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Biphasic Affinity Chromatographic Approach for Deep Tyrosine Phosphoproteome Analysis

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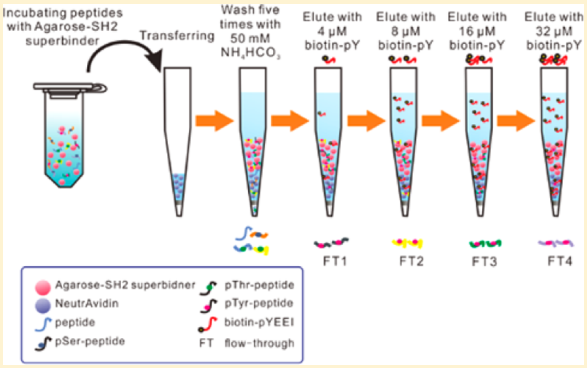
Supporting Information

ABSTRACT: Tyrosine phosphorylation (pTyr) is important for normal physiology and implicated in many human diseases, particularly cancer. Identification of pTyr sites is critical to dissecting signaling pathways and understanding disease pathologies. However, compared with serine/threonine phosphorylation (pSer/pThr), the analysis of pTyr at the proteome level is more challenging due to its low abundance. Here, we developed a biphasic affinity chromatographic approach where Src SH2 superbinder was coupled with NeutrAvidin affinity chromatography, for tyrosine phosphoproteome analysis. With the use of competitive elution agent biotin-pYEEI, this strategy can distinguish high-affinity phosphotyrosyl peptides from low-affinity ones, while the excess competitive agent is readily removed by using NeutrAvidin agarose resin in an integrated tip system. The excellent performance of this system was demonstrated by analyzing tyrosine phosphoproteome of Jurkat cells from which 3,480 unique pTyr sites were identified. The biphasic affinity chromatography method for deep Tyr phosphoproteome analysis is rapid, sensitive, robust, and cost-effective. It is widely applicable to the global analysis of the tyrosine phosphoproteome associated with tyrosine kinase signal transduction.



Protein tyrosine phosphorylation (pTyr) is of central importance in signaling systems in eukaryotic cells as it regulates many important biological events including proliferation, adhesion, differentiation, hormone responses, and immune defense.[1−3] The deregulation of tyrosine phosphorylation has been implicated in human diseases including cancer. Tyrosine kinases, the enzymes catalyzing the phosphorylation of tyrosine residues, are a major class of drug targets, and a number of tyrosine kinase inhibitors have been approved for clinical use.[4−6] Deep Tyr phosphoproteome holds great promise for understanding tyrosine phosphorylation-regulated aberrant signal transduction in human disease. Sensitive methods are highly required for the detection of tyrosine phosphorylation events. Mass spectrometry (MS) has been proved to be an ideal platform for the global identification of proteins and their post-translational modifications (PTMs).[7] Because of the low abundance of tyrosine phosphorylated proteins, the coverage of Tyr phosphoproteome depends on the performance of the pTyr peptide enrichment prior to MS analysis. Typically, pTyr peptides are enriched from protein digest by pan-specific antibodies for pTyr, such as 4G10, P-Tyr-100, and PY-99,[8] for tyrosine phosphoproteome analysis. However, the high cost of these antibodies prohibits their use in a sufficient amount to saturate the pTyr peptides in a sample, resulting in a relatively low coverage of the Tyr phosphoproteome.[9,10] Despite the large number of Tyr kinases in the cell, tyrosine phosphorylation is tightly regulated and maintained at low abundance (accounting for <1% of total phosphorylation events) in the absence of specific stimuli. Hence, a more efficient and cost-effective approach allowing for deeper identification of the Tyr phosphoproteome would facilitate the study of the pTyr-mediated signaling pathways.

