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Novel Modification of Growth Medium Enables Efficient *E. coli* Expression and Simple Purification of an Endotoxin-Free Recombinant Murine Hsp70 Protein

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Heat shock protein 70 kDa (hsp70), a molecular chaperone involved in folding of nascent proteins, has been studied for its ability to activate innate and specific immunity. High purity hsp70 preparation is generally required for immunization experiments, because endotoxins and other immunologically active contaminants may affect immune responses independently of hsp70. We have developed a novel modification of *E. coli*-expression medium that enabled a simple two-step production and purification method for endotoxin-free recombinant hsp70. During Ni–NTA-based affinity purification of hsp70, a contaminating protein from host *E. coli* cells, L-glutamine-D-fructose-6-phosphate aminotransferase (GFAT), was identified. By testing various compounds, supplementation of growth medium with a GFAT metabolite, *N*-acetylglucosamine, was found to reduce GFAT expression and increase the total hsp70 yield five times. The new protocol is based on column purification of His-tagged hsp70 protein produced by *E. coli* with the modified medium, followed by endotoxin removal by Triton X-114 extraction. This approach yielded hsp70 with high purity and minimal endotoxin contamination, making the final product acceptable for immunization experiments. In summary, a simple modification of growth medium allowed production of recombinant mouse hsp70 in high yield and purity, thus compatible with immunological studies. This protocol may be useful for production of other His-tagged proteins expressed in *E. coli*.

Keywords: Affinity purification, endotoxin removal, GlcNAc, hsp70, MALDI–TOF, protein expression

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Heat shock proteins (hsp) have been described to stimulate T cell-mediated immunity as well as the specific antibody responses against the coupled polypeptides [19, 20]. In this process, the hsp moiety is responsible for the receptor-mediated binding of hsp–peptide complex, and delivery of peptide to the proteasome processing followed by presentation of peptide fragments on MHCI molecule to host cytotoxic $CD8^+$ T cells [8, 18].

Isolation of recombinant hsp is the starting point for their testing as potential modulatory molecules in anticancer or anti-infectious disease treatment [6, 7, 23]. However, the production and purification of hsp is a principal limitation for those applications. Tagging of recombinant proteins with a His epitope facilitates affinity purification of the expressed protein using Ni(II), Co(II), or Cu(II) ions on Ni–NTA resins. However, Ni–NTA affinity columns additionally bind other histidine-rich proteins. Removal of such impurities can be achieved by a combination of affinity chromatography with separation techniques, consequently increasing the cost of the product.

Here, we report a simple medium manipulation that enabled isolation of a full-length recombinant mouse hsp70 protein expressed in *E. coli* using a simple two-step purification approach. We noticed that on the Ni–NTA column, the hsp70 was co-purified with L-glutamine-D-fructose-6-phosphate aminotransferase (GFAT; E.C. 2.6.1.16), which has physicochemical properties similar to those of mouse hsp70: molecular mass, pI, and content of hydrophobic amino acids (Waugh's parameter) [22]. Such conditions practically preclude successful removal of GFAT by size-exclusion chromatography, ion-exchange chromatography, or hydrophobic chromatography. Therefore, to downregulate GFAT expression, a supplementation of LB medium with various downstream metabolites was tested. Consequently,

an optimal combination of incubation temperature and *N*-acetyl-D-glucosamine supplementation almost completely removed GFAT from the Ni–NTA-purified fraction. After subsequent endotoxin removal by a successive Triton X-114 extraction, the final hsp70 protein is applicable for various vaccination experiments. This protocol may be useful for improvement of the yield and purity of other His-tagged proteins expressed in *E. coli*.

Materials and Methods

Reagents

All chemicals, unless otherwise specified, were from Sigma (St. Louis, MO, U.S.A.); tissue culture media and reagents were from Gibco (Invitrogen, Carlsbad, CA, U.S.A.).

Preparation of Recombinant Vector for Expression of Murine Hsp70 in *E. coli*

Murine hsp70 cDNA was isolated by RT–PCR using mRNA obtained from BALB/c splenocytes and Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) followed by PCR amplification with proofreading polymerase Phusion (Finzyme, Espoo, Finland) with the following primers: forward, AGCCTTCCAGAAGCAGAGC; reverse, CTAATCCACCTCCTCGATGGT. The PCR product was cloned into pCR II Blunt TOPO cloning vector (Invitrogen) and transformed into *E. coli* TOP10 (Invitrogen) followed by colony screening by restriction analysis of plasmid DNA isolated using the Plasmid Mini Kit (Qiagen, Hilden, Germany). Subsequently, hsp70 cDNA was cloned into the expression vector pET101/D-TOPO (Invitrogen) and the recombinant plasmid was transformed into TOP10 and selected by colony screening followed by identity confirmation by commercial sequencing (Generi Biotech, Hradec Kralove, Czech Republic).

