High Throughput Platform Suitable for Common Proteomic Techniques

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Overview

Purpose

- Using in-well tryptic digestion and MALDI-MS analysis, identify a binding partner to a histidine-tagged protein, in a model system, following purification using a HIS-Select High Capacity (HC) Nickel Coated 96-Well Plate

Methods

- Capture histidine-tagged protein and binding partner in wells of HIS-Select HC plate
- Analyze intact protein by SDS-PAGE and MALDI-MS
- Reduce, alkylate, and tryptically digest captured protein samples for analysis by MALDI-MS

Results

- HIS-Select HC plates captured sufficient protein to perform downstream analysis including SDS-PAGE with Coomassie staining and MALDI-MS
- Various conditions of tryptic digestion yielded sequence coverage of 30-80% for a model protein, which allowed positive identification
- The potential of the HIS-Select HC plates for the study of protein-protein interaction was demonstrated
- The utility of the HIS-Select HC plates as a solid support for reduction, alkylation, and tryptic digestion was demonstrated

Introduction

Immobilized metal affinity chromatography (IMAC) is widely used for the purification and identification of recombinant fusion proteins with histidine-containing tags. A unique HIS-Select HC Nickel Coated 96-Well plate was used to purify microgram quantities of a histidine-tagged protein from a COS-7 lysate in a single step. Upon elution of the protein, characterization was performed including SDS-PAGE and MALDI-MS.

In addition to analysis of intact proteins, proteomics techniques routinely use proteolytic enzyme digestion to generate signature peptides for protein identification. The most commonly used protease in the field is trypsin. Because of its high specificity for cleavage at the carboxyl side of arginine and lysine residues, it is simple to predict the peptides from a digested protein of known sequence. Unknown proteins are typically identified by searching the databases for the m/z ratios of the signature tryptic peptides.

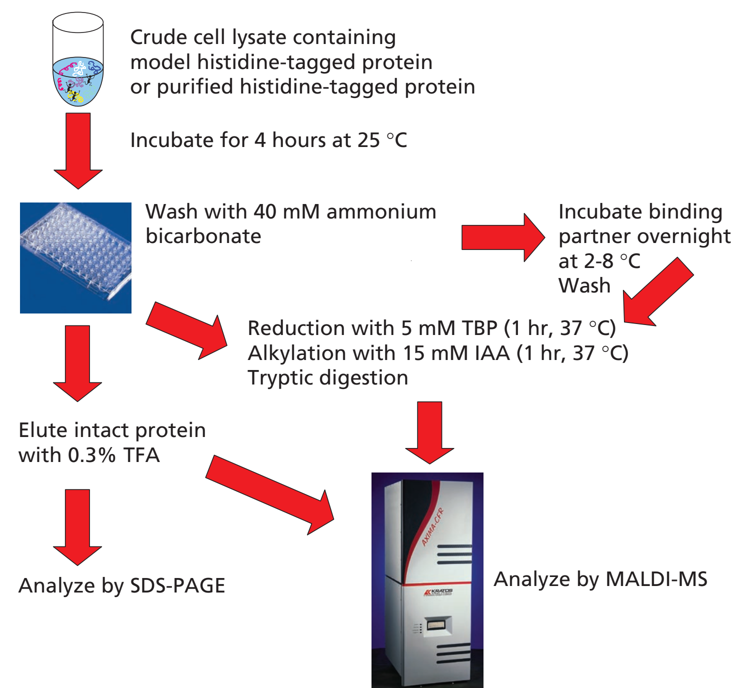
In this poster, a model system was used to demonstrate the ability to positively identify a histidine-tagged protein with known sequence and its binding partner. The two proteins were captured in the wells of a HIS-Select HC plate and the plate then served as a support to perform the reduction, alkylation, and tryptic digestion. The peptide mixture, eluted from the plate, was studied by MALDI-MS. Peptides from the bait protein and the binding partner were matched to individual trypsin digests of each protein.

