Rapid and efficient purification and refolding of a \((\text{His})_6\)-tagged recombinant protein produced in \(E.\) \(\text{coli}\) as inclusion bodies

Summary

This Application Note shows the purification and refolding of a recombinant protein tagged with a \((\text{His})_6\)-tag at its N-terminus. Using a simple but efficient purification and refolding procedure, a protein initially produced as intracellular inclusion body material in \(E.\) \(\text{coli}\) is converting to soluble protein exhibiting the desired activity.

This protocol has been used successfully for several different \((\text{His})_6\)-tagged recombinant proteins. See also reference 12 for further information.

Introduction

Heterologous expression of foreign genes in \(E.\) \(\text{coli}\) can be engineered to lead to either intracellular accumulation of recombinant protein, or to secretion and accumulation in the periplasmic space. While the latter mode of expression is sometimes advantageous in terms of protein folding, solubility and cysteine oxidation, the magnitude of protein production is generally much higher when intracellular expression is used (1).

However, recombinant protein accumulated intracellularly is frequently laid down in the form of inclusion bodies, insoluble aggregates of misfolded protein lacking biological activity (2,3,4,5). The high buoyant density of inclusion bodies facilitate their separation from soluble \(E.\) \(\text{coli}\) proteins and cell debris by differential centrifugation (4,6,7).

Conventional methods for refolding of insoluble recombinant proteins include slow dialysis or dilution into a buffer of near neutral pH (8). Gel filtration, ion exchange or hydrophobic interaction chromatography have in some cases been used (9,10,11) to facilitate the refolding step.

Affinity tagging of the recombinant protein, by for example, the addition of several consecutive histidine residues, opens the possibility of efficient purification and refolding in a single chromatographic step. Since binding of the histidine tract to immobilized divalent metal ion can occur in the presence of chaotropic agent (such as urea or guanidine hydrochloride) at high concentration, \((\text{His})_6\)-tagged inclusion body protein can be solubilized by chaotropic extraction and directly bound to an affinity matrix. Removal of contaminating proteins and refolding by exchange to non-denaturing buffer conditions can then be performed before elution of the protein from the column (12).

A general protocol for the purification and refolding of a \((\text{His})_6\)-tagged recombinant protein produced in \(E.\) \(\text{coli}\) is shown in Fig.1.
Disruption, wash and isolation of inclusion bodies

Resuspend the cell paste from 100 ml culture of E. coli expressing (His)_6-tagged recombinant protein in 4 ml 20 mM Tris-HCl pH 8.0. Disrupt the cells with sonication on ice (e.g., 4 x 10 sec.) and centrifuge at high speed for 10 min. at +4 °C. The pellet, containing the inclusion bodies, is resuspended in 3 ml cold 2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100 pH 8.0 and sonicated as above. Centrifuge at high speed for 10 min. at +4 °C. Subject the pellet to a second round of urea wash. At this stage the pellet material can be washed once in buffer lacking urea and stored frozen for later processing.

Solubilization and sample preparation

Resuspend the pellet in 5 ml 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol pH 8.0. Stir for 30-60 min. in room temperature and centrifugate 15 min. at high speed, +4 °C. Remove remaining particles by passing the sample through a 0.22 µm or 0.45 µm filter.

The optimal concentration of reducing 2-mercaptoethanol (0-5 mM) must be determined experimentally for each individual protein.

Proceed directly with the purification and refolding steps.

Preparation of the column

HiTrap Chelating 1 ml column is washed with 5 ml distilled water using a 5 ml syringe. Load 0.5 ml 0.1 M NiSO_4 and continue to wash with 5 ml distilled water. Equilibrate the column with 5-10 ml 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol pH 8.0.

Purification and refolding

Loading and washing

Load the sample and wash the column with 10 ml 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol pH 8.0. Change the buffer to 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, 6 M urea pH 8.0 and wash with 10 ml.

Refolding

Refolding of the bound protein is performed by the use of a linear 6-0 M urea gradient, starting with the wash buffer above and finishing at one without urea. A gradient volume of 30 ml or higher and a flow rate of 0.1-1 ml/min can be used, while the optimal renaturation rate should be determined experimentally for each protein. Continue to wash with 5 ml of buffer without urea after the gradient has come to its endpoint.

Elution

Elute the refolded recombinant protein using a 10-20 ml linear gradient starting with 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0 and ending with the same buffer including 500 mM imidazole. Fig. 2.
Fractions containing the eluted protein are pooled and subjected to buffer exchange using a HiTrap Desalting or PD-10 column, in order to remove imidazole. The refolded (His)_6-tagged protein is now ready for analysis of biological activity.

The choice of HiTrap column size depends on the amount of expressed protein.

While in this example a HiTrap Chelating 1 ml column is used, a HiTrap Chelating 5 ml is also available and should be used if the expected amount of recombinant protein exceeds 10 mg. For further scaling-up Chelating Sepharose™ Fast Flow is available.

### Analysis

The aggregation state and purity of the refolded (His)_6-tagged recombinant protein eluted from HiTrap Chelating is checked by gelfiltration on Superdex™ 75 HR 10/30, Fig. 3 and SDS-PAGE, Fig.4, respectively.

### Regeneration and storage

Regenerate the column with 5 ml 6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 50 mM EDTA pH 8.0. Wash with 10 ml distilled water followed by 10 ml 20% ethanol. Store the column in 20% ethanol.
References

11. Purification and renaturation of recombinant proteins produced in Escherichia coli as inclusion bodies. Application Note 1B-1112-33, Amersham Biosciences.