Expression of Hsp70 and Metabolite Supplementation

The *E. coli* BL21(DE3) strain was used for expression of recombinant hsp70 protein. The cells were grown at various temperatures in LB medium with 100 μg/ml ampicillin, and after OD_{600} reached 0.8, protein expression was induced by isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM and the cells were grown for another 4 h.

Hexosamine Pathway Metabolites Supplementation

To affect the GFAT level, the LB medium was supplemented with various hexosamine pathway metabolites at the final concentration of 45 mM for the entire cultivation period: *N*-acetyl-D-glucosamine, D-glucosamine, or D-glucosamine-6-phosphate.

Protein Purification

All purification steps were performed on ice or at 4°C. The cells were harvested by centrifugation at 10,000 ×*g*, for 10 min. The pellet was resuspended in a lysis buffer (50 mM $NaH_2PO_4 \cdot 2H_2O$, 300 mM NaCl, 10 mM imidazole, 0.1% v/v Triton X100, 0.5 mg/ml lysozyme, and 100 μM EDTA pH 8.0) supplemented with protease inhibitors phenylmethylsulfonylfluoride (PMSF, final concentration 0.5 mM), leupeptin (0.4 μg/ml), aprotinin (0.5 μg/ml), and 8 ml/g of the pellet, and incubated on a rotator at +4°C for 1 h. Subsequently, the lysate was sonicated 10 times for 10 s with 1-min interruptions on ice followed by centrifugation at 10,000 ×*g* for 10 min. The protein was purified batch-wise under native conditions using Ni–NTA agarose and 1 ml/g of cell pellets, following the manufacturer's instructions (Qiagen); the wash buffer contained 20 mM imidazole and the elution buffer contained 500 mM imidazole. The protein was concentrated using an Amicon Ultra-4 Centrifugal Filter Unit equipped with a 50 kDa cut-off filter (Millipore, Billerica, MA, U.S.A.), transferred to 100 mM Tris-Cl buffer, pH 7.4, and stored on ice. Concentration was determined by the Bradford method.

Endotoxin Removal and Determination

All steps were performed with Pure 300 and Pure 1000 LAL reagent grade pipette tips (Molecular BioProducts, San Diego, CA, U.S.A.). For endotoxin removal, two different approaches were tested: (a) affinity chromatography by columns EndoTrap Red or EndoTrap Blue according to manufacturer's suggestions (Lonza, Walkersville, MD, U.S.A.), or (b) a two-phase separation with Triton X-114 as described elsewhere [1]. In short: Triton X-114 was added to protein sample in a final concentration of 1% (v/v). The entire content was stirred for 30 min, +4°C, and then heated (37°C) for 10 min and centrifuged at 10,000 ×*g*, at 25°C for 10 min. The entire procedure was repeated three times (as recommended by [1]) or repeated until the endotoxin level reached the desired value (<2.5 EU/mg of protein).

The endotoxin level was measured using Pyrotell Limulus Amebocyte Lysate (LAL; sensitivity 0.25 EU/ml of analyzed solution; Associates of Cape Cod, East Falmouth, MA, U.S.A.) according to the manufacturer's instructions. The unit EU describes the biological activity of LPS that varied significantly from one source to another. The FDA established a reference endotoxin standard (EC-2) previously with a potency of 5 EU/ng. The current FDA referenced standard endotoxin (EC-6) has a potency of 10 EU/ng. In a specific experiment, the conversion factor depends on the source of the endotoxin standard included in the LAL assay kit [9].

SDS–PAGE and Western Blot Analyses

Protein samples were separated on 10% SDS–PAGE and stained with Coomassie Blue R-250 or blotted to PVDF membranes (BioRad, Hercules, CA, U.S.A.). The membranes were blocked overnight using the Super Block reagent (Pierce, Rockford, IL, U.S.A.) and developed with HRP-conjugated Anti-V5 (Invitrogen) diluted 1:7,000 in SB plus 0.05% Tween 20 (SB-T) at laboratory temperature for 1 h. The reaction was detected by the SuperSignal West Pico reagents (Pierce) and visualized by exposition on an X-ray film (Kodak, Rochester, NY, U.S.A.).