In addition to the single step purification of the protein of interest from a crude lysate, the multiwell plate offers convenient, high throughput methodology that resins do not. The utility of the plate is not only a tool for purification, but also a high throughput platform for typical proteomic applications used for positive identification of unknown proteins of interest.

Materials

- HIS-Select™ HC Nickel Coated 96-Well plate (S 5563, Sigma-Aldrich)
- HIS-Select™ HC Nickel Affinity Gel (P 6611, Sigma-Aldrich)
- Tributylphosphine (TBP) Stock Solution (T 7567, Sigma-Aldrich)
- Alkylating Reagent, Iodoacetamide (IAA) (A 3221, Sigma-Aldrich)
- Trypsin, Proteomics Grade (T 6567, Sigma-Aldrich)
- α-cyano-4-hydroxycinnamic acid (C 8982, Sigma-Aldrich)
- MALDI calibration standard kit (MS-CAL1, Sigma-Aldrich)
- Model protein containing a patent pending metal affinity histidine tag (expressed in *E. coli* at Sigma-Aldrich)
- Monoclonal antibody directed to the model protein
- ColorBurst™ Electrophoresis Markers (C 4105, Sigma-Aldrich)
- C_{18} Zip Tip™ (Millipore, Inc.)
- All other reagents were purchased from or prepared at Sigma-Aldrich.

Methods



- In-well reduction, alkylation, and tryptic digestion were performed sequentially. Different conditions used included 40 mM ammonium bicarbonate (AB), 40 mM ammonium bicarbonate containing 30% acetonitrile (AB/ACN), 40 mM ammonium bicarbonate containing 100 mM imidazole (AB/imidazole), or 4 M Urea (Urea) in the wells of the HIS-Select HC plate.
- For digestion, trypsin was initially dissolved in 0.1 N HCl and then added at a ratio of 1:80 trypsin to bound target protein (w/w) per well. The plate was allowed to incubate at 37 °C overnight.
- The MALDI matrix was prepared at a concentration of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile (ACN), 0.03% trifluoroacetic acid (TFA).
- When necessary, the peptide samples were desalted before analysis using a C_{18} Zip Tip.™ Peptides were eluted with the MALDI matrix directly onto an uncoated MALDI target.
- MALDI-MS analysis was performed in the reflectron positive ion mode using a Shimadzu-Biotech Axima-CFR+.

Results

Purification of Model Histidine-Tagged Protein from COS-7 Lysate: MALDI-MS

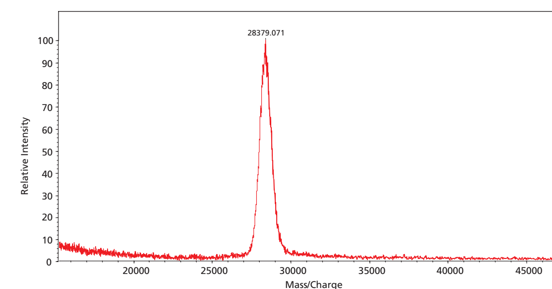


Figure 1. MALDI mass spectrum of affinity purified histidine-tagged protein. The histidine-tagged protein was spiked in COS-7 lysate, affinity purified on the HIS-Select HC plate, and eluted. The sample was zip-tipped prior to analysis by MALDI-MS.

The results gave a single peak at the expected mass for the histidine-tagged protein.

