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Simple Protein Complex Purification and Identification Method for High-Throughput Mapping of Protein Interaction Networks

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Most current methods for purification and identification of protein complexes use endogenous expression of affinity-tagged bait, tandem affinity tag purification of protein complexes followed by specific elution of complexes from beads, and gel separation and in-gel digestion prior to mass spectrometric analysis of protein interactors. We propose a single affinity tag in vitro pull-down assay with denaturing elution, trypsin digestion in organic solvent, and LC−ESI MS/MS protein identification using SEQUEST analysis. Our method is simple and easy to scale-up and automate, making it suitable for high-throughput mapping of protein interaction networks and functional proteomics.

Keywords: protein complex purification • identification • mass spectrometry • mapping protein interaction networks

Introduction

Purification and identification of protein complexes is fundamental to deciphering functions of proteins with known sequence but unknown biological activity. Therefore, establishing the molecular interactions in protein complexes of living cells is one of the major tasks of molecular biology in the post-genomic era.[1] Several approaches have been developed for purifying and identifying protein complexes, such as two-hybrid methods,[2] enzyme assays,[3] or tandem affinity purification (TAP) of protein complexes followed by mass spectrometric analysis.[4] The TAP approach is based on two successive affinity chromatography steps with specific elution combined with a proteolytic cleavage step that enhances elution specificity. The TAP approach is increasingly popular and has been used at the proteome level for to describe the whole yeast interactome.[5] However, TAP and the other approaches are complex and require many steps to get high-quality data.

Expressing the affinity-tagged bait protein in vivo interferes with endogenous expression of the same protein and can significantly change cellular physiology. In addition, the endogenous untagged bait protein interferes with in vivo protein complex formation involving the tagged bait protein, thus decreasing the pull-down yield. Ultimately, to increase the pull-down yield, the endogenous bait expression should be decreased for example, by using the RNAi technology as recently shown by Forler et al.[6] Running SDS-polyacrylamide gels to separate the proteins, followed by band excision, in-gel digestion and mass spectrometric analysis as described by Shevchenko et al.,[7,8] is tedious and difficult to automate. The most commonly used peptide digestion method of MS sample preparation is also quite laborious: multiple steps of urea denaturation and sulfhydryl group reduction followed by carboxymethylation and desalting, during which the samples can undergo significant losses or unwanted modification. To enable a systems biology approach, large data sets need to be generated quickly; therefore, simpler methods are needed for protein complex purification and identification that are easy to scale-up and automate.

In this work, we addressed the limitations of the above approaches by developing a simple protein complex purification and identification method suitable for automated high-throughput protein complex purification. We optimized our method, using the *Shewanella oneidensis* degradosome complex, by directly evaluating the quality of peptide MS identification. Main protein components of this well-known and well-conserved complex have been established in *Escherichia coli* as polynucleotide phosphorylase, (PNP), RNase E and DEAD Box RhlB RNA helicase, and enolase.[9] However, only RNase E and the RNA helicase exhibit physical interactions with PNP,[9] which we used as the bait.

Experimental Section

Chemicals and Materials. We used Affi-Gel 10 (BioRad, Hercules, CA), MagneHis (Promega, Madison, WI), Ni-NTA magnetic beads (Qiagen, Valencia, CA), Talon Metal Affinity Resin (BD Biosciences-Clontech, Palo Alto, CA), and imidazol and other reagents (Sigma, St. Louis, MO).

Gene Cloning. The gene-encoding polynucleotide phosphorylase (PNP, SO1209) in *S. oneidensis* MR-1 was cloned into the pBAD202/D-TOPO (Invitrogen, Carlsbad, CA) expression vector that adds the V5 and hexahistidine affinity epitopes and HP thioredoxin to the C- and N-termini of expressed proteins, respectively. To avoid being N-terminally fused with HP

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thioredoxin, we added a stop codon to the forward PCR primer at the 5′-end after CACC sequence to terminate translation of HP thioredoxin. In addition, the forward primer was designed to amplify not only the protein-coding regions but also the ribosome binding sites to ensure proper initiation of translation for protein synthesis. PCR was carried out in 30 μL containing 60 ng of genomic DNA of *S. oneidensis* MR-1, 30 pmol of each of the forward and reverse primers, and three units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Following initial denaturing at 94 °C for 3 min, the sample was subject to 25 cycles of amplification, consisting of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min per kb of DNA to be amplified. After PCR, 5 μL of resulting PCR products were used for electrophoretic analysis on a 1% agarose gel. They were then purified with a PCR purification kit from Qiagen. The purified PCR products were used for cloning according to the manufacturer's instructions.

