

New Approach to LC/MS Analysis of Tyrosine Phosphorylation using 2-Stage Immunoaffinity Enrichment of pY Proteins and pY Peptides

Anne Zeck, Li Zang and Barry L. Karger
 Barnett Institute, Department of Chemistry and Chemical Biology,
 Northeastern University, Boston, MA, 02115, USA

Introduction

Reversible protein phosphorylation is important for many cellular regulation mechanisms, with more than 30% of the proteome being phosphorylated(1). Phosphorylation analysis can be challenging because of low abundance, partial phosphorylation and that the range of phosphorylation occurrence differs over three orders from serine, threonine to tyrosine (1800:200:1) in eukaryotic cells(1). For analysis of tyrosine phosphorylation, which has important roles in initiating many signaling pathways, affinity enrichment is required. The enrichment has previously been accomplished using either IMAC-LC-MS of the peptide methyl esters(2-3) or a combination of POROS R2/OLIGO R3 micro columns for chromatographic separation followed by highly specific detection of phosphotyrosine immunions by precursor ion scanning(4-6). Compared to these methods, our approach combined the enrichment of pY proteins followed by pY peptides using a small column packed with pY antibody conjugated agarose beads. Enriched pY peptides were then directly submitted for LC-MS analysis. By using a purely antibody-based method, this approach displayed its special advantages such as that no peptide modification was required and high specificity and affinity was achieved for phosphotyrosine peptides. In addition, the 2-stage enrichment significantly decreased the sample dynamic range and complexity, leading to enhancement for pY peptide identification. An on-line coupling system can be established for this whole protocol to avoid sample loss and high-sensitivity detection of pY peptides.

Method

For optimum selection of the pY antibody, which have reasonable affinity to both pY proteins and pY peptides, a direct competitive ELISA method was developed to compare the binding strength of three commercially available pY antibodies (PY99, PY100, 4G10) for pY peptides and pY proteins. High-adsorption 96-well plate (NUNC Maxi sorp) was first coated overnight with 200 μ L 0.2 μ g/L phosphotyrosine conjugated BSA (pY BSA) (Sigma) in carbonate coating buffer (pH 9.6). pY angiotensin II (Calbiochem) or pY BSA with various concentrations was incubated on another plate with the primary antibody (PY99, PY100, and 4G 10) for 30 min. The mixed antibody-antigen solution was then transferred to the coated plate. A secondary antibody (Peroxidase labeled anti-mouse IgG, Vector Laboratories, Inc.) and the substrate were then applied. The whole plate was finally measured using a plate reader (Spectra max, Molecular Devices). The readings were then plotted against the antigen concentration to calculate the binding constants of the measured antibodies. The enrichment of pY ANG II using pY99 antibody bead packed column was monitored using this ELISA method.

ELISA results led us to the selection of pY99 finally. The 2-stage affinity enrichment method for pY proteins followed by pY peptides was applied to relatively small amount of EGF-stimulated A431 cell lysates (1 mg, $\sim 10^7$ cells) (BD Bioscience). It was diluted 20 times with 50 mM Tris buffer (pH 7.4) to lower SDS and β -mecaptoethanol concentration in the original sample. Tyrosine phosphorylated proteins were then immunoaffinity enriched using a short column (5 mm (i.d.) / 5 mm (length) (Tricorn 5/20 column, Amersham) packed with 500 μ L pY99 conjugated agarose beads. The protein enrichment process was monitored using Western blotting. Tyrosine phosphorylated peptides from the tryptic digest of the collected pY proteins were next enriched using the same column and eluted using 0.1% formic acid. The enriched tyrosine phosphorylated peptides were then directly submitted to nanoLC-MS/MS (LCQ DECA) analysis for pY identification.

Results and Discussion

The affinity of pY antibodies towards a pY ANG II and pY BSA were determined using the competitive ELISA method. The midpoints of the respective ELISA curves were the reverse of the affinity constants. The affinity constants measured for pY99, 4G10 and pY100 are listed in Table 1.

