Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies

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Studies of protein–protein interactions are of increasing interest in many areas of biological and biochemical research. Dimerization and other protein–protein interaction motifs that have recently been identified and shown to be important in many regulatory pathways include, for example, the leucine–zipper (1), helix–loop–helix (2), and SH2 and SH3 domains (3). Commonly used analytical methods involve immunoprecipitation or DNA-binding of one of the polypeptides or centrifugation or cross-linking. Protein-affinity chromatography has also been used to identify and purify proteins transiently associated with known polypeptides (4, 5). For proteins whose cDNAs are available the material desirable for protein affinity columns can be obtained most conveniently with the combined use of bacterial T7 RNA polymerase-mediated expression systems and various one-step affinity purification systems (6). The GST fusion system (7) especially, has recently become a popular tool not just for one step purification of the fusion protein but also as a tool to study protein–protein interactions (e.g. 8). In this report we describe a purification method for histidine-tagged recombinant proteins, which would allow the his-tagging system to be used for similar protein interaction studies.

As previously described (9, 10, 11), a histidine-tagged recombinant protein can be purified with commercially available nickel chelating resins (Qiagen, Pharmacia), by eluting bound proteins at low pH (<5.0). We have modified the T7 expression vectors (Figure 1), originally designed in Studier's laboratory (12) to express even toxic proteins at high levels in bacteria, to produce proteins with an N-terminal histidine-tag (available from Novagen Inc., Madison, WI) that can also be cleaved off with thrombin (13) or factor X_a (14). In contrast to previous descriptions (9, 10), we have purified tagged proteins in non-denaturing conditions at physiological pH, utilizing Ni^{2+}–nitrilotriacetic acid agarose (15) and imidazole-containing buffers, as shown for yeast TFIID in Figure 2 (lanes 3–6). The purified protein was shown to be functional in DNA binding (gel shift and DNase I footprint assays) as well as in transcription assays with a specific activity equal or greater than conventionally purified, untagged recombinant yIID (data not shown). Subsequent reduction of the imidazole concentration in the purified protein fraction by dialysis or dilution permits nearly quantitative rebinding to the resin (Figure 2, lane 7). The binding of the his-tagged protein to the resin is resistant to high salt concentrations (Figure 2, lanes 8, 9), anionic detergents (data not shown) and 6M guanidine hydrochloride (10), while it is easily eluted in the buffer of choice containing imidazole at physiological pH (Figure 2, lane 10; 80 mM for 6His, 120 mM for 10His).

Whereas the his-tag purification methodology was previously restricted to certain proteins that are unharmed at low pH, the described procedure permits its use for almost any cloned protein, irrespective of the buffer requirements for functional activity. Furthermore, the modified methodology represents a potentially powerful tool for rapid protein affinity chromatography, due to the following characteristics: (i) The purification or re-purification can be done in native conditions. (ii) The resin has a high capacity (2–10 mg tagged protein bound on 1 ml resin) allowing for rapid working up of many samples in batch. (iii) As the tag is short (in contrast to the GST system), protease cleavage is in many cases not necessary for functional tests of the recombinant protein. (iv) The system allows anchorage of the protein to the resin at a single defined point. (v) When used for protein affinity chromatography, the protein complex can also be allowed to form in solution before being purified with nickel resin, similar to immunoprecipitations. (vi) In contrast to most antibody–antigen interactions, however, the His–tag–nickel interactions are such that that we have the choice to elute only the associated protein (by salt or detergents) or the entire complex in native form (by imidazole or EDTA) for subsequent functional analysis. Although a number of polypeptide species in crude mammalian extracts have been found to bind at similar stringency as tagged proteins to the chelating resin, these can be identified readily by appropriate controls and even eliminated by passage of the extract through a control nickel column in the absence of the his-tagged protein. Given the ease with which high amounts of recombinant proteins can be expressed and purified, the described system permits a direct mutational analysis of protein interaction domains that will improve our understanding of the molecular basis for the transient association of proteins involved in regulatory loops and pathways.

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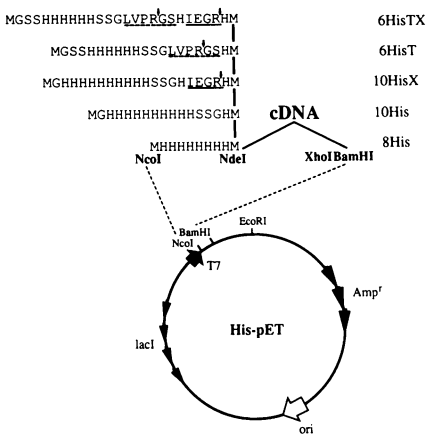

Figure 1. Design of the histidine tag encoding linkers cloned into the bacterial expression vector pET 11d (12). Tags containing 6 or 10 histidines and cleavage sites for the specific proteases thrombin (solid line) and factor X_a (dashed line) are inserted between the *Nco*I and *Bam*HI sites resulting in vectors 6HisTX-pET11, 6HisT-pET11, 10HisX-pET11 and 10His-pET11. cDNAs can be inserted with their first ATG at the *Nde*I site and their 3′-end at the *Xho*I or *Bam*HI sites.

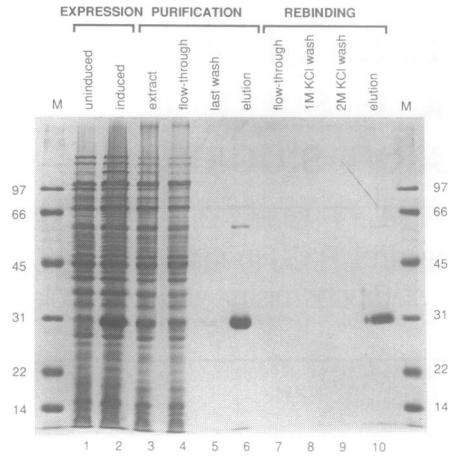

Figure 2. Coomassie-blue stained SDS−PAGE demonstrating expression, one-step purification, and rebinding of His-tagged yeast TFIID (16). Expression (lanes 1, 2) of yIID within the 6HisT-pET11 vector in BL21 (DE3) plysS bacteria according to published procedures (12). Bacterial extract (lane 3) was made by repeated sonication in a buffer containing 10 mM Tris.HCl (pH 7.9), 10% glycerol, 0.5 M NaCl, 0.1% NP40, 5 mM DTT, and protease inhibitors and a subsequent 10 min spin at 10,000 g. Imidazole.HCl (pH 7.9) was added to the extract to a concentration of 1 mM before loading onto a Ni^{2+}−NTA column (Qiagen, 1 ml per mg of expressed protein) at a flowrate of 5 to 10 column volumes per hour (lane 4). The column was then washed with about 20 column volumes of BC100 (20% glycerol, 20 mM Tris.HCl (pH 7.9), 100 mM KCl, 5 mM DTT and 0.5 mM PMSF) containing 20 mM imidazole.HCl (last wash fraction in lane 5) before being eluted with BC100 containing 80 mM imidazole.HCl (lane 6). The eluate was diluted 10-fold in BC100 and loaded onto an identical column (lane 7). Two high salt washes with BC1000 (lane 8) and BC2000 (lane 9) demonstrate binding is salt resistant, but the bound protein can be recovered in BC100 containing 80 mM imidazole.HCl (lane 10).