Protein Expression and Purification. PNP was expressed in 1 L of *S. oneidensis* MR-1 cell culture incubated at 25 °C overnight in 2-L Fernbach culturing flasks after induction with 0.2% arabinose at O. D. 600 = 0.6−0.9 in LB medium containing 50 μg/mL kanamycin. The cells were harvested by centrifugation for 10 min at 7000 rpm in 1-L Kendro rotors on a RC5B Plus Sorvall centrifuge for 10 min and re-suspended in 1:3 v/w of the lysis buffer (20 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mg/mL lysozyme, 5 mM mercaptoethanol, 10 μg/mL RNase A [bovine pancreas] Roche 100−134, 5 μg/mL DNase [bovine pancreas grade II] Roche 104−159), flash frozen in liquid nitrogen, and stored at −80 °C. The frozen cell suspensions in 50-mL Falcon tubes were quickly thawed at 37 °C and then sonicated at 0 °C on ice using a small probe on a sonic dismembrator (Model 100, Fisher) in three 1-min cycles at the maximum power setting. The cell lysates were spun down on a Beckman ultracentrifuge L8−70M with a 50.2 Ti rotor at 40 000 rpm (∼193 000 × *g*) for 1 h, and protein samples were purified using 1.5 mL Ni-NTA microcolumns (Qiagen). The purified protein was then concentrated in 10 000 MWCO centrifugal filter devices, Amicon Ultra-4 and −15 (Millipore) to 3 mg/mL, and dialyzed against 20 mM HEPES, pH 7.5 containing 0.150 mM sodium chloride overnight at 4 °C. Homogeneity of the purified PNP was more than 95% evaluated by 4−12% precast Tris-Bicine polyacrylamide gels (Invitrogen) using In−Vision, HisTag stain (Invitrogen) and Coomassie Blue stain (Gel Code Blue Stain Reagent, Pierce, Rockford, IL) for specific and nonspecific protein staining, respectively.

Preparation of the Host Cell Lysate. *S. oneidensis* MR1 cells were grown on LB at 30 °C overnight. Cells were spun at 8000 rpm for 8 min at 4 °C in a Beckman Avanti J-25 centrifuge using a JLA 10.5 rotor and carbon-fiber secondary-tube holders. A cell pellet from one 40-mL centrifuge tube was used to determine wet weight biomass after decanting the supernatant. The cells were re-suspended in lysis buffer (20 mM Tris HCl, pH 7.5, 150 mM sodium chloride, 5% glycerol, 0.05% NP 40) 1:1 w/v and stored at −80 °C. The frozen cell suspension was thawed in a 37 °C warm-water bath, and the rest of the procedure was performed at 4 °C. The cell suspension was passed through the French Pressure cell (GlenMills, Inc.) twice, then centrifuged using a L8-M ultracentrifuge (Beckman Coulter) at 40 000 rpm in 50.2 Ti rotor (∼193 000 × *g*) for 1 h to remove cell debris. The cleared supernatant was preincubated for 1 h at 4 °C with the same number of blank beads to be used in pull-down experiments with the bait.

Covalent Binding of Baits to Agarose Beads. AffiGel (Bio-Rad) was washed three times with 10 volumes of ice-cold water. The agarose gel slurry was then adjusted to 50% slurry v/v with coupling buffer (50 mM HEPES, pH 7.5, 150 mM NaCl). AffiGel was then added to a protein solution of 1.5 mg/mL and incubated for 4 h at 4 °C. Control beads were made concurrently using 100 mM ethanolamine, pH 9.0. The amount of protein bound to the beads was measured by differential Bradford assays before and after coupling.

Binding of Baits to Ni^{2+} Affinity Beads. Ni^{2+} beads in 1:1 v/v suspension of binding buffer were exposed to purified dialyzed bait protein in the ratio of 1:5 v/w overnight at 4 °C in the binding buffer (20 mM Tris buffered saline, pH 7.5, containing 5% glycerol and 0.05% NP-40). After determining the bound protein using a Bradford assay before and after binding, the volume of bead suspension was adjusted to contain 1 mg of protein per milliliter of bead suspension.

