Efficient, rapid protein purification and on-column cleavage using GSTrap FF columns

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Abstract

A purification strategy involving the production of soluble, purified proteins in a single step would be an invaluable resource and indispensable in many laboratory environments. This Application Note describes a flexible, efficient, and rapid protein purification scheme for the purification of GST-tagged proteins using GSTrap™ columns and PreScission™ Protease. The method describes a purification technique that can be applied to reproducibly isolate a variety of diverse proteins of differing function, structure, and chemical nature. This strategy is performed on a single column, yielding "native" proteins (cleaved fusion proteins) in high yield with a high level of enrichment in the 95–97% purity range.

Introduction

The purification strategy described here utilizes the technologies of the pGEX-6x vector system, coding for a GST-tagged protein linked with PreScission Protease proteolytic site, in conjunction with GSTrap FF affinity columns, and PreScission Protease enzyme. This allows expression and purification of desired target proteins in high yields with high levels of enrichment. Several proteins, which had previously required complex multi-step purification methods, and often yielded poorly purified products, were selected and assayed. These proteins were sub-cloned, expressed, purified, and cleaved on-column yielding highly pure product. The aim of this strategy is to produce highly pure, "native" protein samples suitable for downstream biochemical assays and structural studies, specifically protein crystallographic analysis.

A flexible method for the purification of diverse gene products

For this method, two unrelated proteins, Pur-α and TLP40, were selected in order to determine the versatility and efficiency of the purification protocol.

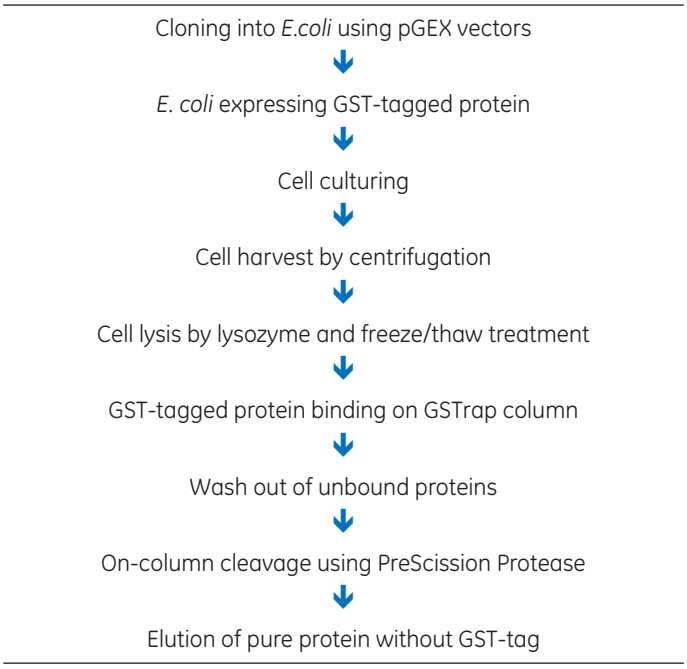

Fig. 1. General purification and on-column cleavage protocol for GST-tagged proteins.

Pur-α is involved in transcription regulation

Pur-α is sequence-specific, single-stranded DNA and RNA binding protein that binds to purine-rich promoter regions with a consensus $(GGN)_n$ sequence (1). Pur-α has been shown to function in transcriptional regulation by interacting

with selective promoter response elements such as: myelin basic protein (MBP) regulatory motif (2); neuronal nicotinic acetylcholine receptor (NAChR) genes (3); single-stranded cyclic AMP response element-binding protein (ssCRE-BP; 4); calmodulin response element (CaMRE) neuropeptide Y (5); and human immunodeficiency virus 1 (HIV-1) TAT protein via activation of the TAT-responsive DNA element (*upTar*) of the major late promoter of the JC virus (6). In addition, Pur-α has been shown to enhance transcription through an RNA element by transactivating HIV-1 through TAR RNA (7).

TLP40 is involved in photosynthesis regulation

TLP40 is an eukaryotic plant protein involved in photosystem II regulation. It is a cyclophillin-like protein with *cis-trans* isomerase activity directed to proline residues, typical for immunophillins (8). Biochemical investigations characterizing the physiological role of TLP40 elucidated the interaction with an integral membrane protein phosphatase. These characteristics strongly suggests that TLP40 is a regulator/modulator of phosphorylation states of photosystem II proteins (9).

