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Purification, Preparation, and Use of Chaperone–Peptide Complexes for Tumor Immunotherapy

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Abstract

The molecular chaperone heat-shock protein 70 (Hsp70) possesses immune stimulatory properties that have been employed in the preparation of anticancer vaccines. Hsp70 binds antigenic peptides in the cytoplasm of cancer cells. Hsp70 thus serves as a convenient, non-discriminating transporter of antigens and can protect the peptides during internalization by APC and cross presentation to T lymphocytes. We describe a method for purifying Hsp70–peptide complexes that can be used to prepare molecular chaperone-based vaccines, involving sequential gel fi ltration, ion exchange, and af fi nity chromatography

Keywords

Affinity chromatography; Chaperone; Heat-shock protein; HSP70; Lipopolysaccharide; Vaccine

1. Introduction

Intracellular heat-shock proteins (HSP) play an essential role in protein folding and quality control (1, 2) . They apparently recognize topological features in unfolded proteins and can bind to a wide array of small peptides and unfolded polypeptide sequences (3) . This property suggested that such proteins might be employed to bind polypeptides that contain within their sequence antigenic determinants (4) . Indeed, this approach has been used to prepare anticancer vaccines using molecular chaperones such as HSP including Hsp70, Hsp90, Hsp110, and GRP170 (5– 7) . We have concentrated in this report on Hsp70-derived vaccines.

The Hsp70 family contains 12 members including cytoplasmic proteins such as hspa1, ER-resident protein Grp78/BiP, and mitochondrially localized mortalin (1, 8). Some of these Hsp70 proteins such as Hspa1a, Hspa1b, and Grp78 are strongly induced by stress while others are expressed constitutively (1). The Hsp70 family proteins appear to be deployed in the front line in the process of housekeeping folding on ribosomes as well as during stress

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and their precise functions are likely similar, differences being more to do with contrasting transcriptional stimulus and cellular location (9). Biochemically purified Hsp70 used to prepare cancer vaccines will likely contain multiple Hsp70 family members (10– 13).

Purification of Hsp70 can employ its strong, but reversible, association with adenosine nucleotides for affinity chromatography purposes (14). Hsp70 proteins all contain an N-terminal adenosine nucleotide-binding domain and a C-terminal peptide-binding domain. Hsp70 binds to its clients only when associated with ADP and binding is reversed when ATP is exchanged for ADP (9). HSP-interacting polypeptides are referred to as “clients” rather than substrates, as HSPs, strictly speaking, are not enzymes (3, 9). Avid binding to ADP has the convenient implication that Hsp70 can be purified in association with interacting peptides. The peptide-binding domain of Hsp70 can enclose 7-mer peptide sequences and it has been shown in vivo that Hsp70 binds preferentially to peptides of 8–26 mer (15) . Thus Hsp70 seems an ideal molecule to chaperone processed peptides in the cytosol and transport them into antigen-presenting cells.

2. Materials

2.1. Source Materials for Hsp70

1. Human melanoma A375 cells or MC-38/MUC1 murine cells were used as source material for the cell-derived Hsp70 and grown in low-endotoxin medium (see Note 1). Alternatively, Hsp70 for metabolic studies was prepared from minced mouse liver essentially as described for cell lines below. Recombinant hsp70.1 protein was prepared from *Escherichia coli* bacteria BL-21 by standard methodology and purified as below (16).
2. 15 cm tissue culture dishes.
3. Both mammalian cell types were grown in Dulbecco's Modi fi ed Eagle's Medium mixed with 10% fetal bovine serum and supplemented with 100 IU/ml penicillin and 100 μ g/ml streptomycin.

2.2. Common Equipment and Reagent

1. Dounce Homogenizer.
2. Eppendorf centrifuge.
3. Spectrophotometer (Beckmann Model DU-7).
4. Gradient maker.
5. Fraction Collector with drop counter (Bio Rad).
6. PD10 chromatography columns (GE Healthcare).

[1]If Hsp70 vaccine is to be prepared from tumors in vivo, tumor material is rapidly resected, dissected free of non-tumor tissues, and snap frozen in liquid nitrogen. Collected tumor tissue is then weighed, rapidly thawed, and lysed by Dounce as in step 2 in the Methods section. For procedures involving tissue culture cells, levels of Hsp70 can be increased by prior heat shock. Cells are exposed to 1 h at 43°C and allowed to recover for 6 h at 37°C. This treatment leads to an approximate doubling in intracellular Hsp70 levels.