Protein Complex Purification. Aliquots of bead suspension containing either covalently or affinity-immobilized protein in the amount of 10−20 μg were washed with binding buffer three times and exposed to the host cell lysate for 1 h at 4 °C. The beads were then washed three times with binding buffer, and protein interactors were subsequently eluted from the beads using denaturing eluant (8 M urea, 0.8% sarcosyl, or 40% acetonitrile). If sarcosyl elution was employed, then cold precipitation with 80% v/v ethanol overnight was used to remove sarcosyl from the pull-down sample. The protein mixture was then digested by trypsin and submitted to MS analysis.

Trypsin Digestion after Denaturation by 8 M Urea. The protein mixture was re-suspended in 500 μL final volume of 50 mM Tris buffer, pH 7.4. Solid urea was added to 8 M and 1 M fresh DTT solution was added to 5 mM final concentration and incubated at room temperature for 30 min. Then the 50 mM Tris HCl buffer was added to a final concentration of 2 M urea. Trypsin (Promega) was added at 1:50 w/w, and the mixture was incubated overnight at 37 °C. Finally, the samples were cleaned on a C_{18} microplate (Supelco-Sigma, St. Louis, MO) and forwarded for ESI−LC MS/MS.

Trypsin Digestion in 40% Acetonitrile. Trypsin digestion in acetonitrile was performed according to the procedure of Russell et al.[10] in 40% acetonitrile, 25 mM ammonium bicarbonate, and pH 8.0 at 37 °C for 1 h. Acetonitrile was then evaporated and the samples were forwarded for ESI−LC MS/MS.

Automated LC−ESI MS/MS Analysis. Samples were analyzed on a fully automated LC MS/MS system using an Agilent 1100 series capillary HPLC system (Wilmington, DE) interfaced to a Finnigan LCQ Deca XP ion trap mass spectrometer (Thermo, San Jose, CA). An 8-μL sample was loaded onto an in-house prepared reversed-phase capillary column (360 μm o.d. × 150 μm i.d., 30 cm length, 5 μm, Jupiter C_{18} Phenomenex, Torrence, CA) using mobile phase A (0.2% acetic acid and 0.05% trifluoroacetic acid in water) at a flow rate of 3 μL/min. After 5 min, peptides were eluted with a 60-min linear gradient of 0−85% mobile phase B (90% acetonitrile and 0.1% trifluoroacetic acid in water). This was followed by a 5-min linear gradient from 85 to 95% to flush the column of highly retained species and a 5-min linear gradient back to starting conditions where the column was allowed to re-equilibrate for 40 min prior to another injection. Eluting peptides were introduced to the ion trap mass spectrometer using an in-house designed electrospray ionization (ESI) source and emitter without a sheath gas.

The capillary temperature and spray voltage were 200 °C and 2.5 kV, respectively. Samples were analyzed over a mass (m/z) range of 400−2000 followed by MS/MS analysis of the three most abundant ions from the MS spectra using a collision energy setting of 45%. Dynamic exclusion was used to discriminate against previously analyzed ions.

SEQUEST Analysis of Peptides. Tandem mass spectra were analyzed by SEQUEST (Bioworks 3.0, Thermo, San Jose, CA),[11] which performs analyses by cross-correlating experimentally acquired mass spectra with theoretical idealized mass spectra generated from a database of protein sequences. These idealized spectra are weighted largely with *b* and *y* fragment ions; that is, fragments resulting from the amide-linkage bond from the N- and C-termini, respectively. For these analyses, no enzyme rule restrictions were applied to the possible cleavage points available for peptide generation from the initial proteins, allowing identifications resulting from nontryptic cleavage to be observed as well. The peptide mass tolerance was 3.0, and the fragment ion tolerance was 0.0. The FASTA protein database searched against was generated by TIGR (http://www.tigr.org) of the *S. oneidensis* MR-1 project containing an annotated protein list. Only the peptides passing the X_{corr} defined by Washburn et al.[12] were considered.