MALDI–TOF Analyses

Protein identity was confirmed by peptide mass fingerprinting of SDS–PAGE-resolved samples using a Microflex MALDI–TOF LRF20 mass spectrometer (Bruker Daltonik, Bremen, Germany) as described elsewhere [16, 17]. In brief, protein bands from Coomassie-stained gels were excised, destained, and, after previous reduction/alkylation, in-gel digested by 0.5 μM raffinose-modified trypsin at 37°C for 12 h. MALDI probes were prepared using an MSP AnchorChip 600/96 microScout target (Bruker Daltonik) and a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA (1:2, v/v) as a matrix. Measurements were performed in positive-ion reflectron mode. Protein identification from obtained mass spectra was achieved using the program Mascot Server 2.2 (Matrix Science, London, U.K.);

searches were performed against a nonredundant protein database (MSDB; version August 31, 2006).

Determination of ATPase Activity of Recombinant Hsp70

ATPase activity was determined by the established spectrophotometric method with malachite green, essentially as described previously [3, 5, 12]. The reaction mixture containing 10 μg of hsp70 and 1 mM ATP in 50 mM HEPES, 2 mM Mg^{2+}, pH 7.2, was incubated at 37°C for 30–60 min. The phosphatase reaction was quenched by malachite green reagent, and after development at 37°C for 10 min, the absorbance at 660 nm was measured against a blank prepared without adding of the hsp70. Calibration was made in the range 1–10 nmol phosphate using KH_2PO_4.

RESULTS AND DISCUSSION

We have cloned murine hsp70 cDNA into expression vector pET101 (designated pET101-hsp70). Sequencing confirmed cloning of the entire coding region (GenBank Accession No. NM_010479) and in-frame added C-terminal fused His-tag (for affinity purification) and V5 tag (for detection by monoclonal anti-V5 antibody). As estimated by the ProtParam tool (http://www.expasy.ch), a theoretical molecular mass of the fusion protein is 73.6 kDa and pI=5.89.

Hsp70 was Purified on Affinity Ni–NTA Column Together with GFAT

The recombinant hsp70 protein was expressed in *E. coli* BL21(DE3) as an intracellularly localized protein without any signs of inclusion bodies formation for at least 4 h after the induction with IPTG. After a pilot-scale Ni–NTA affinity purification of hsp70 under native conditions, two closely localized bands were present on Coomassie blue-stained SDS–PAGE gels (Fig. 1). The identity of both bands was confirmed by MALDI–TOF MS and subsequent database search. The top band corresponded to the cloned mouse hsp70 (MSDB Accession No. Q61696). The closely located bottom band was identified as GFAT (MSDB Accession No. Q8XEG2), an aminotransferase involved in the hexosamine pathway of GlcNAc synthesis in *E. coli* cells. Parameters of the identification were as follows: hsp70 – 14 peptides identified, sequence coverage was 38%, and probability-based MOWSE score 83; GFAT – 13 peptides identified, sequence coverage was 36%, and probability-based MOWSE score 84. Mature GFAT contains 608 amino acids with a theoretical molecular mass of 66.8 kDa and pI=5.6. This protein is co-purified on affinity Ni–NTA column probably because of its relatively high histidine content (24 per one GFAT molecule).

Similar molecular mass and pI of both proteins, recombinant hsp70 and GFAT, precluded effective separation either by size-exclusion chromatography or ion-exchange chromatography. Furthermore, the nonpolar amino acids composition was analyzed. Waugh's parameter, *NPS* (larger nonpolar side-chains), for both proteins is similar (*NPS* for hsp70 ~ 0.309 and *NPS* for GFAT ~ 0.349) indicating that both proteins have roughly a 30–35% content of nonpolar residues [21, 22]. Properties of GFAT were so similar to those of hsp70 that it precluded use of hydrophobic chromatography as an alternative purification approach.

