HSP70 PROTEIN PURIFICATION

Buffer/Reagents:

LB media
1M IPTG (Sigma I-5502)
1xSDS gel loading buffer
TEK_{50} Lysis Buffer
20 mM Tris pH 7.2, 5 mM $MgCl_2$, 100 mM NaCl Buffer
10 % glycerol + 25 mM ATP
Lysozyme (Sigma L-6876)
Protease inhibitors:
Leupeptin (L-0649)
Pepstatin A (P-4265)
PMSF (P-7626)
BL21 cells, pMS119Hsp70

Materials:

Sorvall GS-3 centrifuge
500ml centrifuge bottles
sonicator with microtip
polypropylene Oakridge centrifuge tubes
Sorvall SA-600 rotor
Resource Q column XK 26 (Amersham)
Dialysis tubing
Beckman ultracentrifuge & Ti60.1 rotor & tubes
Methanol/dry ice bath
DEAE sepharose XK16 column (Amersham)
Pharmacia FPLC
20 ml ATP agarose column (Sigma A-2767)
Superdex S200 HR 10/30 column (Amersham)
Millipore concentrator

Cell Protein Extract Preparation:

1. Prepare a bacterial plate with the bacteria containing the expression plasmid and incubate at 37°C overnight.
2. Inoculate a 25ml LB/amp(200µg/ml) culture from the bacteria plate and incubate overnight at 37°C.
3. Pour the 25 ml culture into a 2-liter flask containing 1-liter LB/amp (200 µg/ml). Incubate at 37°C for 2-4 hours (until A_{595} = 0.5-0.9).
4. Remove 1 ml aliquot of ***uninduced*** cells, microfuge 1min and resuspend pellet in 100 µl of 1x SDS gel loading buffer. Store at -20°C.
5. Induce cells for overexpression by adding IPTG to a final concentration of 1 mM. Incubate for 4-6 hours at 37°C. 1 liter: add 1 ml of 1M IPTG stock
6. Remove a 1 ml sample of cells after IPTG incubation (4-6 hr), microfuge 1min and resuspend in 100 µl of 1xSDS sample buffer. Store at -20°C.

It is useful at this point to run an SDS/PAGE gel to determine the amount of protein induction. Using a 10-well gel, run samples of 1, 3, 5, 10 µl of both uninduced and induced cells. For HSP70 or proteins 60-90kd in size, it is helpful to use 1 µg BSA as a marker.

7. Pre-weigh the centrifuge bottle that you will use to collect the cells (500 ml – 1 liter) and record weight. Collect cells by spinning in centrifuge at 5 Krpm for 10 minutes.
8. Re suspend pellet in 25 ml TEK$_{50}$. If using more than one bottle is used to collect cells from the same flask, combine cells into one bottle at this point. Spin at 5,000 rpm for 10 min. Pour off supernatant and weigh bottle to determine the weight of the cells.

Option: Either quick-freeze the pellet in dry ice/MeOH and store at 70°C--or--continue with protocol.

9. Determine volume of lysis buffer (TEK$_{50}$) required for 3 ml/g of cells.
10. Resuspend cells in one-half of that volume (keep cells on ice). Handle cells gently to avoid causing air bubbles which will oxidize the protein. In the other half of lysis buffer, dissolve 1 mg/ml lysozyme (for the total volume of lysis buffer). Gently transfer cells to a 50 ml screw-cap conical tube and add lysozyme solution. Mix gently.
11. Add protease inhibitors:

Inhibitor:	*Final concentration:*	*Stock solution:*
Leupeptin A	1 µg/ml	1 mg/ml in dH$_2$0
Pepstatin A	1µg/ml	1 mg/ml in EtOH
PMSF	0.5 mM final volume	1 mg/ml in EtOH

12. Incubate cells on ice for 30 minutes (slowly invert several times every 10 min.).
13. Freeze/thaw cells three times in MeOH/dry-ice and 30°C baths. Do not completely thaw cells in 30°C bath; only thaw until there is a small sliver of frozen cells remaining--then immediately put cells in dry ice bath. Upon final thaw, put cells on wet ice when there is a small sliver of frozen cells remaining.

Option: Keep cells frozen upon 3rd dry ice freeze and store at -70°C--or--continue with protocol.