Results and Discussion

Pull-Downs Using Covalently- and Affinity-Bound Bait. We first compared pull-downs using covalently- and affinity-bound PNP, SO1209. Both covalent binding and nickel-hexahistidine interaction satisfied the requirement of strong binding of the bait to the beads; that is, resistance to denaturing conditions created by application of 8 M urea, 0.8% sarcosyl, and 40% acetonitrile. We selected AffiGel as the matrix for covalent binding because of its low nonspecific protein background. We compared multiple beads for this purpose using 8 M urea as the denaturing elution reagent. The AffiGel agarose beads provided superior binding capacity (~10 mg of protein per milliliter of beads) as well as very low protein background with bacterial lysate. Immobilized metal affinity chromatography (IMAC) is the only affinity method in which the affinant remains to be bound to the beads under strongly denaturing conditions.[13] Therefore, we compared the multiple nickel and cobalt beads listed in the Materials and Methods section for their binding capacity, nonspecific protein interaction, and pull-down efficiency (results not shown). The best matrix following those criteria appeared to be the MagneHis product from Promega. In Figure 1 and Table 1 we compared pull-down results for PNP (SO1209) using the bait immobilized on the AffiGel and MagneHis beads in identical duplicate experiments. Only the bait SO1209; its most abundant interactor, RNase E (SO2785); and one of the most abundant nonspecific interactors, the translation elongation factor Tu (SO2017), are shown in Figure 1. All data that passed Washburn et al.'s X_{corr} criteria in SEQUEST analysis[12] are shown in Table 1. Interestingly, we observed that the intensity of a band on the silver-stained gel does not correspond to the intensity of peaks and quality of data from the MS/MS experiment. For example, the RNase E is not visible on the silver-stained gel made from the 8-M urea elution of the AffiGel, yet it is unambiguously identified in the MS spectra of the same pull-down. Therefore, it was more prudent to base our pull-down analysis directly on the MS data as opposed to comparing silver-stained gels.

It is clear that both the AffiGel and the MagneHis beads provide clean and reproducible pull-down results with minimal background of nonspecific proteins. AffiGel has a cleaner background than the MagneHis. The number of identified peptides for PNP and RNase E that passed Washburn et al.'s X_{corr} criteria in SEQUEST analysis was well above both the background and all nonspecific interactions. Although the AffiGel provides a cleaner background, MagneHis beads provide

