Overview

The purpose of this study is to evaluate the IMAC technology without any pre-chemical modification. The protocol of three commercially available IMAC kits:Millipore $ZipTip_{MC}$, Eprogen IPAC beads and Pierce Swellgel Chelated Disks, were optimized. The optimized method was evaluated for selectivity, reproducibility, sensitivity and recovery. Eprogen IPAC beads are the most selective of the three kits. Both IPAC and Swellgel are reproducible. $ZipTip_{MC}$ and IPAC beads are sensitive to 5 pmol of sample. The minimum amount of sample that can be used with the Swellgel kit is 25 pmol.

Introduction

Reversible protein phosphorylation plays a central role in regulating cell function. Identification of protein phosphorylation sites can assist in understanding the mechanism of a biological pathway and as a result, aid in drug discovery. There are a number of techniques available to identify sites of phosphorylation. One of the key steps in a number of techniques towards phosphorylation site identification is to isolate and enrich phosphopeptides from a proteolytic digest. IMAC has been widely used for this purpose. There have been a number of new commercially available IMAC kits for phosphopeptide enrichment and isolation each with strengths and weaknesses. We have evaluated the selectivity, reproducibility and sensitivity of three commercially available IMAC kits, Millipore $ZipTip_{MC}$, Eprogen IPAC beads and Pierce Swellgel Gallium Chelated Disks, for phosphopeptide enrichment (Figure 1).

Method

Material:

The standard mixture listed in Table 1 and 2 and all chemicals were purchased from Sigma. β-casein was digested in 50 mM NH_4HCO_3 pH 7.8 with trypsin at 37°C for 4 hours. Metal solutions, 200 mM $CuSO_4$, 200 mM $NiCl_2$, 200 mM $FeCl_3$ 10 mM HCl, 100 mM $GaCl_2$ and elution solutions, 0.3 N NH_4OH, K_2HPO_4 pH 8.5, $NH_4H_2PO_4$ pH 4.8 and

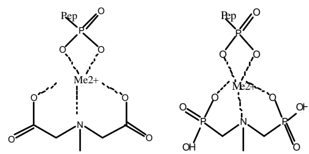

Figure 1: Chemical structure of IMAC and IPAC resin

Table 1: Standard mixture for the study.

Peptides	Sequence	# of P sites	Mass mono
pSer616-IRS-1	Ac-GYMPMpSPGVAPZKC-NH2	1	1571.7
Ins Receptor Frag 1154-1165	TRDIpYETDpYpYRK	3	1861.67
monophosphopeptide	FQpSEEQQQTEDELQDK	1	2061.8
RII 81-99, pSer95, Ala97	DLDVPIPGRFDRRVpSVAAE	1	2191.08
tetraphosphopeptide	RELEELNVPGEIVEpSLpSpSpSEESLTR	4	3121.26
4 peptide A	DLWQK	0	689.36
4 peptide B	AKPSY(hP)(hP)TYK	0	1183.6
4 peptide C	YRPPGFSPFR	0	1223.63
4 peptide D	YGGFLRRIRPKLK	0	1603.99

9.0, Na_2HPO_4 pH 8.5, or NH_4HCO_3 pH 9.0 were prepared by Biogen Media Prep lab. CRC with lower filter was purchased from USB.

Optimized protocol:

IPAC: 10 µL Beads were charged with Fe^{3+} in a CRC with a lower filter. 20 µL (5-20 pmol) of sample was diluted 1:1 in 2.5% acetic acid and loaded on the beads and bound for 15 minutes. The beads were sequentially washed with 0.1% acetic and 30% acetonitrile. Elute with 10 µL 200 mM K_2HPO_4 pH 8.5. For every step, the solvent was removed from the beads by spinning down the CRC using a capsulefuge. The elution was cleaned by C_{18} ZipTip before MALDI analysis.

$ZipTip_{MC}$: $ZipTip_{MC}$ was charged with Ni^{2+}. 20 µL (5-20 pmol) of sample was diluted 1:1 in 2.5% acetic acid and aspirated and dispensed 10X. The tip was sequentially washed with 0.1% acetic and 10% acetonitrile 0.1% acetic. The enriched phosphopeptides were eluted from the tip with 10 µL 0.3 N NH_4OH with 20% MeCN. The elution was cleaned by C_{18} ZipTip before MALDI analysis.

Swellgel: 40 µL (25-40 pmol) of sample after dilution of 1:1 in 5% acetic acid was loaded and bound to the disk for 5 minutes. The disk was washed with 0.1% acetic and 10% acetonitrile 0.1% acetic. Elute with 25 µL 100 mM NH_4HCO_3 pH 9.0. For every step, the solvent was removed from the disk by spinning down the CRC using a capsulefuge. The eluent was cleaned by C18 ZipTip prior to MALDI analysis.

MALDI-TOF MS: The C_{18} ZipTip cleaned samples were spotted with DHB as the matrix. MALDI-MS was acquired using a ABI-Perseptive Voyager DE-STR in the reflector mode with an accelerating voltage of 20 kV.

HPLC quantification: The standard curve and recovery was determined from the UV trace of the eluent separated over a 300 µm X 50 mm Michrom C18 Magic bullet column using an Dionex Ultimate.

Results and Discussion

All experiments were run in triplicate.

Best metal for charging

The recommended protocol was tested on the standard mixture shown in table 1. The results indicate that each kit is specially favored to a different metal (Table 2).

Table 2: Identify optimal metal for charging IMAC. Each star represents the enrichment of one phosphopeptide. Five phosphopeptides were in the mixture. Swellgel is charged with Ga^{3+}.