Recently, we developed a method using a Src Homology 2 (SH2) domain-derived pTyr-superbinder as the affinity reagent for the pTyr peptide enrichment.[10] The superbinder SH2 was engineered by introducing three point mutations to its pTyr-binding pocket. The triple mutated SH2 domain possesses nano- to micromolar affinities to pTyr-containing peptides.[11] The markedly enhanced affinity made the superbinder almost pan-specific to phosphorylated tyrosine residue. We have demonstrated that this superbinder enabled deeper and broader

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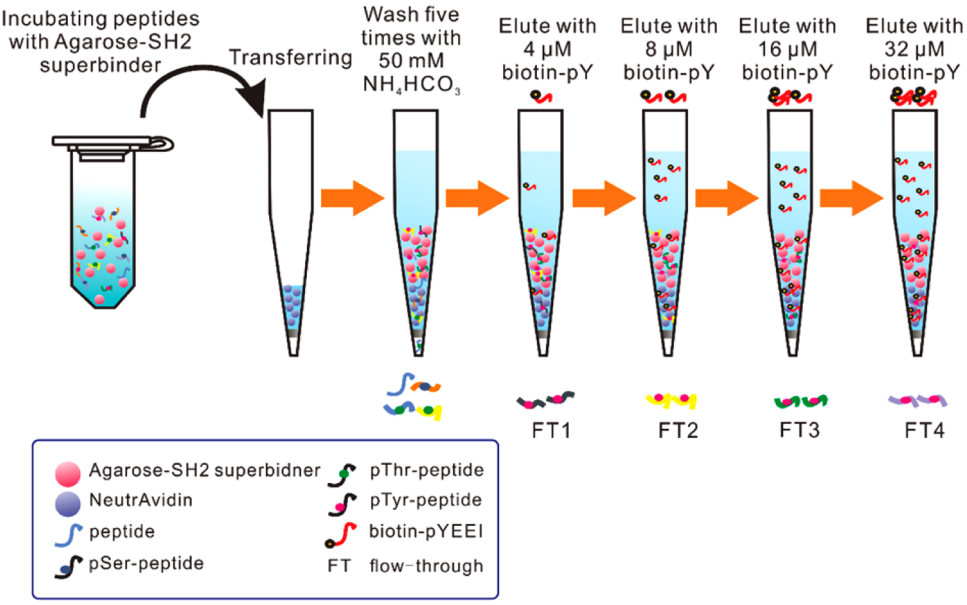

Figure 1. Workflow for deep analysis of Tyr phosphoproteome by using biphasic affinity chromatographic approach. The sample to be enriched is first incubated with the Sepharose beads with immobilized Src superbinder at 4 °C overnight with rotation. Then, the beads are transferred into a 1 mL pipet tip prepacked with NeutrAvidin agarose resins. Finally, the biphasic tip is washed with 50 mM NH_4HCO_3 buffer and sequentially eluted with 4, 8, 16, and 32 μM competitive agent (biotin-pYEEI) in NH_4HCO_3 buffer. All the flow throughs (Fs) are collected for further analysis.

coverage of the Tyr phosphoproteome than conventional pan-specific antiphosphotyrosine antibodies.[10] Indeed, we identified ~20,000 distinct pTyr peptides and >10,000 pTyr sites from nine human cell lines, achieving unprecedented deep coverage for tyrosine phosphoproteome.[10]

The SH2 domain is a sequence-specific phosphotyrosine-binding module presented in many signaling molecules. There are 120 SH2 domains encoded by the human genome, and each SH2 domain binds a unique spectrum of tyrosine phosphorylated sites.[12,13] Because SH2 domains are what the cell uses to respond to changes in tyrosine phosphorylation during signaling, the binding pTyr sites of different SH2 domains can provide a wealth of information about the mechanisms and status of pTyr signaling. With the markedly enhanced binding affinity, almost all pTyr peptides could be bound to the SH2 superbinder. Nevertheless, LC-MS/MS analysis following one-step elution of all bound peptides by TFA would result in the loss of information in binding specificity and affinity for the identified pTyr sites. It is known that the SH2 superbinder and the wild-type SH2 domain have similar specificity toward pTyr peptides despite large differences in affinity.[10] We hypothesize that the bound pTyr peptides could be sequentially eluted on the basis of their binding affinity. Because progressive elution of the bound pTyr peptides based on affinity would provide another dimension of separation prior to LC-MS/MS analysis, it could lead to deeper and broader coverage of the Tyr phosphoproteome. The superbinder used in this study is derived from Src SH2 domains. pTyr-Glu-Glu-Ile (pYEEI) was found to have the highest affinity to Src SH2 domains by screening of a degenerate phosphopeptide library.[14−16] Therefore, we utilized a biotinylated pTyr peptide with the sequence of biotin-pYEEI as a competitive elution agent. To prevent the interference of the competing reagent on the detection of the enriched pTyr peptides, a biphasic affinity chromatographic approach was developed to enrich and fractionate pTyr peptides. It was found that the pTyr peptides were successively released in accordance with their binding affinity to the superbinder SH2 domain. A total of 7,227 pTyr peptides and 3,480 unique pTyr sites were identified by the sequential elution for affinity purification from only 2 mg of pervanadate-treated Jurkat cell lysate digest.