7. Sephadex G25 (Sigma) swollen in endotoxin-free double-distilled H_2O 24 h prior to use.
8. DE-52 diethyaminoethyl (DEAE) cellulose (Whatman) pre-equilibrated in buffer B overnight.
9. Adenosine diphosphate (ADP)-agarose (Sigma).
10. Dulbecco's phosphate-buffered saline (pH 7.4): Commercially obtained.
11. Liquid handling procedures: All liquid handling was carried out with plastic pipettes with low endotoxin ratings. Likewise all chemicals and liquids were low in endotoxins.

2.3. Hsp70 Purification

1. 20× Buffer B (2 L): 400 mM Tris base (96.8 g), 400 mM Sodium chloride (48 g NaCl), 2.0 mM EDTA (1.68 g), make up to 2 L with H_2O, and titrate to pH 7.5 with glacial acetic acid.
2. Glycerol.
3. Phenylmethylsulfonyl fl uoride (PMSF): 10 mg/ml in ethanol.
4. Pepstatin: 1 mg/ml in methanol.
5. Leupeptin: 10 mg/ml in H_2O.
6. Dithiothreitol Stock (0.5 M): 0.5 M Dithiothreitol (7.7125 g), make up to 100 ml with H_2O.
7. 300 mM $MgCl_2$ stock solution (100 ml): 300 mM $MgCl_2$ (2.856 g), make up to 100 ml with H_2O.
8. Lysis Buffer B (100 ml): Dilute 5 ml 20× buffer B in 100 ml H_2O, add 0.1 ml 0.5 M Dithiothreitol, 1 ml PMSF stock, 0.1 ml pepstatin stock, 0.1 ml leupeptin stock.
9. Buffer B elution (500 ml): Dilute 25 ml 20× buffer B in 500 ml H_2O, add 0.5 ml 0.5 M dithiothreitol, 5 ml PMSF stock, 0.5 ml pepstatin stock, 0.5 ml leupeptin stock.
10. Buffer B high salt (500 ml): Dilute 25 ml 20× buffer B in 100 ml H_2O, add 0.5 ml 0.5 M dithiothreitol, NaCl to 350 mM (9.9 g), 5 ml PMSF stock, 0.5 ml pepstatin stock, 0.5 ml leupeptin stock.
11. Buffer D (2 L): 100 ml 20× buffer B, 20 ml 300 mM stock $MgCl_2$, make up to 2 L with H_2O.
12. Buffer D high salt (0.5 M NaCl): 500 ml Buffer D, 0.5 M NaCl (15 g).
13. Buffer D + 1 mM GTP: 100 ml Buffer D, 1 mM GTP (52.3 mg), adjust to pH 7.5.
14. Buffer D + 3 mM ADP: 100 ml Buffer D, 3 mM ADP (128.16 mg), adjust to pH 7.5.

2.4. LPS Removal

1. Polymyxin-B-sepharose columns (END-X, associates of Cape Cod, Inc.)
2. 1% Sodium deoxycholate.
3. Limulus Amoebocyte Lysate (LAL) assay (BioWhittacker).

2.5. Hsp70 ATPase Activity

1. Malachite green: 0.081% w/v. Store at 4°C.
2. Polyvinyl alcohol: 2.3% w/v. Store at 4°C.
3. Ammonium heptamolybdate tetrahydrate: 5.7% w/v in 6 M HCl. Store at 4°C.
4. Malachite Green Reagent: Mix the Malachite green, polyvinyl alcohol (2.3% w/v), and ammonium heptamolybdate tetrahydrate with H_2O in the ratio 2:1:1:2.
5. 2.5 M Adenosine triphosphate (ATP).
6. Sodium Citrate: 34% in H_2O.

2.6. Lowry Assay

1. Copper-tartrate–carbonate (CTC): Add 20% sodium tartrate to a solution of 0.2% copper sulfate-tartrate to a fi nal concentration of 0.1% copper sulfate, 0.2% potassium tartrate, and 10% sodium carbonate.
2. 10% sodium dodecyl sulfate (SDS).
3. 0.8 M sodium hydroxide.
4. 2 M Folin-Ciocalteu phenol reagent (Sigma).
5. Lowry Reagent A: Mix equal parts of the CTC, NaOH, SDS, and H_2O.
6. Lowry reagent B: Dilute 1 volume of Folin-Ciocalteu phenol reagent with fi ve volumes of distilled water.
7. Bovine serum albumin.