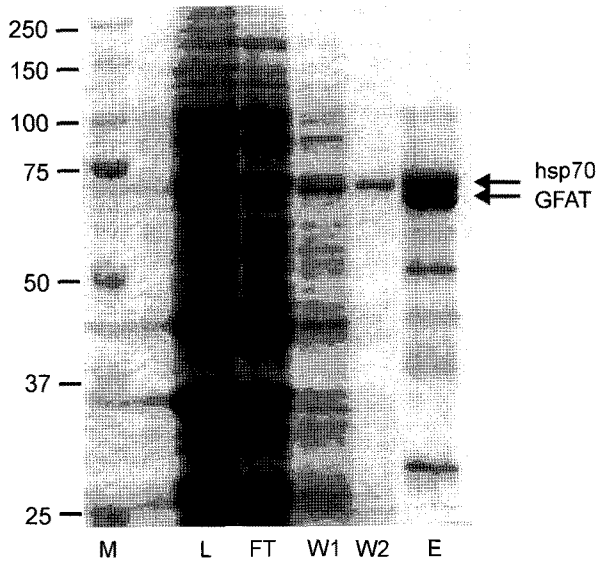


Fig. 1. Purification of recombinant hsp70 using Ni–NTA column yields two closely located bands corresponding to hsp70 and GFAT.

Recombinant mouse hsp70 comprising a C-terminally fused His-tag was purified from bacterial lysate (L) using an Ni–NTA column under native conditions. Flow through (FT), initial wash (W1), terminal wash (W2), and eluate (E) fractions were analyzed by SDS–PAGE with Coomassie blue R-250 staining. In fraction E, two closely located bands were identified by MS: mouse hsp70 (upper arrow) and GFAT (lower arrow). M indicates molecular mass marker.

GFAT Production was Minimized by Growth-Medium Supplementation with GlcNAc, Which Additionally Increased the Yield of Hsp70

One possibility of how to reduce the GFAT expression was to test the effect of reduced cultivation temperatures during the entire expression experiment (before and after the induction by IPTG). The *E. coli* protein expression profile was determined in response to growth temperatures 27, 30, and 37°C followed by a small-scale Ni–NTA purification. No significant reduction of GFAT moiety was observed at all temperatures tested, although a slight increase in the yield was noted at 30°C (Fig. 2A). GFAT participates in β1,6-GlcNAc-branched glycan biosynthesis, specifically in the hexosamine pathway. It catalyzes the conversion of D-fructose-6-phosphate to D-glucosamine-6-phosphate. Thus, one potential approach to how to reduce the GFAT transcription was to supplement the growth medium with metabolites downstream of the GFAT reaction in the hexosamine pathway (Scheme 1). Three potentially downregulating metabolites

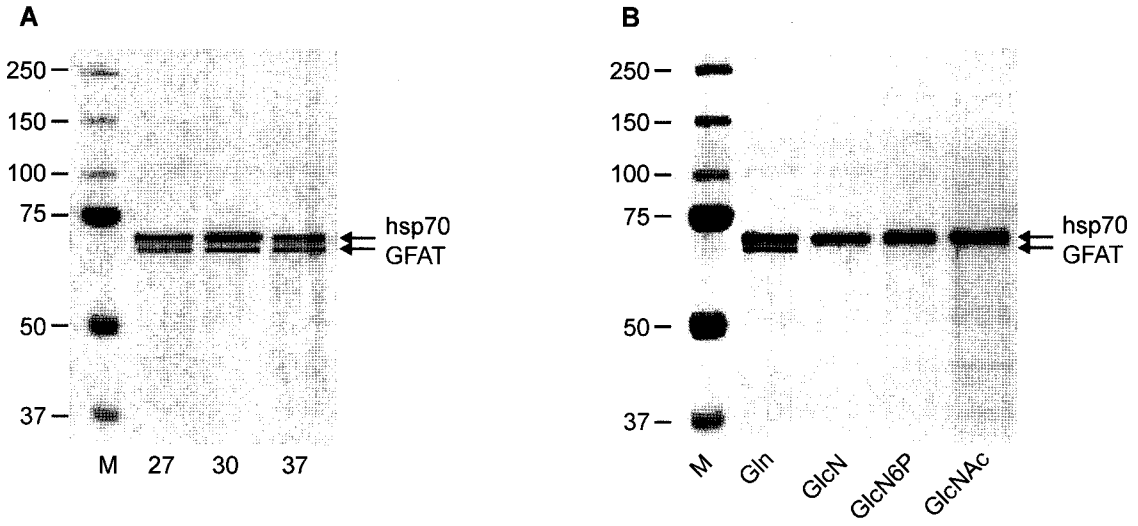
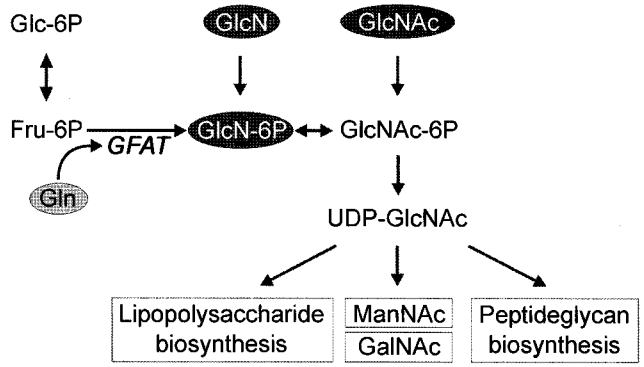