EXPERIMENTAL SECTION

Chemicals and Materials. Formic acid (FA) was obtained from Fluka (Buches, Germany). Acetonitrile (ACN, HPLC-grade) was purchased from Merck (Darmstadt, Germany). NeutrAvidin agarose resin was obtained from Thermo Fisher Scientific (Waltham, MA, USA). CNBr-activated Sepharose 4B was purchased from GE (Princeton, NJ, USA). Daisogel ODS-AQ (5 μm) was purchased from DAISO Chemical CO., Ltd. (Osaka, Japan). Biotin-pYEEI was synthesized from ChinaPeptides (Shanghai, China). Fused-silica capillaries with 200 and 75 μm i.d. were purchased from Polymicro Technologies (Phoenix, AZ, USA). All of the water in the experiments was purified by a Milli-Q system from Millipore Co. (Bedford, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Expression, Purification, and Immobilization of the Src Superbinder. We performed the expression and purification of Src superbinder according to Kaneko et al.[11] The Src superbinder SH2 domain was immobilized on CNBr-activated Sepharose 4B following the manufacture's protocol (GE Healthcare), and the final concentration for the immobilized Src superbinder was 1 mg of protein per mL of medium.

Cell Culture, Protein Extraction, and Protein Digestion. Jurkat cells were cultured in RPMI 1640 medium with 10% neonatal bovine serum, 100 units/mL penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were collected by centrifugation, washed three times with PBS, and treated with 1 mM pervanadate (freshly prepared by mixing 1 mM orthovanadate with 1 mM hydrogen peroxide) in a tube for 15 min at 37 °C. The procedures for cell

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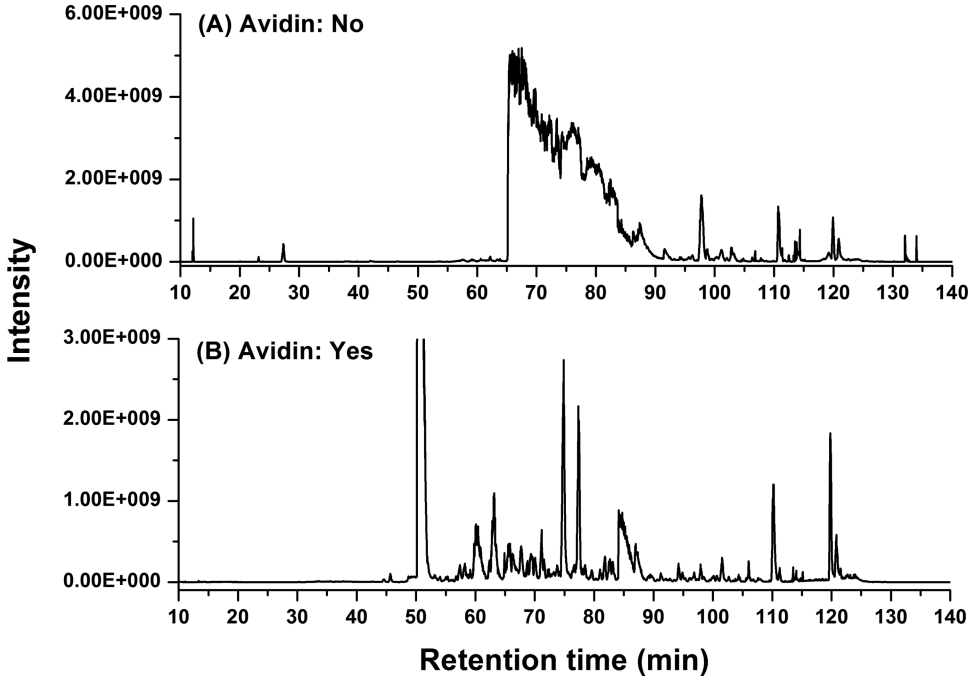

