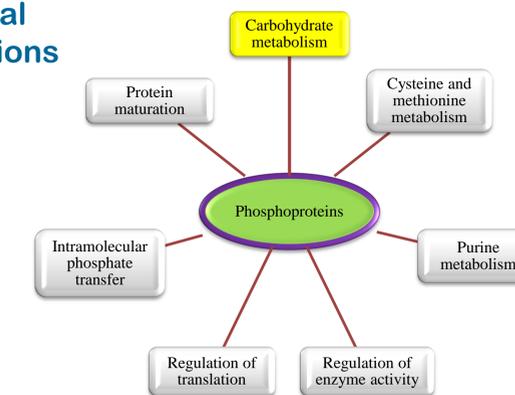


Fig. 5: The *E. coli* phosphoproteome involved in biological processes.

- The phosphoproteins observed in this study play important biological roles, particularly in the central metabolism.
- Consistent with previous studies, the phosphoproteins identified in our study are mainly involved in carbohydrate metabolism.

Conclusions

- The automated IMAC system enhances analytical performance and reduces labor costs.
- The employment of a large-capacity IMAC column increases peptide loading amount and reduces the wash time.
- The automated IMAC system can be readily adapted to other applications.
- Our data enrich the understanding of *E. coli* phosphoproteome.
- The phosphoproteome is essential to a variety of biological processes, particularly carbohydrate metabolism.

Acknowledgements

Portions of this research were funded by the U. S. Department of Energy Office of Biological and Environmental Research (DOE/BER) as part of the Genome Sciences Program Biofuels Scientific Focus Area project and Environmental Molecular Sciences Laboratory (EMSL) intramural research and capability development projects.

Work was performed in EMSL, a DOE/BER national scientific user facility located at Pacific Northwest National Laboratory.

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