NOTE

Single step affinity purification for fungal proteomics.


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Running title: Protein affinity purification.
A single-step protein affinity purification protocol is described using *Aspergillus nidulans*. Detailed protocols are provided for cell breakage, affinity purification and, depending on the application, methods for protein release from affinity beads. Examples are provided defining the utility of the approaches which should be widely applicable.

Over 50 years ago, Richards *et al.* found that cleavage of RNase A by subtilisin resulted in two peptides, the S-peptide (residues 1-20) and the S-protein (residues 21-124), which bind tightly to each other with high affinity (dissociation constant ~10^{-9} M) (1, 7, 8). Because of this high affinity, and the small size of the S-peptide (which can be reduced to 15 aa and still bind with high affinity) this interaction has been used as the basis for protein affinity purification (2).

We previously described methods for generating C-terminal GFP-tagged constructs using fusion PCR and pyrG^{AF} as the nutritional transformation marker (11). To maximize the utility of the C-terminal GFP-tagging primers, we have generated new base cassettes which, using the very same primers, allows the generation of endogenous C-terminally S-tagged versions of proteins of interest (4) and (http://www.fgsc.net/plasmid/atagging.htm). The focus of this Note is to outline protocols for efficient protein extraction and single step S-tag affinity purification using *Aspergillus nidulans* as the model system. The methods should be appropriate for other cell types.

Using previously described methods (5, 6, 11), the S-tag is targeted via homologous recombination in frame at the 3’ or 5’ end of target genes. Diagnostic PCR and western blot analysis are used to confirm gene replacement and expression of the correct sized S-tagged protein. Samples for this diagnostic analysis are generated from static Petri dish liquid cultures (Protocol 1A). Cells are harvested and lyophilized overnight (3) and Osherov and May, Fungal Genetics Newsletter#45 (http://www.fgsc.net/fgn45/45osherov.html). The lyophilized cells are broken (Protocol 1A, Video 1) to generate small scale protein samples for western blot analysis (Protocol 1B) or DNA for diagnostic PCR (Protocol 1C).
Once confirmed, S-tagged strains are grown (typically after one outcross) in a shaken liquid flask culture (Protocol 2) to generate samples for protein extraction and affinity purification. The filamentous mode of growth of many fungi facilitates rapid harvesting by filtration followed by freezing in liquid nitrogen (1000 ml cultures can be harvested and frozen in less than one minute). The frozen cells are then lyophilized. A 1000 ml culture typically yields 0.7-1.0 g of dried cell mass. The lyophilized cells may be stored at -80°C before protein extraction but are re-lyophilized for 30 min before processing.

Breaking lyophilized cells is simple, quick and efficient. It is achieved by grinding in a mortar and pestle at room temperature (Video 2) until the lyophilized cells are a homogenous fine powder. For protein extraction (Protocol 2) the cell powder is mixed directly with cold HK extraction buffer and subsequent steps are carried out at 4°C. The powder is thoroughly mixed by vortexing (Video 2). The cell extract is cleared by two rounds of centrifugation harvesting the supernatant. Protein concentration is typically 20-25 mg/ml measured using a Bradford protein assay. Importantly, this method of generating protein extracts maintains sensitive biochemical activities (Fig. 1A) and has also been utilized successfully for Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Escherichia coli cells (Fig. 1B). This method has advantages in that cell breakage and efficient protein extraction is very rapid and no specialized instrumentation is required.

To minimize proteolysis, protein extraction and affinity purification are completed sequentially without delay. For affinity purification (Protocol 3) a batch wise protocol is used utilizing S-Protein agarose beads. After one hour incubation, beads are collected by centrifugation and washed extensively (Protocol 3).

It is possible to release purified proteins from the S-Protein beads using different methods depending on the experimental demands. For mass spectrometry (MS) analysis, purified proteins are released by boiling in SDS-sample buffer (Protocol 3A). The released proteins are run through SDS-PAGE. To identify the main proteins purified, the gel is stained using Coomassie blue and visible bands can be excised and processed for MS analysis using...
standard methodologies (Fig. 2). Alternatively, to identify all proteins purified, including those of low abundance, 1/10 of the purified sample is run on an analytical SDS PAGE gel and silver stained to record the pattern of proteins purified (Fig 3A). For MS analysis the remaining 9/10th is subjected to preparative SDS-PAGE but the proteins are run through the stacking gel and just into the separating gel. This results in all purified proteins being compressed into a tight series of bands (Fig. 3B). The total protein bands are excised as one and processed for MS in the normal manner. Using this second approach all proteins purified can be identified in a single MS analysis.

To release native biochemically active proteins, elution with free S-peptide is employed (Protocol 3B) enabling biochemical analysis of purified proteins in solution.

Enzymatic assays can also be carried out utilizing the purified protein still bound to the beads. For such on-bead assays the washed beads are equilibrated in the appropriate assay buffer. We have confirmed that cell breakage at room temperature after lyophilization is compatible with biochemical activity. For example, comparable NIMA kinase activity was purified from extracts generated from lyophilized cells as from extracts generated by the more normal method of grinding frozen non-lyophilized cells in liquid nitrogen (Fig.1B). Notably however, significantly more total protein was extracted using the lyophilization based breakage (48% more in this experiment).

We have also used in vitro phosphatase treatment of purified S-tagged proteins on beads to confirm mobility shifts observed upon SDS PAGE are caused by phosphorylation (9, 10).

We have successfully utilized the methods described for purification of a wide range of S-tagged proteins demonstrating their general applicability. If necessary, conditions of cell growth and buffer stringencies during purification can readily be optimized on a protein-by-protein basis.
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References

Figure Legends

FIG 1. Extraction of proteins from *A. nidulans*, yeast and bacterial cells after lyophilization. (A) Protein extracts were generated from frozen mycelia before (Conventional) or after lyophilization. The activity of the NIMA protein kinase was determined by the ability of S-tag affinity purified NIMA (eluted with S-peptide) to phosphorylate β-casien in standard protein kinase assays. The radioactivity shown in the autoradiograph was quantified by ImageQuant (Molecular Dynamics). (B) Cells of the indicated organisms were grown and frozen in liquid nitrogen then lyophilized. Equal weights of each sample were extracted using HK or SDS sample buffers and the extracted protein run through SDS PAGE and the gel stained with Coomassie Blue.

FIG 2 Identification of SONA co-purifying partners after S-tag affinity purification. Endogenously S-tagged SONA was affinity purified along with a control extract from a strain not expressing an S-tagged protein and the purified proteins separated using SDS PAGE. After Coomassie Blue staining the indicated bands were excised and identified using LC-MS-MS.

FIG 3 SDS PAGE conditions for identification of all purified proteins. (A) 1/10th of the indicated S-tagged proteins purified from cells arrested in G2 or mitosis were run in SDS PAGE and the gel stained with silver to record the pattern of proteins purified. (B) To identify the proteins purified in a single MS analysis, the remaining purified samples were run just into the separating gel during SDS PAGE and the gel stained with Coomassie blue. A single gel fragment containing all purified proteins can then be excised and processed for MS analysis.
Figure 3