Figure 2. Base peak chromatograms for RPLC analysis of the enriched pTyr peptides (a) without NeutrAvidin and (b) with NeutrAvidin by using single-step competitive elution method (where, for example, 6.00E+009 represents 6.00×10^9).

lysis and trypsin digestion were performed according to Humphrey et al.[17]

Phosphotyrosyl Peptide Enrichment. The protein digest was subjected to enrichment with immobilized titanium(IV) ion affinity chromatography (Ti^{4+}-IMAC) for phosphopeptides as previously reported.[18] The enriched phosphopeptides from 2 mg of initial cell lysate were then dissolved in ice-cold immunoaffinity purification (IAP) buffer containing 50 mM MOPS-NaOH (pH 7.2), 10 mM Na_2HPO_4, and 50 mM NaCl. The sample was incubated with immobilized Src superbinder (300 μg) at 4 °C overnight with rotation. For the application of Src superbinder based sequential competitive elution strategy (as shown in Figure 1), a sieve plate with 2 mm diameter was packed into a tip (1 mL), washed with 80% ACN containing 0.1% TFA and 50 mM NH_4HCO_3 buffer (pH 7.2), respectively. Then, 500 μL of NeutrAvidin agarose resin was packed into the tip and washed with 50 mM NH_4HCO_3 buffer (the binding capacity was ~40 nmol of biotin-pYEEI per milliliter of resin, according to the specification of NeutrAvidin resins that the binding capacity was 10 μg of biotin/mL). Another sieve plate with 5 mm diameter was packed into the tip above the NeutrAvidin. Finally, the immobilized Src superbinder Sepharose beads with the captured pTyr peptides were packed into the tip. This biphasic tip was washed five times with 400 μL of NH_4HCO_3 buffer and sequentially eluted with 4, 8, 16, and 32 μM competitive agent (biotin-pYEEI) in NH_4HCO_3 buffer (300 μL). All of the eluents were collected, lyophilized, and stored at −30 °C for further analysis.

For the single-step competitive elution strategy, all of the steps were identical with these mentioned above, except the elution step using 30 μM competitive agent (400 μL). Another single-step competitive elution strategy was performed similarly except for the use of NeutrAvidin. For the trifluoroacetic acid (TFA) elution strategy, the NeutrAvidin agarose resin was absent, and the elution step was performed using 0.2% TFA (400 μL). For comparison, the experiments using samples without being subject to Ti^{4+}-IMAC enrichment were also performed. These samples were desalted by OASIS HLB column and then incubated with immobilized Src superbinder. After being washed with 50 mM NH_4HCO_3 buffer, the bound peptides were finally eluted with either 30 μM biotin-pYEEI or 0.2% TFA.

MS Analysis, Database Searching, and Data Analysis. Peptides were loaded onto a 14 cm capillary column with a 75 μm inner diameter, packed in-house with C18 particles (3 μm, 120 Å). For the RPLC separation, 0.1% FA in water and in 80% acetonitrile were used as buffers A and B. Peptides were eluted with a gradient of 4−35% buffer B over 85 min followed by 35−45% buffer B over 10 min, resulting in approximately 95 min gradients, at a flow rate of 0.3 μL/min. The MS analysis was performed on a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific), with one full scan (400−2000 m/z, R = 70,000 at 200 m/z) at a target of 1e6 ions with max IT of 120 ms. And the ions were fragmented by higher energy collisional dissociation (NCE 28%), followed by 10 data-dependent MS/MS scans with a resolution of 35,000 at 200 m/z (target 5e5 ions with max IT of 100 ms; isolation window, 2.0 m/z). Dynamic exclusion (30 s) was on.