	$ZipTip_{MC}$	IPAC
Cu^{2+}	++++	-
Ni^{2+}	++++	-
Fe^{3+}	+++	++++
Ga^{3+}	-	-

Elution of phosphopeptides

From all of the elution buffers we have tested, the elution buffers listed below gave the best complete recovery of phosphopeptides and strongest MALDI-MS signal for each kit (Table 3)

$ZipTip_{MC}$: 0.3 N NH_4OH
IPAC: 200 mM K_2HPO_4 pH 8.5
Swellgel: 100 mM NH_4HCO_3 pH 9.0

Table 3: Results from optimized protocols

	P-Pep	non-P-Pep
$ZipTip_{MC}$	4 out of 5	1 out of 4
IPAC	4 out of 5	0 out of 4
Swellgel	5 out of 5	1 out of 4

Sensitivity

Using the optimized protocol, each kit was tested for sensitivity. Sensitivity is defined as the lowest amount of sample that shows the optimal recovery of phosphopeptides.

$ZipTip_{MC}$: 5 pmol
IPAC beads: 5 µL beads, 5 pmol
Swellgel disk: 25 µL resin, 25 pmol

For IPAC and Swellgel, if the resin was packed differently, such as in a microtip, the sensitivity could be lower because less beads could be packed.

Reproducibility

For reproducibility, each kit was tested by three parallel experiments (Table 4). The reproducibility issues with $ZipTip_{MC}$ likely arise from quality control.

Table 4: Reproducibility results

Method	$ZipTip_{MC}$	IPAC	Swellgel
Reproducibility	no	yes	yes

Selectivity

A known problem with IMAC methods is that the acidic peptides can also be enriched together with the phosphopeptides. It is also known that Ni^{2+} binds His-Tag and perhaps histidine-containing peptides. Each optimized protocol was further tested for selectivity by adding the histidine and acidic peptides shown in table 5. Selectivity is represented in Figure 2 by calculating the percentage of the signal in each MALDI spectrum that arises from a phosphopeptide or non-phosphopeptide. The total signal from these peptides is represented as 100%. The first bar shows the results from the starting material, before any enrichment. The data shows that the kits do enrich phosphopeptides. IPAC shows the best selectivity over $ZipTip_{MC}$ and Swellgel disk. All three kits show recovery of one of the acidic peptides after enrichment. Swellgel shows recovery of the histidine containing peptides.

Table 5: Histidine and acidic peptides added to standard mixture

Peptide	Sequence	Mono Mass
5-peptide A	RVYV**H**PI	882.51
5-peptide B	RVYV**H**PF	916.49
5-peptide C	RVYI**H**PF	930.51
5-peptide D	DRVYV**H**PF**H**L	1281.66
5-peptide E	DRVYI**H**PF**H**L	1295.68
ACTH 18-39	RPVKVYPNGA**E**D**E**SA**E**AFPL**E**F	2464.19
ACTH 7-38	FRWGKPVGKKRRPVKVYPNGA**E**D**E**SA**E**AFPL**E**F	3656.92

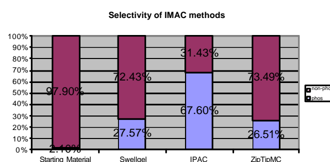

Figure 2: Selectivity of IMAC kits.

Recovery

Monophosphopeptide shows medium response in both MALDI and UV. A standard curve was run with 2.5, 5, 10, 20 and 25 pmol of monophospeptide (figure 3) and recovery was estimated based on the recovery of this phosphopeptide. Both Swellgel and IPAC showed approximately 60% recovery of monophospeptide after enrichment

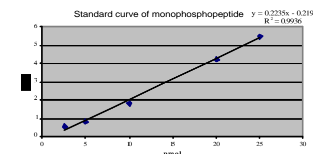

Figure 3:Standard curve of monophospep

Evaluation of the optimized protocols using tryptic digest of β-casein

Of the three methods, the protocol of IPAC beads with Fe^{3+} seems to show the best result for phosphopeptide enrichment. Figure 4 shows: **A**: Tryptic digest of β-casein with phosphopeptides labeled as 1 and 4. **B**: One of the phosphopeptides was enriched using $ZipTip_{MC}$ with Ni^{2+}; **C**: Both phosphopeptides were enriched using IPAC beads with Fe^{3+}; **D**: Both phosphopeptides were enriched with Swellgel but the signals are weaker that IPAC. The peaks not labeled in B, C and D correspond to acidic peptides from the Tryptic digest of β-casein.

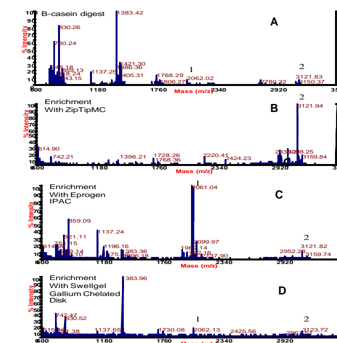

Figure 4: MALDI-MS of tryptic digest of β-casein with and without IMAC enrichment

Conclusions

Optimized protocols were developed for $ZipTIp_{MC}$, IPAC and Swellgel. Of the three methods, IPAC beads charged with Fe^{3+} shows the best selectivity and is reproducible. The biggest limitation of $ZipTip_{MC}$ is that the tips are not reproducible. Two limitations of Swellgel are that the disk contains 25 µL of resin which requires a minimum of 25 pmol samples for loading and the enrichment of histidine containing peptides. All three methods show in addition to the enrichment of phosphopeptides, the enrichment of acidic peptides.

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Evaluation of Commercially Available IMAC Kits: Millipore $ZipTip_{MC}$, Eprogen IPAC beads and Pierce Swellgel Gallium Chelated Disks

Azita Kaffashan and Chenhui Zeng
Department of Analytical Biochemistry, Biogen Inc, Cambridge, MA 02142