2.7. Immunoblot Assay

1. Mini-gel protein electrophoresis apparatus with 200 v power source (BioRad, CA).
2. Western blotting, dry transfer apparatus for minigel (BioRad).
3. Polyvinylidene fl uoride (PVDF) membranes (Millipore).
4. 2× Laemmli sample Buffer (Sigma Chemicals).
5. Tris glycine buffer, polyacrylamide solutions for electrophoresis, see (17).
6. Towbin's electrophoretic transfer buffer: Tris base 30.3 g, Glycine 144 g, Double-distilled water 1.0 L.

7. Anti-Hsp70 antibody (rabbit polyclonal Ab 46) was prepared in-house using a synthetic peptide from the C-terminal 20 amino acids of human Hsp70.1 (18). Antibodies were diluted 1/10,000 in phosphate-buffered saline for immunoblot.
8. Biotin/avidin/alkaline phosphatase reagents (Vector Laboratories Inc.)

3. Methods

3.1. Hsp70–Peptide Complex Purification by Ion Exchange and Immunoaffinity Chromatography

1. Grow 15 dishes of cells in 15 cm tissues culture dishes to con fl uence (see Note 1).
2. Scrape into 12 ml of Lysis buffer B (approx. 3 g of cells).
3. Lyse cells in 20 strokes of Dounce (tight pestle).
4. Spin for 30 min at 15,000 × *g* to sediment debris.
5. Elute through a 55 ml G-25 column pre-equilibrated with buffer B, collecting 2.0 ml fractions.
6. Measure OD 280 and combine fractions in the fi rst peak.
7. Take a sample for protein assay by the Lowry method (see Subheading 3.2.4)and adjust protein concentration to 10 mg/ml.
8. Add glycerol to 5% and layer onto the 2 × 20 cm DE52 column.
9. Wash column with 200 ml of buffer B.
10. Elute with a 20–350 mM NaCl gradient (600 ml total volume taking 8 ml fractions). Elution is carried out with a gradient maker, beginning with the low-salt buffer (elution buffer B) and introducing high-salt buffer (Buffer B high salt).
11. Measure OD 280 on fractions.
12. Assay cell lysate, fl ow through, and every fourth sample by 10% SDS-PAGE using Coomassie Blue staining and Hsp70 immunoblot (see Subheading 3.2.5).
13. Collect main Hsp70 peak for ADP-agarose affinity chromatography (see Note 2).
14. Adjust to 3.0 mM $MgCl_2$ from the 300 mM stock.
15. Apply sample to 10 ml ADP-agarose affinity column and leave for 1 h.
16. Hook up the column to a fraction collector and elute with, sequentially, 40 ml Buffer D (160 drop fractions), 20 ml Buffer D high salt (80 drop fractions), 20 ml buffer D (low salt wash), 20 ml Buffer D + 1 mM GTP (80 drop fractions), and 40 ml Buffer D + 3.0 mM ADP (80 drop fractions).

[2]Client proteins/peptides rapidly dissociate from Hsp70 in the presence of ATP while ADP locks the client in place in the peptide-binding domain. Purification of Hsp70–peptide antigen complexes is thus carried out using ADP-agarose. ATP levels in solution can be rapidly decreased by treatment with the bacterial ATPase *apyrase* .

17. Hsp70 in the fractions is detected by immunoblot (see Subheading 3.2.5)and Hsp70-containing fractions are pooled and loaded onto a PD-10 column pre-equilibrated with ice-cold PBS. Hsp70 was eluted in the void volume.
18. Concentrations of Hsp70 in the preparation are determined by the Lowry protein assay (see Subheading 3.2.4).
19. Hsp70–peptide complexes are frozen at −80°C prior to use for tumor immunization.
20. Vaccine doses of 1.5–3.0 μ g of Hsp70–peptide complex were employed in vivo as described (13).

3.2. Testing the Purified Hsp70

For use in vaccine preparation, it is important to ascertain that the Hsp70–peptide complexes are fee from low-molecular-weight impurities. Hsp70 can bind avidly to endotoxins such as LPS (see Note 3). Therefore great care is taken to avoid introducing LPS into the purified Hsp70 preparations. After purification, the Hsp70 is tested for endotoxin contamination by the LAL assay purchased from BioWhittacker (Walkersville MD). In our work, contaminated preparations are routinely discarded. However it is possible to rescue the Hsp70 preparations by sequential elution through polymyxin-B-sepharose columns, until the preparations are low in endotoxins (see Subheading 3.2.1 and see Note 4).