The raw data files generated by the Q-Exactive were analyzed with software MaxQuant version 1.3.0.5, which was developed by Matthias Mann's laboratory.[19] The MS/MS spectra were searched against the Human Uniprot FASTA database. Trypsin specificity (KR/P, DE) was adopted, and up to two missed cleavage sites were allowed. Carbamidomethyl (C) was chosen for fixed modifications, and oxidation on methionine; phospho (S, T, Y) were set as variable modifications. The mass tolerances for the precursor ions and fragment ions were set to 10 ppm and 0.05 Da, respectively. The maximum false-discovery rate (FDR) was set to 1% for both the peptides and proteins. All of the phosphorylation sites reported in this study met the combined cutoff values of localization probability > 0.75 and ΔPTM score ≥ 5. Sequence logos were automatically

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generated by the WebLogo (http://weblogo.berkeley.edu/logo.cgi).[20]

RESULTS AND DISCUSSION

In this study we aimed to fractionate the pTyr peptides according to their binding affinity to Src SH2 superbinder. This was achieved by eluting the bound pTyr peptides with increased concentrations of a competing agent. The peptide pYEEI was found to have the strongest affinity to the Src SH2 domain by screening a degenerate phosphopeptide library.[14−16] Because the SH2 superbinder and the wild-type SH2 domain have similar specificity toward pTyr peptides, pYEEI would be a good competing reagent for selective elution of bound pTyr peptides. However, the presence of pYEEI in the eluted sample would interfere with subsequent LC-MS/MS analysis of pTyr peptides. To solve this problem, we used biotin-pYEEI as the competing agent. After elution, the biotin-pYEEI could be removed by immobilized NeutrAvidin. Thus, in this approach two affinity purifications are performed. To facilitate this fractionation procedure, we designed the biphasic affinity chromatography scheme as shown in Figure 1. The sample to be enriched was first incubated with the Sepharose beads with immobilized Src superbinder at 4 °C overnight with rotation. After the pTyr peptides were bound to the superbinder, the beads were transferred into a 1 mL pipet tip prepacked with NeutrAvidin agarose resins at the end of the tip. In this way, a biphasic affinity tip, where the superbinder was at the top and the avidin was at the bottom, was formed. When the biotin-pYEEI was used to elute the bound pTyr peptides from the superbinder beads, the excess biotin-pYEEI was directly captured by the avidin at the bottom of the tip. In this way, the enriched pTyr peptides would be free of biotin-pYEEI. Using the biphasic affinity tip, the fractionation of the pTyr peptides could be conveniently achieved by elution with increasing concentrations of competitive agent biotin-pYEEI.

We first investigated the performance of this approach to enrich pTyr peptides from the complex peptide mixture. Tryptic digest of the total cell lysate of Jurkat cells was first subjected to purification with immobilized titanium(IV) ion affinity chromatography (Ti^{4+}-IMAC) to deplete unphosphorylated peptides. The resulting phosphopeptides, with the majority of them being pSer/pThr peptides as they were the highly abundant ones, were then subjected to the biphasic affinity purification scheme to enrich the low-abundance pTyr peptides. The biphasic affinity tip was thoroughly washed to remove pSer/pThr peptides and residual nonphosphorylated peptides. The bound pTyr peptides were then eluted by 30 μM biotin-pYEEI and analyzed by LC-MS/MS. This resulted in the identification of 2,467 unique peptides (Supporting Information Table S1), of which 2,194 (88.93%) were pTyr peptides, indicating good specificity. We also investigated if the avidin purification could be omitted. As shown in Figure 2A, the biotin-pYEEI was observed to be eluted from 65 to 90 min for the RPLC analysis of the sample without avidin purification. Because of serious interference of the biotin-pYEEI in the sample, only 1,300 pTyr peptides were identified without NeutrAvidin. As shown in Figure 2B, the biphasic affinity chromatography could effectively remove the interference of biotin-pYEEI, resulting in a 69% increase in pTyr peptide identification. Clearly the biphasic affinity tip with NeutrAvidin was preferred for competitive elution.

Instead of competitive elution, the bound peptides could also be eluted by TFA, which resulted in the identification of 3,034 unique peptides where 2,553 (84.15%) were pTyr peptides. Compared with TFA elution, higher specificity was obtained by the competitive elution. However, a slightly lower number of pTyr peptides was identified by the latter approach. Similar results were obtained from replicate runs (Supporting Information Table S1). Combining the pTyr sites identified from two replicate runs for each method, the competition method recapitulated 82% of the pTyr sites identified by the TFA elution method. The number of identified pTyr sites did not increase with a further increase of the biotin-pYEEI concentration, indicating that 30 μM competitive peptide was sufficient to elute the bound pTyr peptides from the superbinder (Supporting Information Table S1). A significant advantage of the competitive approach is its high specificity. When the crude protein digest without Ti^{4+}-IMAC enrichment was directly subjected to the biphasic affinity chromatography purification, high specificity (>72%) was still achieved (Supporting Information Table S1). In contrast, for the TFA elution method, the specificity for pTyr identification was 44% with the majority of identified peptides found to be unphosphorylated.