Purification of Model Histidine-Tagged Protein from COS-7 Lysate: SDS-PAGE

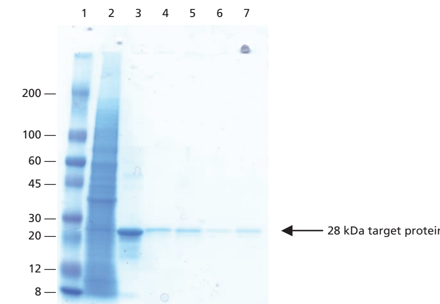


Figure 2. SDS-PAGE 4-20% Tris glycine gel of the affinity purified histidine-tagged protein stained with EZBlue. Lane 1 contains ColorBurst markers. Lane 2 contains the COS-7 lysate spiked with purified histidine-tagged protein (0.1 mg/ml, see lane 3) and lane 4 contains 5 μg of the histidine-tagged protein affinity purified from COS-7 on the HIS-Select HC plate. Lane 3 contains the histidine-tagged protein affinity purified from *E. coli* on the HIS-Select agarose and lane 5 is the captured histidine-tagged protein on the plate of material that was previously purified on the HIS-Select agarose. Lanes 6 and 7 contain the histidine-tagged protein after reduction and alkylation, respectively, performed while the protein was still bound to the plate. The loads in lanes 4-7 represent 5% of the protein captured per well. Samples eluted after capture in lanes 4-7 contained approximately 6 μg of protein per well by a BCA protein assay.

The SDS-PAGE gel demonstrates the specificity of the plates for a histidine-tagged protein. Additionally, the gel illustrates that once captured, the histidine-tagged protein remains bound to the plate after reduction and alkylation.

Tryptic Digestion of Purified Model Histidine-Tagged Protein on HIS-Select HC Plates

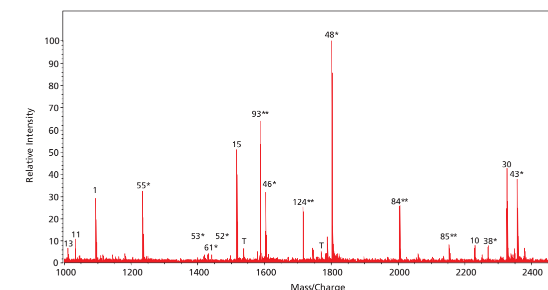


Figure 3. MALDI mass spectrum of a tryptic digest of the histidine-tagged protein affinity purified from a COS-7 lysate. Reduction, alkylation, and tryptic digestion were performed in the wells of the HIS-Select plate. The tryptic digestion was performed in 40 mM ammonium bicarbonate containing 30% acetonitrile. The peptide numbers designated with * and ** are the peptides resulting from a single partial cleavage and a second partial cleavage, respectively. T represents autolytic tryptic peptides of the proteomics grade trypsin.

Upon tryptic digestion, 80% sequence coverage was obtained, including peptide number 55* which contains an alkylated cysteine.

Observed Peptides of the Histidine-Tagged Protein from Various Tryptic Digestion Conditions

Theoretical Peptides		Observed Peptides				
Peptide No.	MW	AB/imidazole	AB/ACN	AB	Urea	AB-wash (no zip-tip)
13	1013					x
27	1027					
11	1033	x	x	x	x	x
1	1095					x
5	1139					x
4	1150					x
15	1517	x	x	x	x	x
10	2230	x		x		x
30	2327	x	x	x	x	x
Partial Cleavages (1)						
36*	1012					x
60*	1183	x				x
55*	1235					x
52*	1442					x
46*	1603					x
48*	1803	x	x	x	x	x
49*	2080				x	
38*	2269	x	x	x		x
43*	2357	x	x		x	x
total % sequence coverage		60	33	45	58	80

Table 1. Model histidine-tagged protein theoretical tryptic peptide fragments in the mass range illustrated in the spectrum given in the figures. The masses of the cysteine containing peptides reflect alkylation with iodoacetamide. The peptide numbers designated with * are peptides resulting from a single partial cleavage.

