HIP PURIFICATION

Materials/Reagents:

LB/kan media (50mL and 1L with each having 30mg/L kan.)
pET28a-Hip plasmid
BL21/DE3 competent cells
LB/kan plates (2)
IPTG (1mL of 1M)
Lysozyme
Leupeptin and Pepstatin A
Dialysis tubing
TEN_{100} buffer (20mM tris pH 7.4, 0.1mM EDTA, 100mM NaCl)
Ni^{+2} agarose column.
Binding buffer (5mM imidazole, 0.5M NaCl, 20mM Tris-HCl pH 7.9)
Wash Buffer (60mM imidazole, 0.5M NaCl, 20mM Tris-HCl pH 7.9)
Elution buffer (1M imidazole, 0.5M NaCl, 20mM Tris-HCl pH 7.9)
Charge buffer (50mM $NiSO_4$)
Imidazol
Glycerol
Millipore concentrators.

Procedure:

Overexpression

1) (day 1) Transform 100ng of the pET28a-Hip plasmid into BL21/DE3 competent cells and plate on an LB/kan plate.
2) (day 2) Pick a single colony of the transformation and streak several times onto another LB/kan plate.
3) (day 3) Pick a single colony off of the streaked plate and place into 50mL LB/kan and incubate overnight at 37°C.
4) (day 4) Take the overnight culture and inoculate it to a 40X dilution in 1L LB/kan.
5) Grow at 37°C in a shaker until the $O.D._{595}$ is 0.5-0.9 (around 2 hours).
6) Take a 1mL sample and save as an uninduced sample.
7) Induce the cells with a final concentration of 1mM IPTG.
8) Grow at 30°C in a shaker for 4-6 hours.
9) Take a 1mL sample (induced).
10) Harvest cells by spinning them down at 5,000 rpm for 20 minutes.
11) Remove the supernatant. Pellet can be frozen at -80°C for use later.

Test for overexpression

1) Take the uninduced and induced sample and spin at max rpm for 1 minute.
2) Aspirate the supernatant.

3) Resuspend the pellets in 1x SDS sample buffer (100μL for the uninduced and 200μL for the induced)
4) Load 10μL each on a SDS-PAGE gel.
5) Look for overexpression at the predicted size. There should be a much larger band in the overexpresion lane.

Cell lysis

1) Resuspend cells in Ten_{100} buffer at 3ml per 1g of cells.
2) Add lysozyme to 0.5mg/mL concentration.
3) Add leupeptin and pepstatin A to concentrations of 1μg/μL.
4) Put on a nurator in the cold room for 30 minutes.
5) Freeze and thaw 4X in a methanol bath and 37°C water bath.
6) Sonicate 30X in 3X cycles (using 30-40% duty cycle and 3-4 output control) or until the viscosity is greatly reduced.
7) Centrifuge at 32000rcf for 60 minutes
8) Take the supernatant to load onto a column.

Chromotography

1) First separate the lysate over a DEAE column using a salt gradient from 0mM NaCl to 500mM NaCl over 1000mL of elution.
2) Run gels with uninduced and induced samples along with 16μL of each odd fraction.
3) Stain by coomassie and identify fractions containing Hip.
4) Pool fractions.
5) Pour Ni^{+2} agarose into an empty column (about 10mL bed volume).
6) Put the plunger part-way in and run water through it until the beads settle.
7) Push the plunger all the way down to just above the bead line.
8) Wash by running 5x of the bed volume of 20% ethanol.
9) Wash 5x volume ddwater.
10) Pump 5x volume charged buffer through.
11) Wash with 5x volume binding buffer.
12) Recirculate the pooled fractions overnight (meaning the pump pushes the pooled fractions through the column and back into the container containing the pooled fractions. The fractions thus are continuously going through the column.)
13) Wash the column with 50ml wash buffer and collect with the recirculation
14) Elute with 10 ml elution buffer and collect.
15) Wash with 50mL binding buffer and collect.
16) Concentrate down to 10mL using the Millipore concentrators (MW 30,000).
17) Do dialysis overnight in TEN_{100} for two nights (one buffer change, 2L each).

18) Recirculate the previous sample solution that you recirculated and perform the procedure again
19) Resolve the samples after dialysis over the Resource Q column using a salt gradient of 0mM NaCl to 500mM NaCl and elution volume of 350mL.
20) Run odd samples on a gel as described before and identify fractions that contain Hip.
21) Concentrate the protein using Millipore concentrators.

Troubleshooting/Critical Parameters:

If overexpression is not achieved, try lowering the overexpression temperature. Also, the overnight and initial dilutions can be performed at lower temperatures. IPTG concentration can also be increased. The indicated parameters should work. See protocols for FPLC use and programming for column help.

Reference:

pET system manual (Novagen)

Submitted by:

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