To achieve wider coverage of the Tyr phosphoproteome, we used stepwise competitive elution to fractionate the bound pTyr peptides in the biphasic affinity purification protocol. The same sample, i.e., the phosphopeptides enriched from the digest of the total cell lysate of Jurkat cells by Ti^{4+}-IMAC, was loaded onto the superbinder sepharose beads. The weakly bound peptides were eluted by washing with low concentration (4 μM) of biotin-pYEEI. More strongly bound peptides were eluted stepwise with 8, 16, and 32 μM biotin-pYEEI. Each fraction was separately collected and analyzed by LC-MS/MS. After database searching, 3,480 unique pTyr sites were identified in two replicate MS runs from 2 mg of initial Jurkat cell lysate. We compared the number of identified unique pTyr sites in different fractions (Figure 3A). It was found that most

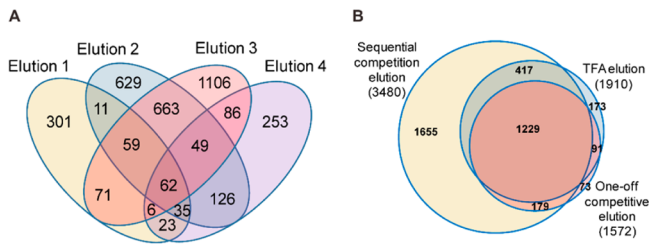

Figure 3. Venn diagrams illustrating (a) the unique pTyr sites found in the four sequential elutions using competitive biotin-pYEEI. (b) Overlap of pTyr sites identified by sequential competitive elution, one-step competitive elution and conventional one-step elution using TFA. All sites have a localization probability > 0.75 and ΔPTM score ≥ 5.

pTyr sites, 1,467 (42.2%), were identified in fraction 2. Only 62 (1.8%) were detected in all four fractions, indicating excellent resolution of fractionation. For deep analysis of tyrosine phosphoproteome, separation and fractionation approaches to further reduce the sample complexity are equally important with specific enrichment. As shown in Figure 3B, compared with the single-step competitive elution method, 2.2-fold more pTyr sites were identified by the fractionation method, which indicated the coverage was improved significantly. We also found that >85% of sites identified by the TFA elution could also be identified by the fractionation method, indicating that

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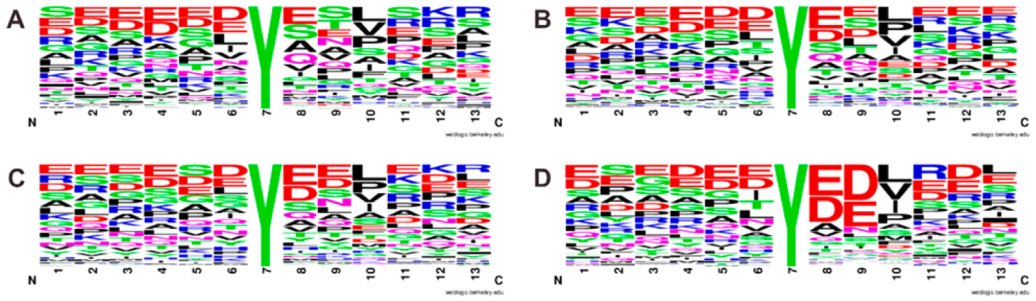

Figure 4. Phosphotyrosyl motif enrichment analysis for the pTyr sites identified from the four sequential competitive elutions (frequency plot of all amino acids flanking the phosphotyrosine site).

the stepwise elution with the competitive peptide was fairly complete.