Ordering information

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Code No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiTrap Chelating</td>
<td>5 x 1 ml</td>
<td>17-0408-01</td>
</tr>
<tr>
<td>HiTrap Chelating</td>
<td>1 x 5 ml</td>
<td>17-0409-01</td>
</tr>
<tr>
<td>HisTrap</td>
<td>1 kit</td>
<td>17-1880-01</td>
</tr>
<tr>
<td>HiTrap Desalting</td>
<td>5 x 5 ml</td>
<td>17-1408-01</td>
</tr>
<tr>
<td>PD-10 Column</td>
<td>30</td>
<td>17-0851-01</td>
</tr>
<tr>
<td>Chelating Sepharose Fast Flow</td>
<td>50 ml</td>
<td>17-0575-01</td>
</tr>
<tr>
<td>Superdex™ 75 HR 10/30</td>
<td>1</td>
<td>17-1047-01</td>
</tr>
<tr>
<td>Superdex 200 HR 10/30</td>
<td>1</td>
<td>17-1088-01</td>
</tr>
<tr>
<td>HiLoad™ 16/60 Superdex 30 pg</td>
<td>1</td>
<td>17-1139-01</td>
</tr>
<tr>
<td>HiLoad 26/60 Superdex 30 pg</td>
<td>1</td>
<td>17-1140-01</td>
</tr>
<tr>
<td>HiLoad 16/60 Superdex 75 pg</td>
<td>1</td>
<td>17-1068-01</td>
</tr>
<tr>
<td>HiLoad 26/60 Superdex 75 pg</td>
<td>1</td>
<td>17-1170-01</td>
</tr>
<tr>
<td>HiLoad 16/60 Superdex 200 pg</td>
<td>1</td>
<td>17-1069-01</td>
</tr>
<tr>
<td>HiLoad 26/60 Superdex 200 pg</td>
<td>1</td>
<td>17-1171-01</td>
</tr>
<tr>
<td>XK 16/20 column</td>
<td>1</td>
<td>18-8773-01</td>
</tr>
<tr>
<td>XK 16/40 column</td>
<td>1</td>
<td>18-8774-01</td>
</tr>
<tr>
<td>XK 26/20 column</td>
<td>1</td>
<td>18-1000-72</td>
</tr>
<tr>
<td>XK 26/40 column</td>
<td>1</td>
<td>18-8768-01</td>
</tr>
<tr>
<td>ÄKTA™ FPLC®</td>
<td>1</td>
<td>18-1118-67</td>
</tr>
<tr>
<td>PhastSystem 120 VAC</td>
<td>1</td>
<td>18-1018-23</td>
</tr>
<tr>
<td>PhastSystem 220 VAC</td>
<td>1</td>
<td>18-1018-24</td>
</tr>
<tr>
<td>PhastGel Gradient 10–15</td>
<td>10</td>
<td>17-0540-01</td>
</tr>
<tr>
<td>PhastGel Buffer Strips SDS</td>
<td>20</td>
<td>17-0516-01</td>
</tr>
</tbody>
</table>

to order:

Asia Pacific: Tel: +852 2811 8693 Fax: +852 2811 5251
Australia: Tel: +61 2 9899 5152 Fax: +61 2 9899 7511
Austria: Tel: 01 576 0616 20 Fax: 01 576 0616 27
Belgium: Tel: 0800 73 888 Fax: 03 272 1637
Canada: Tel: 1 800 463 5800 Fax: 1 800 567 1000
Central, East, South East Europe: Tel: +43 1 982 3826 Fax: +43 1 858 3327
Denmark: Tel: 45 16 2400 Fax: 45 16 2424
Finland: Tel: 09 512 3940 Fax: 09 512 1710
France: Tel: 01 46 35 67 00 Fax: 01 46 35 56 23
Germany: Tel: 0766 4903 401 Fax: 0766 4903 405
Italy: Tel: 02 2732 21 Fax: 02 2730 213
Japan: Tel: 81 3 5331 9336 Fax: 81 3 5331 9370
Latin America: Tel: +55 11 3667 5700 Fax: +55 11 3667 87 99
Middle East and Africa: Tel: +20 (1) 96 00 687 Fax: +20 (1) 96 00 693
Netherlands: Tel: 0165 580 410 Fax: 0165 580 401
Norway: Tel: 2318 5800 Fax: 2318 6800
Portugal: Tel: 01 417 7035 Fax: 01 417 31 04
Russian Federation: Tel: +7 (095) 230 6327 Fax: +7 (095) 230 6377
South East Asia: Tel: 60 3 724 2080 Fax: 60 3 724 2900
Spain: Tel: 93 594 49 50 Fax: 93 594 49 55
Sweden: Tel: 018 16 40 09 Fax: 018 73 24 44
Switzerland: Tel: 01 802 81 50 Fax: 01 802 81 51
UK: Tel: 0800 616 928 Fax: 0800 616 927
USA: Tel: +1 800 526 3593 Fax: +1 800 329 3593

HiTrap, Sepharose, Superdex, FPLC, PhastGel, PhastSystem and ÄKTA are trademarks of Amersham Biosciences or its subsidiaries. Amersham is a trademark of Nycomed Amersham plc.
Tris and Triton are trademarks of Union Carbide Chemicals and Plastics Co. Amersham Biosciences Biotechnology 30, 0E-751 84 Upssala, Sweden.
Amersham Biosciences UK Limited Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England. Amersham Biosciences Inc. 800 Centennial Avenue, PO Box 1327, Piscataway, NJ 08855 USA. Amersham Biosciences Europe GmbH Munzinger Strasse 9, D-79111 Freiburg, Germany. All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Biosciences group that supplies them. A copy of these terms and conditions is available on request. © Amersham Biosciences 1999 - All rights reserved.