Cell growth, protein over-expression, and cell harvesting

The genes encoding for Pur-α and TLP40 protein were independently sub-cloned into the pGEX-6P-1 expression vector system and transformed into *E. coli* BL21 over expressing cell lines. Bacteria were grown at 30ºC in LB medium supplemented with 100 µg/ml Carbenicillin in TUNAIR™ shaking flasks. Cells were grown until OD_{600} = 1.0, and GST-tagged protein synthesis was induced with 1.0 mM IPTG (final concentration), and grown for 3–4 h. Cells were harvested by centrifugation (3000 × g at 4°C for 30 min), the pellet was resuspended and washed with TB buffer (9.1 mM HEPES, 55 mM $MgCl_2$, 15 mM $CaCl_2$, 250 mM KCl adjusted to pH 6.7), centrifuged, and frozen at -80°C.

Cell lysis

The cell pellet containing GST-tagged protein was resuspended in PBS lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 adjusted to pH 7.4) supplemented with lysozyme (1 mg/ml, Sigma), Complete™ Protease Inhibitor Cocktail (1 tablet, Roche), 10 mM $MgCl_2$, and DNAse I (10 U/ml, Roche). Cells were effectively lysed by repeated (3×) freeze (-170°C)/thawing (30°C). The lysate was centrifuged at 70 000 × g for 25 min at 4°C. Clarified lysate was then ultracentrifuged at 300 000 × g for 60 min at 4°C to remove insoluble proteins, cell fragments, and membrane components. The supernatant was applied to a 50 ml Superloop™ for loading onto a GSTrap FF column.

GST-tagged protein binding

The following described purification procedure was performed on an ÄKTAexplorer™ 100 chromatography system. The GST-tagged protein containing supernatant was loaded on two pre-equilibrated GSTrap FF 5 ml columns in series with PBS, pH 7.4 as binding buffer using a Superloop. Sample loading of the column was run at a reduced flow rate (1 ml/min) to optimize the GST affinity media (Glutathione Sepharose™ 4 Fast Flow) binding interactions on the column. Following GST-tagged protein binding to the column, bound material was washed with PBS, pH 7.4 until the baseline returned. Once the baseline had stabilized, buffer was substituted with PreScission Protease buffer(50 mM Tris HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0).

PreScission buffer was used to wash the GST-tagged protein more stringently, bound to the GSTrap FF column, and to pre-equilibrate the column, altering the buffer composition required for efficient PreScission Protease cleavage. PreScission buffer equilibration was continued until both the UV absorbance baseline and the conductivity baseline stabilized, after which the buffer flow was stopped.

On-column GST-tagged protein cleavage with PreScission Protease

With GST-tagged protein effectively bound to the GSTrap FF column, washed, and equilibrated in PreScission buffer, the cleavage reaction could be initiated. Exploiting the combined characteristics of the PreScission Protease and the GSTrap FF column, a simple, and rapid on-column GST-tagged protein cleavage can be performed. The reaction proceeds with high efficiency at 4°C, and is specific and reproducible. The enzyme contains a non-cleavable GST-affinity tag for optimum on-column cleavage, and the column remains on-line, connected to the purification system, eliminating the loss of any material.

Proteolytic digestion requires 2 units enzyme/100 µg of bound GST-tagged protein. PreScission Protease can be diluted in PreScission buffer according to the size of the GSTrap FF column (for example, 4.5 ml for a 5 ml column) and manually injected onto the column at an increased flow rate of 5-7 ml/min. This increased flow rate promotes a reduced affinity interaction time of the PreScission Protease enzyme with the GSTrap FF media allowing for uniformly distributed enzyme throughout the entire column. Following injection, the column is in a closed flow status where efficient PreScission Protease binding occurs, and ideally, even distribution of the enzyme throughout the column is achieved. The system is incubated on-line for 12–16 h at 4°C.

Elution of cleaved purified protein

Prior to elution, a 1 ml GSTrap FF column (pre-equilibrated with PreScission buffer) was connected downstream to the GSTrap FF proteolytic cleavage column. This tandem column scheme has several functions including acting as a volume delay to route any cleaved material upon flow start-up, minimizing the loss of cleaved product. This allows for a short baseline recalibration before peak elution. The GSTrap FF affinity column also acts as a filter to capture any released cleaved GST protein, uncleaved GST-tagged protein and unbound PreScission Protease.

Cleaved protein elution occurs immediately upon flow start-up with PreScission buffer. The elution peak containing the cleaved target protein was eluted first through the 1 ml buffering GSTrap column, then to the detector and fraction collector. The peak profile containing the target protein was somewhat asymmetric in shape due to diffusion effects from the incubation time, and freely bound protein. Following target protein elution and return of absorbance baseline, a GST affinity competitor (reduced glutathione) was applied to elute GST, unbound GST-tagged protein, and PreScission Protease. A buffer containing reduced glutathione (50 mM Tris-HCl and 10 mM reduced glutathione, pH 8.0) was applied in a one-step gradient (100%) to elute the GST-affinity peak.