In addition to endotoxins, purified Hsp70 may also contain other ions or molecules such as ADP or high salt that could affect the biological properties of the chaperone in an immune setting. It is therefore optimal to exchange purified Hsp70 into the fi nal buffer used in experiment by gel fi ltration (see Subheading 3.2.2).

Some of the procedures described above could also lead to inactivation of the Hsp70. To test for this possibility, one Hsp70 biochemical function that can be readily assayed is ATPase activity. ATPase (phosphatase) activity can be measured by the Malachite Green colorimetric assay (see Subheading 3.2.3)(19). ATPase activity in suspect preparations is compared to that of standard, purified Hsp70 in order to check for inactivation. Ability of Hsp70 to bind peptide antigens may be impaired if ATPase activity is compromised.

3.2.1. LPS Removal from Hsp70 Preparations

1. 0.5 ml polymyxin-B-sepharose columns are washed sequentially with 10 ml of deoxycholate and 10 ml of phosphate-buffered saline.
2. 100 μ l of Hsp70 from ATP agarose elution is loaded onto the polymyxin-B-sepharose column.
3. Column is eluted with 5 ml phosphate-buffered saline, taking 0.5 ml fractions.

[3]Hsp70 can bind avidly to endotoxins such as *E. coli* -derived LPS (22, 23). It is thus advisable to test all Hsp70 vaccine preparations for endotoxins using the LAL test as in Subheading 3.2 . In addition, as with all purified protein preparations, it is advisable to remove chemicals used in the purification by buffer exchange. This can be done conveniently by dialysis or gel fi ltration.
[4]It should be noted that while polymyxin-B can bind avidly to *E. coli* LPS, it does not remove all types of endotoxin and, for example, LPS from *S. Minnesota* does not bind to this agent (24).

4. Fractions are assayed for endotoxin contamination by the LAL assay.

3.2.2. Buffer Exchange of Purified Hsp70

1. PD-10 column is washed with 25 ml endotoxin-free phosphate-buffered saline.
2. Hsp70 fractions are loaded onto the column and eluted with 10 ml phosphate-buffered saline, taking 1.0 ml fractions.
3. Fractions are tested for Hsp70 by immunoblot on 5% SDS-PAGE gels (see Subheading 3.2.5). Hsp70 elutes in the void volume (fractions 1–3).

3.2.3. Hsp70 ATPase Activity (20)

1. Add 15 μ l of the Hsp70 preparation under test (approx 1.0 μ M) to an Eppendorf centrifuge tube.
2. Add 10 μ l of the ATP solution.
3. After 30-min equilibration at 37°C, add 80 μ l of malachite green reagent and incubate for 1 h.
4. Add 10 μ l of 34% sodium citrate to halt the reaction.
5. Mix thoroughly and measure OD at 620 nm.
6. Compare absorbance to that of purified Hsp70.

3.2.4. Lowry Assay for Protein Concentration (21)

1. Take 20 μ l aliquot of column eluate.
2. Add 20 μ l of reagent A, mix, and stand for 10 min at room temperature.
3. Add 10 μ l of reagent B and mix.
4. At 30 min, read the absorbance at 750 nm using a spectrophotometer.
5. Determine protein concentration by reference to a standard curve determined using bovine serum albumin concentrations from 5.0, 20, 50, 100, 200, to 250 μ M BSA.

3.2.5. Immunoblot Assay for Purified Hsp70 in Column Fractions

1. Fractions are diluted 1:1 with 2× Laemmli buffer and boiled at 100°C for 5 min.
2. A 25 μ l sample is loaded onto the gel sandwich for discontinuous SDS-PAGE, using 5% stacking gel and 10% separating gel, and subjected to electrophoresis until the Bromophenol Blue tracking dye reaches the bottom of the sandwich.
3. Separating gels are removed, soaked in transfer buffer, and proteins then transferred electrophoretically onto PVDF membranes for 30 min using standard conditions (18).
4. Membranes are blocked in 5% condensed milk and then incubated with anti-Hsp70 antibodies overnight with shaking at 25°C.

5. Hsp70 bands are detected using a tiered biotin–avidin–alkaline phosphatase ampli fi cation system.

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