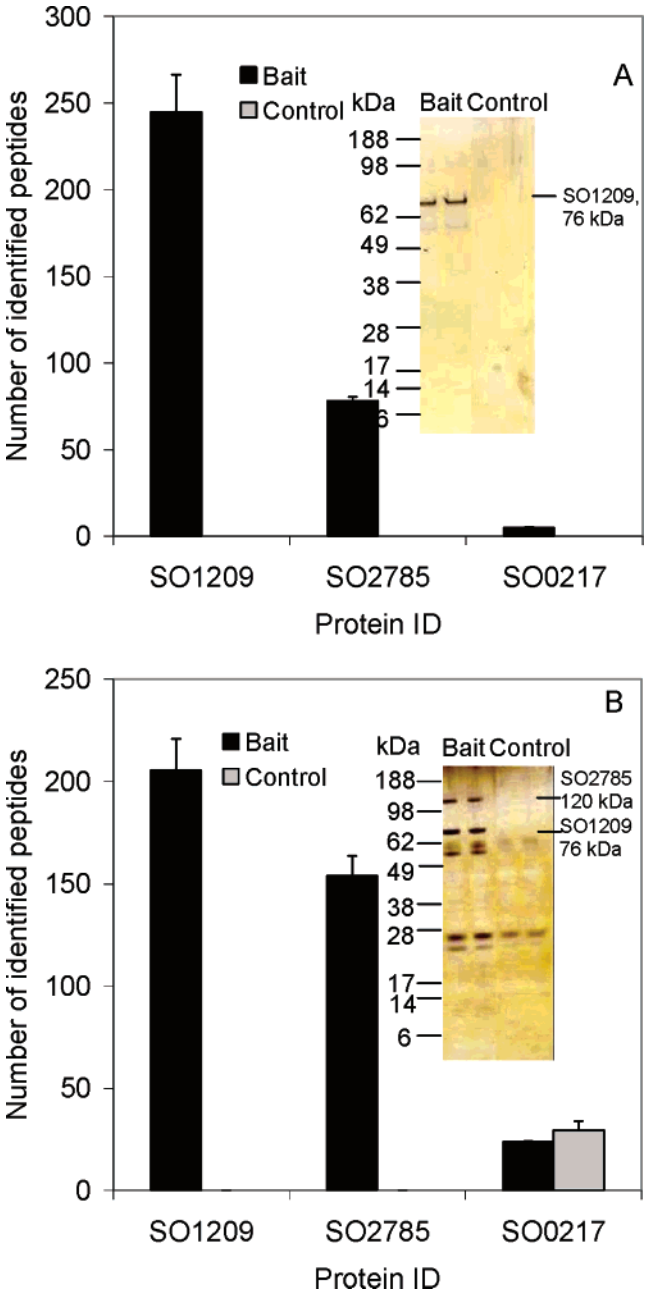

Figure 1. Comparison of SO1209 pull-downs using bait covalently bound to AffiGel (A) with those using bait bound to Ni^{2+} MagneHis (B) beads via a hexahistidine affinity tag. Immobilized bait, 10 μg, was exposed to 1 mL of the fresh cell lysate, 5 mg/mL from *S. oneidensis*, incubated for 1 h at 4 °C and then eluted with 50 μL of 8 M urea for 30 min. Twenty percent (10 μL) of the eluted sample was used for analysis on 4−12% gradient SDS polyacrylamide gels, and the rest was used for LC−ESI MS/MS analysis. The data were analyzed by SEQUEST analysis as described above. SO1209, PNP is the bait; SO2875, RNase E, the specific interactor; and SO0217, translation elongation factor Tu, is the nonspecific interactor. Average data from two parallel experiments are shown.

Table 1. LCQ−ESI MS/MS Data from SO1209 Pull-Downs Using AffiGel and MagneHis after SEQUEST Analysis Using X_{corr} According to Washburn et al.[12a]

protein/ gene ID	protein name	average mass [kDa]	average number of identified peptides: AffiGel bait	AffiGel control	MagneHis bait	MagneHis control
SO2785	Ribonuclease E	120.5	78 ± 2		154 ± 10	
SO1209	Polyribonucleotide phosphorylase	75.8	245 ± 22		206 ± 15	
SO 0217	Translation elongation factor Tu	43.3	5 ± 0		24 ± 1	30 ± 4
SO4054	510-methylenetetrahydrofolate reductase	33.1			7 ± 3	6 ± 4
SO4755	ParB family protein	32.5			8 ± 1	12 ± 7
SO2852	Transcriptional regulator GntR family	25.0			31 ± 2	25 ± 2
SO0624	Catabolite gene activator	23.7			3 ± 2	2 ± 0
SO3417	Peptidyl-prolyl cis-trans isomerase SlyD	23.5			21 ± 3	21 ± 8
SO0231	Ribosomal protein L3	22.5			1 ± 1	3 ± 0
SO3940	Ribosomal protein L13	15.7			8 ± 3	0
SO3930	Ribosomal protein S6	15.0			1 ± 1	0
SO3537	Ribosomal protein S20	9.8		4 ± 0		0

[a] Average of two parallel experiments are shown.

a significantly higher peptide score for RNase E with a decreasing score for the bait protein. In addition, the MagneHis beads allow magnetic separation that is easy to automate. AffiGel beads would require filtration technologies downstream of the process that are much more difficult to automate than the phase separations by magnet. Therefore, we selected the MagneHis beads for further optimization.

Optimizing Elution Conditions. Selecting the right denaturing conditions for elution of protein interactors from MagneHis beads is essential to successful pull-downs. The main goal was to achieve maximum yield of specific interactors while minimizing the elution of both the nonspecific interactors and the bait protein. We used three different denaturing elution buffers of decreasing stringency: 8 M urea, 0.8% sarcosyl, and 40% acetonitrile. Figure 2 suggests that the relative amount of eluted bait based on the MS data is the highest for the urea elution. Peptide scores for the bait are lower when sarcosyl and acetonitrile elutions are used. At the same time, the ratios of specific interactor/bait RNase E, SO2785/PNP, and SO1209 are much higher for those elutions compared to the urea elution. This is preferred because the identity of the weakly bound, low-abundance protein interactors must not be overshadowed by bait protein signal. Our data show the optimal elution using 40% acetonitrile, which provides not only high interactor scores but also minimizes elution of nonspecific interactors. Virtually no specific proteins SO1209, SO2785, and SO0407 were identified in the samples prepared from the control beads with no bait. When 20% acetonitrile elution was employed, the stringency appeared to be too low to elute sufficient amounts of the interactor from the beads. We also evaluated acetonitrile containing 0.1% trifluoroacetic acid elution. This condition significantly increased bait elution most likely caused by disruption of hexahistidine-nickel binding by low pH (data not shown).