According to the mechanism of competitive elution, the weakly bound peptides would be first eluted with the initial elution buffer containing low concentration of biotin-pYEEI, and more strongly bound peptides would be eluted in the next round of wash with higher concentration of elution agent. Since the binding of Src superbinder to pTyr peptides is dependent on the primary sequence around the pTyr, we compared the amino acid residues around the pTyr sites identified in the four sequential competitive elutions to elucidate the characteristic distribution. First, we investigated the consensus sequences containing pTyr sites using WebLogo software for the four fractions.[20] Considering the weakly bound peptides may not be completely replaced by the elution agent and still appeared in the latter eluent, we separately uploaded the new identified pTyr sites from each elution compared to the results in the former elutions with a 13 amino acid sequence window to WebLogo and obtained the frequency plots as shown in Figure 4. An apparent increase in frequencies for Asp (D) and Glu (E), C-terminal to the pY residue (pY + 1, + 2), were observed in the four sequential elutions, indicating these two residues are dominant determinants of strong binding affinity to the Src superbinder. Besides, sequences containing Leu (L)/Val (V)/Pro (P)/Ile (I) occupied a great proportion at position of pY + 3 for the strong binding. Consequently, the Src superbinder preferred phosphopeptides with the pY−[D/E]−[D/E]−[L/I/V/P] motif. This motif is highly consistent with the binding motif of wild-type Src SH2 domain, which confirms that superbinder SH2 has the same specificity regardless of its markedly enhanced binding affinity.

The pTyr mediates the assembly of protein complexes essential to intracellular kinase signaling. The Comprehensive Resource of Mammalian Protein Complexes (CORUM) is a database of manually curated and validated mammalian protein complexes.[21] The pTyr sites identified in this study were mapped to the complexes in CORUM. Among 1,565 complexes in the CORUM, 538 (34.4%) contain at least 1 pTyr sites (Supporting Information Figure S1). Some complexes (98, 6.3%) have more than five pTyr sites. This indicated that tyrosine kinases play a significant role in regulating the function or assembling of protein complex. The fractionation of pTyr peptides by the Src SH2 superbinder enabled the separation of the pTyr sites into different categories. We then investigated if any macromolecular complexes enriched with pTyr proteins with different site categories. It was found a total of 86 complexes were enriched with p-value < 0.05 (hypergeometric test) (Supporting Information Table S3). Interestingly, the 11 complexes with p-value < 0.001 were observed for pTyr proteins identified from elution 4 with the strongest binding sites. These included the emerin complex 1, emerin regulatory complex, emerin complex 24, emerin complex 25, emerin complex 52, ASCOM complex, emerin complex 32, SNW1 complex, PLCB1-PARD3-PARD6A complex, nogo−potassium channel complex, and LMO4-gp130 complex. Among them, >50% complexes were involved in Emery−Dreifuss muscular dystrophy (EDMD) and found to be enriched with an average p-value less than 0.000001 (Supporting Information Table S4). It is known that EDMD is caused by loss of emerin, a hyper-tyrosine-phosphorylated protein, in which 13 pTyr sites (Supporting Information Figure S2) have been identified to date.[22] In this study, we identified 13 pTyr sites; all of them were the same as the reported 13 pTyr sites, providing an excellent example of the comprehensiveness of our phosphoproteome analysis.

CONCLUSIONS

Comprehensive study of Tyr phosphoproteome is challenging because of the low abundance of pTyr relative to pSer and pThr. Here we presented a Src SH2 superbinder based sequential competitive elution strategy for deep Tyr phosphoproteome analysis. This strategy is accomplished by applying a biphasic affinity approach in which a competitive biotinylated pTyr peptide (biotin-pYEEI) was used to elute pTyr peptides bound to the Src superbinder−agarose beads and the excess competitive agents in the eluents were removed by NeutrAvidin agarose resin in an integrated tip system. As a high-throughput approach to identify tyrosine phosphoproteome with deep coverage and to distinguish high-affinity pTyr peptides from low-affinity ones, this strategy can be a powerful and cost-effective tool to shed light on the function of eukaryotic proteomes regulated by tyrosine phosphorylation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b04288.

Plots of distributions of complexes, amino acid sequences of emerin, number of pS/T/Y sites and peptides identified using different listed elution strategies, and enriched complexes from sequential elutions and specifically from elution 4 (PDF)

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Notes

The authors declare no competing financial interest.

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