Table 1 Affinity Constants Measured for Three Popular pY Antibodies in High-Salt Buffer and Water

Antibody	K_{aff} for pY-ANG II [M^{-1}]		K_{aff} for pY-BSA [M^{-1}]	
	TBS buffer	Water	TBS buffer	Water

PY99 (Santa Cruz)	0.4·10 ⁶	3·10 ⁶	0.28·10 ⁹	14·10 ⁶
PY100 (Cell Signaling)	0.6·10 ⁶	n.d.	0.032·10 ⁹	n.d.
4G10 (Upstate)	0.7·10 ⁶	0.62·10 ⁶	0.35·10 ⁹	n.d.

TBS buffer: 150 mM NaCl, 50 mM TRIS salt

The affinity constants against pY ANG II for the 3 antibodies were similar, at the micromolar level. However, the affinity of pY100 was observed to have 10 times weaker binding with pY BSA. In addition, it was found that the salt concentration highly affected the binding strength between these pY antibodies and antigens. As shown in Table 2, pY99 demonstrates nearly 10 times stronger binding against pY ANG II and pY BSA in water compared to TBS buffer.

The competitive ELISA method was further applied to monitor the affinity enrichment process of pY ANG II. The amount of pY ANG II in every fraction (100µL) collected during enrichment using an antibody bead packed column was measured using the ELISA method and plotted against fraction number, as shown in Figure 1. It was clearly revealed that this pY peptide was successfully enriched in the 3 elution fractions.

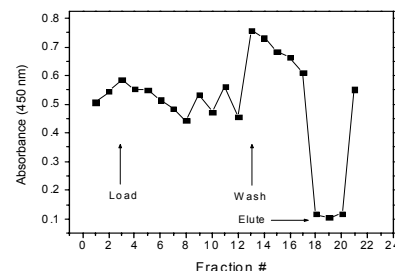


Figure 1 pY ANG II Affinity Enrichment Monitored using Competitive ELISA

Table 2 PY Peptides Identified From 1 mg EGF Stimulated A431 Cell Lysates

Number	Proteins	Phosphorylation Site
1	EGF Receptor	Tyr #1172*
2	EGF Receptor	Tyr #1110*
3	CRK like Protein	Tyr #207*
4	Tetraphosphatase	Tyr #92
5	Fatty acid-CoA ligase 4	Tyr #82

*: known pY sites

the EGF receptor. The sample was highly simplified using the 2-stage affinity enrichment method with less background observed.

Conclusion

An antibody-based pY peptide enrichment method was developed and its performance was examined using a relatively small amount of sample (1 mg total cell lysates). Five pY peptides were identified, including three known pY sites and two new sites. Affinity constants for three popular commercially available antibodies (pY99, pY100, 4G10) were measured using a new competitive ELISA method. High salt was found to affect the multivalent binding between antibodies and pY ANG II, pY BSA, leading to weaker binding in the high-salt buffer.

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The 2-stage affinity enrichment method using pY99 antibody was applied to 1 mg EGF-stimulated A431 cell lysates. The pY peptides enriched were then split into two injections for nanoLC-MS/MS analysis. Five pY peptides were identified, including 3 known and 2 new pY sites, as listed in Table 2. The MALDI-MS spectrum of enriched pY peptides sample was shown in Figure 2, revealing two enriched pY peptides from

4700 Reflector Spec #1[BP = 866.9, 6023]

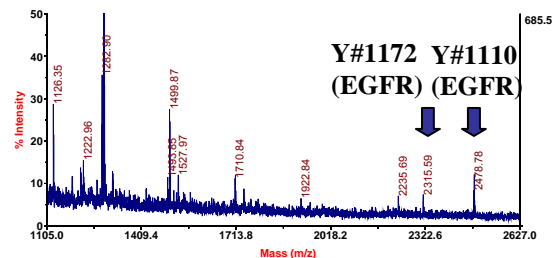


Figure 2 MALDI-MS Spectrum of Enriched pY Peptides from EGF-Stimulated A431 Cell Lysates