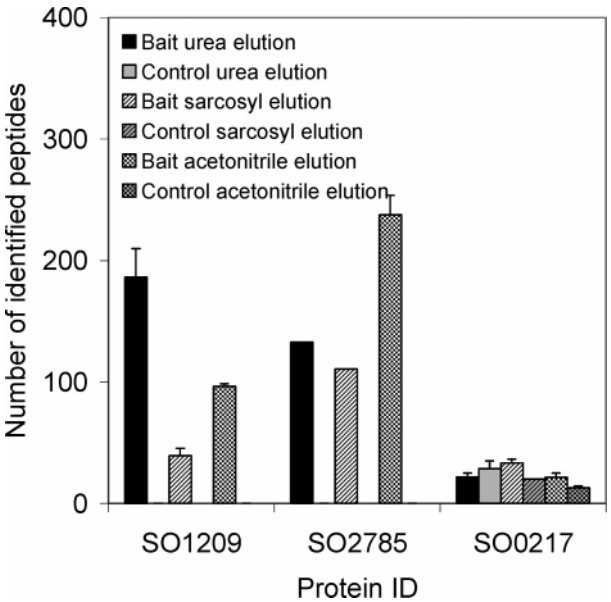

Figure 2. Optimization of pull-down elution conditions. Immobilized bait was exposed to the lysate, and samples were eluted from beads by 30-min incubation with 50 μL of 8 M urea, 0.8% sarcosyl, or 40% acetonitrile. Sarcosyl was removed by precipitation with cold 80% ethanol prior to MS sample processing. Averages of two experiments are shown.

Comparing Trypsin Digestions. Trypsin digestion in 40% aqueous acetonitrile provides a very simple alternative to the complex and tedious procedure involving denaturation by 8 M urea. We compared the two procedures directly by the number of identified peptides in LC−ESI MS/MS spectra in pull-down samples prepared at the same time with equal amounts of the PNP bait protein on the MagneHis beads. Sarcosyl elution was used for both digestion procedures to avoid influencing the data by elution conditions.

Figure 3 shows that the digestion in the aqueous acetonitrile is superior to that of urea-driven trypsin digestion. In this particular experiment we also observed significant peptide scores for RNA helicase, SO0407, another interactor of PNP in bacterial degradosome,[14] suggesting that we are able to observe also weak interactors with this improved procedure. This result is very important because it allows us to streamline our protein complex purification and identification procedure. Using the 40% acetonitrile elution procedure in conjunction with trypsin digestion in the same solvent can provide a seamless transition from protein complex purification to protein complex identification.

Optimizing Bait Amount. Bait amount drives the equilibrium of protein complex formation from endogenously formed protein complexes to those on bait-covered beads. Therefore, this is another critical parameter in the in vitro pull-down procedure. In Figure 3, we show the dependence of pull-down results on bait (PNP, SO1209) amount bound to MagneHis

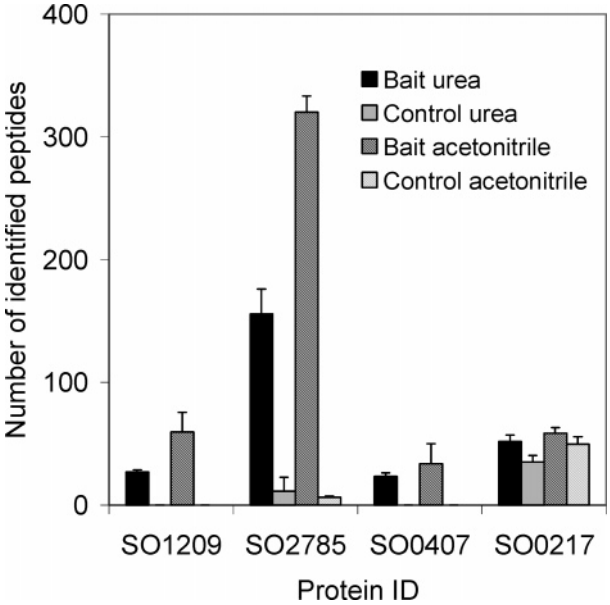

Figure 3. Trypsin digestion in 40% acetonitrile yields better MS data than digestion using 8 M urea for protein denaturation. Pull-downs were performed as above and eluted with 0.8% sarcosyl. The eluted protein samples were precipitated by saturation with cold ethanol to 80%. The samples were then digested with trypsin following the urea denaturation protocol or in 40% acetonitrile (Materials and Methods). Averages of two experiments are shown.