14. Sonicate cells to ensure complete lysis. If available, use a sonicator with a microtip. Use speed setting at #5 and set timer on "hold". Insert the microtip very close to the bottom of the tube (to avoid bubbles forming when microtip is near the top of the sample). Turn sonicator switch on for 5-10 seconds. Immediately put cells back on ice for about 30 seconds. Repeat until cell lysate is very watery in texture (4-5 times). Avoid sonicating the sample for intervals of longer than 10 seconds as the microtip will become quite warm.
15. Transfer cell lysate to a polypropylene Oakridge centrifuge tube. Spin extract in Sorvall SA-600 rotor at 12,000 rpm for 30 min. (4°C).
16. Ultracentrifuge supernatant. Transfer supernatant to a Beckman Ti60.1 ultracentrifuge tube. Centrifuge in Ti60.1 rotor (chilled to 4°C) at 35,000 rpm (123,000xg) for 1 hour at 4°C. Transfer supernatant to a 50 ml conical tube and keep on ice.

Option: Quick-freeze in dry-ice/MeOH and store at -70°C--or--continue with protocol

17. Load protein extract onto 200ml DEAE sepharose column on the FPLC. Elute the protein with a 50-500 mM NaCl gradient over 6 column volumes. Pool the fractions containing Hsp70 (SDS page will do for this). If you are not using a DnaK(-) strain, be careful not to pool fractions containing DnaK (elutes at a higher salt and above Hsp70).

Samples with Hsp70 are run several times over an ATP-agarose (Sigma) column as follows:

18. Recirculate the pooled samples over a 20 ml ATP agarose column for at least 4 hours at 4°C. The ATP agarose column should first be equilibrated with 20 mM Tris pH 7.2, 5 mM $MgCl_2$, 100 mM NaCl buffer first. It is necessary that Mg^{++} is included for proper sample binding
19. Wash the column with 150 ml of 20 mM Tris pH 7.2, 5 mM, $MgCl_2$, 100 mM NaCl. Save the first 30 ml to add to the circulating sample. Discard the remaining 120 ml.
20. Slowly load 10 ml (1ml/min) of 20 mM Tris pH 7.2, 5 mM $MgCl_2$, 100 mM NaCl buffer + 10 % glycerol + 25 mM ATP to the column. After 4 mls have been loaded, begin collecting into a 50 ml conical tube. Finish loading the remaining solution and then flush with 20 mM Tris pH 7.2, 5 mM $MgCl_2$, 100 mM NaCl buffer until a total 50 ml has been collected in the conical tube. If you are doing this for the first time it is better to collect 10 ml each and run them on the SDS page.
21. Save 35 ml and recirculate the remaining sample.
22. After repeating the column 3 times, run a gel with the three 35 ml collections and the remaining circulating sample. Hsp70 should be seen in the 35 ml collections but not in the circulating sample. If Hsp70 remains in the circulating sample, then repeat the column.
23. When the circulating sample is free of Hsp70, proceed to concentrate the 35 ml fractions (to a volume of 10 ml or less; depending on the amount of protein, concentration steps might take long time).
24. Dialyze the remaining sample and concentrate in 20mM Tris pH 7.2, 50mM NaCl, 1 mM EDTA on a spinner plate in the cold room for 2 days. Then change to new buffer and dialyze for 1 more day. This step is crucial to get rid of ATP.
25. Load the post dialysis Hsp70 over a Resource Q column (Amersham) and elute with 100-400 mM NaCl gradient over 6 column volumes.
26. The protein may be pure at this point. If not, concentrate the appropriate fraction using a Millipore BioMax concentrator to less then 500uM then resolve over a Superdex 200 column (Amersham).
27. Pool the appropriate fractions, concentrate and determine the protein concentration.

LB media

<u>1M IPTG</u> (Sigma I-5502)
(dissolve in dH_2O)

1xSDS gel loading buffer

TEK_{50} Lysis Buffer:
20 mM Tris pH 8.0
0.1 mM EDTA
50 mM KCl

Lysozyme (Sigma L-6876)
(dissolve in dH_2O)

<u>ATP agarose column Buffer</u>
20 mM Tris pH 7.2
5 mM $MgCl_2$
100 mM NaCl

10 % glycerol
25 mM ATP

<u>Dialysis Buffer</u>
20mM Tris pH 7.2
50mM NaCl
1 mM EDTA

protease inhibitors:

Inhibitor:	*Catalog #:*	*Final concentration:*	*Stock solution:*

Leupeptin A	Sigma #L-2884	1 µg/ml	1 mg/ml in dH_20
Pepstatin A	Sigma #P-4265	1µg/ml	1 mg/ml in EtOH
PMSF	Sigma #P-7626	0.5 mM final volume	1 mg/ml in EtOH