Protein-protein Interaction Study After Tryptic Digestion

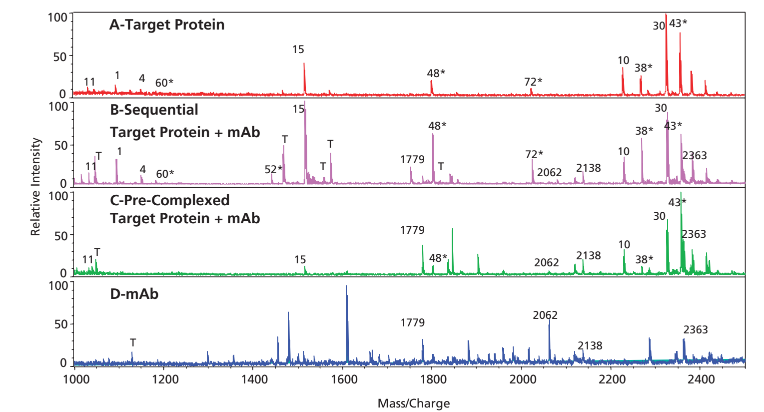


Figure 4. MALDI mass spectra of **A)** tryptic digest of affinity captured histidine-tagged target protein after reduction, alkylation, and tryptic digestion in the wells of the HIS-Select HC plate in 40 mM ammonium bicarbonate. (No wash steps between the reduction, alkylation, and tryptic digestion.) **B)** Tryptic digest of sequentially captured histidine-tagged target protein and a monoclonal antibody (mAb) directed to the protein. Reduction and alkylation were performed in 40 mM ammonium bicarbonate. The sample was zip-tipped prior to MALDI-MS analysis. **C)** Tryptic digest of captured histidine-tagged protein pre-complexed with a monoclonal antibody directed to the protein. Reduction and alkylation were performed in 40 mM ammonium bicarbonate containing 30% acetonitrile. The sample was zip-tipped prior to MALDI-MS analysis. **D)** Solution tryptic digest of monoclonal antibody directed to the histidine-tagged protein following reduction and alkylation in 40 mM ammonium bicarbonate. The peptide numbers designated with * and ** are the peptides resulting from a single partial cleavage and a second partial cleavage, respectively. T represents autolytic tryptic peptides of trypsin.

The data demonstrates that signature tryptic peptides from the histidine-tagged protein were obtained. Additionally, when comparing the captured digest to the solution digest of the antibody to the protein, matching peaks were identified.

Expanded Window from 2000-2300 Daltons from Figure 4

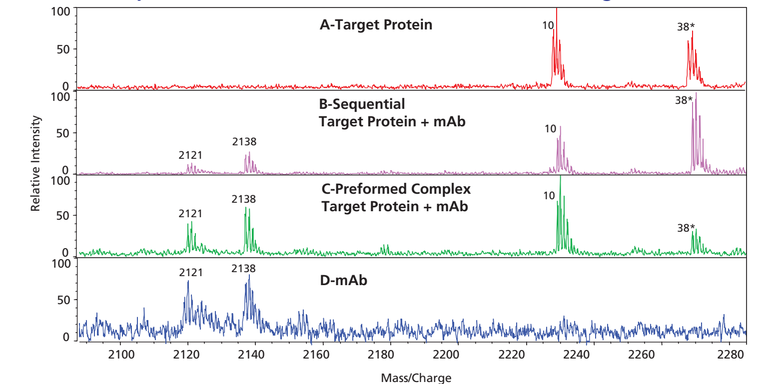


Figure 5. Region of MALDI spectrum expanded to demonstrate the overlapping peaks of Figure 4 (A-D).

This region demonstrates two peptides from each of the proteins captured in the well of the plate.

Conclusions

- HIS-Select HC plates are capable of capturing microgram quantities of protein per well, providing ample material to perform downstream analysis including SDS-PAGE and MALDI-MS
- 30-80% sequence coverage was demonstrated upon tryptic digestion of a model histidine-tagged protein in the wells of the HIS-Select HC plates after affintiy purification from COS-7 lysate in the wells
- The potential of the plates for the study of protein-protein interaction was demonstrated both sequentially and with preformed complexes
- The plates are a useful platform for high throughput purification of a histidine-tagged protein and interacting proteins and are compatible with in-well reduction, alkylation, and tryptic digestion prior to analysis by MALDI-MS