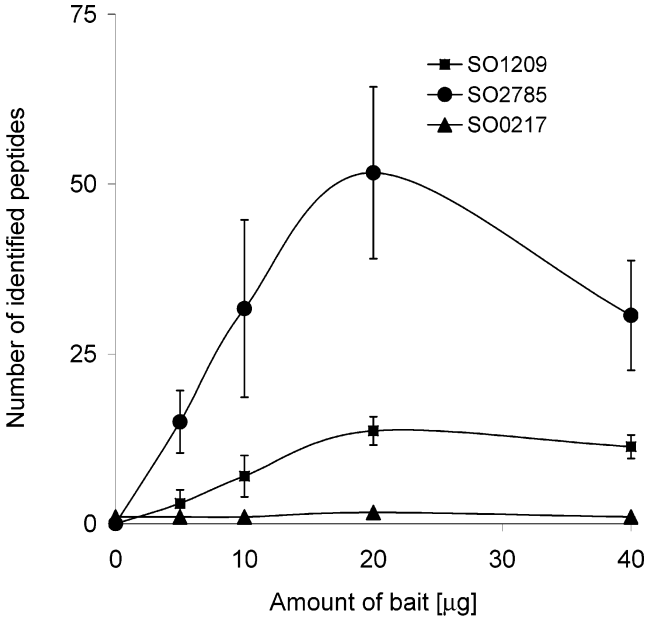

Figure 4. Optimization of the amount of bait used for pull-down experiments. Pull-downs were performed with MagneHis beads, as in Figure 1, eluted with 40% acetonitrile and digested in the same solvent. In averaged data from three experiments of SO1209 PNP, the bait; SO2785 RNase E, the interactor; SO0217; and translation elongation factor Tu, the nonspecific proteins are shown.

beads. The optimal amount of bait for PNP pull-downs appears to be 20 μg. While RNase E and PNP show dramatic dependence on the amount of bait bound to the beads, the nonspecific interactor SO 2017 does not. Similar results were observed for other nonspecific protein interactors (data not shown). This result was typical for nonspecific protein interactors in our pull-down experiments. If the scores obtained from MS data are directly proportional to the protein amounts of identified proteins, our result suggests that only the amount of the specific interactor in the eluate is changing with changing amount of the bait on beads.

Other Pull-Down Optimizations. Many other parameters affect the quality of data from the described in vitro pull-down procedure. Preparation of the host cell lysate is one of the most important parameters. First, the host cell lysate has to be free of any particles that could attach to the beads and elute under denaturing conditions. Therefore, high-speed centrifugation with the centrifuge at 100 000 $\times$ g and higher is essential for obtaining quality pull-down data. We also investigated whether the presence of a detergent in cell lysate affects the pull-down results. Both silver-stained gels and MS data indicated that the detergent can significantly decrease the nonspecific background but also can influence the abundance of the interactors found in the pull-down elution. We performed pull-down experiments with lysate from *S. oneidensis* MR1 using AffiGel with and without bait and using no detergent, 0.05% NP-40, or 0.05% of octylglucosyl (results not shown). The lowest background was observed with use of the 0.05% NP-40.

We have tested other conditions for appropriate preparation and handling of the host cell lysate using our successful pull-down experiments by directly comparing silver-stained gels as well as MS data. We observed that the data obtained from using the fresh lysate were comparable to those obtained from lysate that was frozen and thawed. The optimal concentration of host cell lysate was found to be 5 mg/mL. Higher concentration of the lysate provided higher amounts of interactors, but the background was also much higher, thus complicating sorting of specific and nonspecific interactors by LC−MS/MS analysis. Lower concentration of the lysate provided insufficient amount of proteins for identification.