The overall purification and on-column cleavage chromatogram can be seen in Figure 2.

Columns: GSTrap 2 × 5 ml columns in series
Sample: TLP40 (loaded 50 ml from 2 L culture)
Buffer A: PBS, pH 7.4
Buffer B: PreScission buffer, pH 8.0
Buffer C: Reduced glutathione buffer, pH 8.0
System: ÄKTAexplorer 100

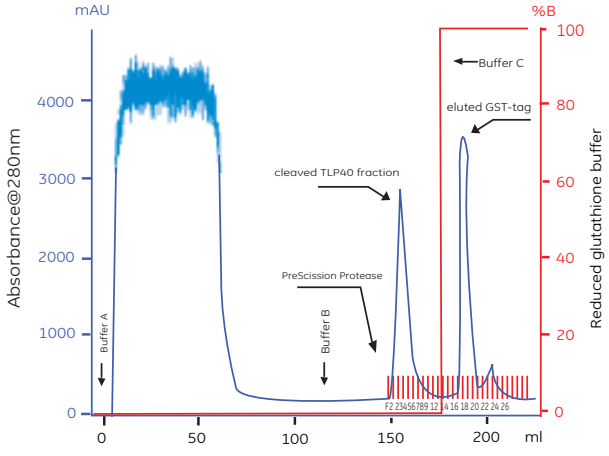

Fig 2. Purification and on-column cleavage of GST-TLP40 fusion protein using GSTrap and PreScission Protease.

Determination of purified target protein

Purification stages and chromatography profiles were analyzed by SDS-PAGE to assay the overall purity and enrichment of the desired target protein, Figure 3. Overall, the final cleaved target protein product was highly enriched with very few contaminants. An estimate of the overall purity is in the range of 95%-97% from a single column purification strategy.

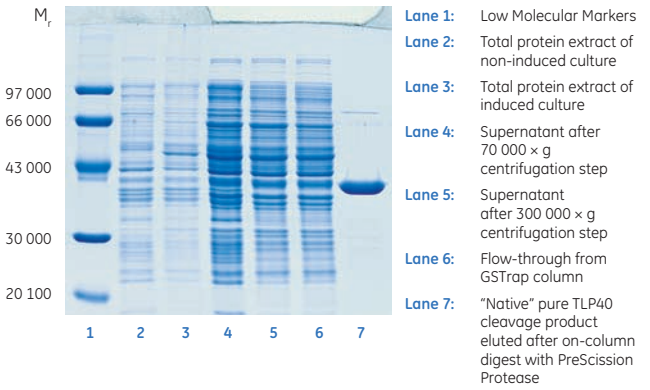

Fig 3. SDS-PAGE analysis of the steps during the purification. Samples were separated on a 3.5%–12% polyacrylamide gel with Coomassie™ staining.

Conclusions

The protein purification strategy described here is a simple and rapid method for the isolation of soluble "native" proteins. The GST-tagged protein binding and on-column cleavage assay is greatly facilitated by GSTrap affinity columns and PreScission Protease enzymes allowing for the purification strategy to be performed reproducibly and routinely. The protein yields are highly pure and suitable for downstream processes. This purification strategy exploits the advantages of using the pGEX-6P-1 expression vector system in concert with the engineered PreScission Protease enzyme with its highly specific proteolytic digestion site and uncleavable GST-affinity tag bound to the highly specific binding GSTrap column. With minimal background contaminants, no protease contamination, and reproducible results, this method can be regarded as a standard for protein expression and purification trials. A further support for the effectiveness of this strategy invokes a more demanding criterion to judge a protein's purity; that is, for it to be able to crystallize. In one of the above test cases, the highly purified protein product crystallizes, reflecting the highly pure and homogeneous state of the protein.

This method for the purification of "native" proteins is characterized by a simple and rapid strategy with excellent yields.

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Ordering information

Product	Quantity	Code No.
GSTrap FF	2 × 1 ml	17-5130-02
GSTrap FF	5 × 1 ml	17-5130-01
GSTrap FF	100 × 1 ml*	17-5130-05
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Related literature	Code No.
Recombinant Protein Purification Handbook, Principles and methods	18-1142-75
Affinity Chromatography, Columns and Media Selection Guide	18-1121-86
Affinity Chromatography Handbook, Principles and Methods	18-1022-29
HiTrap Column Guide	18-1129-81

Related products	Quantity	Code No.
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
Glutathione Sepharose 4 Fast Flow	100 ml	17-5132-02
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PreScission Protease	500 units	27-0843-01
GST Detection Module	1 kit	27-4590-01
GST 96-Well Detection Module	5 plates	27-4592-01
Anti-GST Antibody	0.5 ml	27-4577-01
Superloop, 50 ml	1	18-1113-82

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