Another question is how to provide an appropriate control for the pull-down experiment. We observed that the elution profile of the proteins eluted from the beads by denaturing elution strongly depends on what is bound to the beads. Often, the control beads that have no protein bound would capture more nonspecific interactors from the host cell lysate than the beads with a well-folded active protein bound to them. On the other hand, if we denatured the bait protein on the beads by boiling for a few minutes, the denaturing elution of pull-downs from such beads showed a very complex profile of proteins released from the beads. These results demonstrated the importance of correct folding of immobilized proteins for pull-down experiments and may explain why partially denatured baits have higher numbers of nonspecific interactors. The nonspecific background can further be decreased by preincubation of the lysate with the same amounts of beads free of bait protein that are used for the pull-down procedure with the bait.

Conclusions

Affinity tag-based purification of protein complexes followed by identification using mass spectrometry remains one of the main approaches used for elucidation of protein function.[15] Among the methods published to date, tandem affinity purification (TAP) of in vivo formed protein complexes followed by denaturing 1D or 2D gel separation, excising the gel pieces with separated proteins, and analyzing them by mass spectrometry is the most frequently used protein complex purifica-

tion and identification method.[16] There are two obvious limitations of this method, however: (1) endogenous bait competition with the affinity-tagged bait for protein complexes and (2) laborious MS sample preparation.

We address these limitations in the work presented here by developing a simple protein complex purification and identification procedure based on strong binding of bait to beads in combination with denaturing elution followed by the liquid LC−ESI MS/MS-mediated protein identification. Instead of transfecting cells of interest with a gene of interest encoding an affinity tag, we express and purify affinity tagged protein in a suitable expression system; in our case, *E. coli*. Purified bait protein is bound to beads, and the equilibrium of protein complex formation is driven to favor exogenous over endogenous complex formation via a large excess of bait protein on the bead surface. This method in conjunction with the specific elution from beads has been used and validated for multiple protein complexes mainly using glutathione S-transferase as the affinity tag and the glutathione elution from the beads under native conditions.[17−19] In contrast to this approach, we used strong covalent or affinity binding of the bait to the beads in combination with denaturing elution to prevent release of large amounts of protein bait from the beads that would otherwise overwhelm signals of weak interactors in MS spectra. MagneHis beads appeared to be the most suitable matrix in this procedure allowing its subsequent automation.

Circumventing the tedious gel cutting and the inefficient in-gel digestion procedure in the analysis of protein complexes via single-dimensional liquid chromatography-tandem MS/MS spectrometry has been published by McCormack et al.[20] An improvement of this method called the multidimensional protein identification technology (MudPIT) extended its applicability to large protein complexes and has been confirmed as an alternative to gel-based protein separation.[21] This method was recently used for successful TAP-based pathway identification in yeast, while still employing the complex multistep protein digestion procedure based on the urea denaturation, sulfhydryl reduction followed by carboxymethylation of free sulfhydryls.[22] Our simplified in vitro pull-down procedure provided such clean pull-down samples that we obtained good quality MS data without applying MudPIT. Similarly clean and unambiguous data were obtained for other bacterial complexes such as DNA-dependent RNA polymerase (SO0256) and dissimilatory sulfite reductase (SO2379) from *S. oneidensis* and other complexes from S. oneidensis (data not shown).

One of the most critical improvements of our procedure was substituting the complex multistep protein digestion procedure in MS sample preparation with single-step trypsin digestion in 40% acetonitrile. Indeed, we obtained even better protein IDs for protein complexes using digestion in acetonitrile than using the urea denaturation-driven trypsin digestion. In combination with denaturing elution of protein interactors from MagneHis beads using the same solvent, we streamlined the protein complex identification procedure all the way from the protein complex purification to MS/MS identification.

We have developed a simple exogenous protein complex purification and identification method that is easy to automate and scale-up into a high-throughput format. We are currently validating an automated version of the method that could be added seamlessly downstream of some of the recently developed automated protein expression and purification processes.[23−25] If those processes could be coupled, then the bottlenecks for large-scale data generation for functional proteomics could be eliminated. The greatest advantages of our approach are that it is simple, straightforward, and easily applicable at a proteomic scale to any organism or tissue cell extract. However, the expression of soluble proteins of a host proteome in a foreign expression system and their subsequent purification to homogeneity will remain the greatest limitation of this approach. This limitation can be alleviated by expression of well-defined soluble functional domains of "difficult" proteins or by overexpressing the proteins directly in the host cells.

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