Fig. 2. Effect of various temperatures and hexosamine-pathway metabolites on the proportion between hsp70 and GFAT expressed in transformed *E. coli.*

The hsp70 protein expression was analyzed at three different growth temperatures (27, 30, and 37°C). Parallel cell cultures were grown until OD_{600} reached 0.8 and then the expression was induced with 1 mM IPTG for 4 h. Hsp70 protein was isolated using an Ni–NTA column, and eluted fractions were analyzed by 10% SDS–PAGE stained with Coomassie blue R-250 (**A**). The upper arrow indicates hsp70, the lower arrow indicates GFAT. In subsequent experiment, the effect of four different hexosamine pathway metabolites was tested in parallel cultures: the LB medium was supplemented either with D-glucosamine (GlcN), or D-glucosamine-6-phosphate (GlcN-6P), or *N*-acetyl-D-glucosamine (GlcNAc) at 45 mM final concentration during the entire experiment. As the control, the LB medium was supplemented with L-glutamine (Gln). The transformed *E. coli* cells were grown until OD_{600} reached 0.8 and then protein expression was induced with 1 mM IPTG for 4 h. The recombinant hsp70 was isolated and analyzed as above (**B**).

(D-glucosamine, D-glucosamine-6-phosphate, or GlcNAc) and one potentially upregulating metabolite (L-glutamine) as a control were tested in separate cultures followed by Ni–NTA affinity purification (Fig. 2B). From the downregulating metabolites tested, GlcNAc was the most effective one followed by D-glucosamine-6-phosphate and D-glucosamine. Furthermore, the supplementation of the growth medium with GlcNAc increased the cell density reached after the IPTG induction period (4 h) without any observed evidence of hsp70 degradation during cultivation (Fig. 3). The increase in the total hsp70 protein yield was about five times.

After optimizing the culture conditions, we tested upscaling of the purification process using a 3.5 l bioreactor (MBR; Picatech Huber AG, Luzern, Switzerland). The apparatus



Scheme 1. Involvement of GFAT in the hexosamine pathway (*E. coli*).

GFAT is an aminotransferase converting D-fructose-6-phosphate (Fru-6P) to D-glucosamine-6-phosphate (GlcN-6P). It requires L-glutamine (Gln) as the amino group source. An increased intracellular concentration of all metabolites behind this reaction, such as GlcN-6P, *N*-acetyl-D-glucosamine (GlcNAc), or GlcN (depicted in dark ellipses), represents a potential basis for downregulation of GFAT expression.

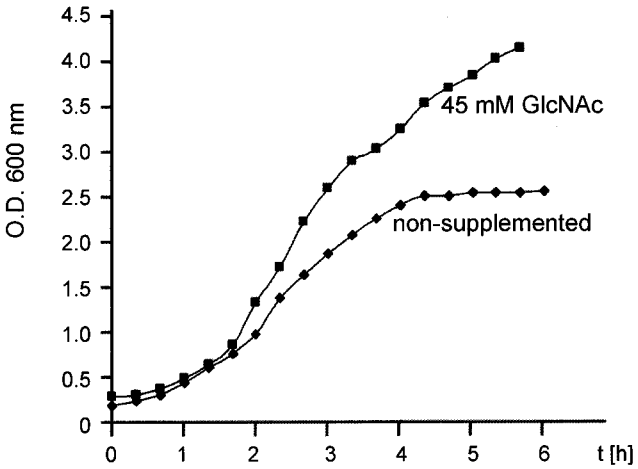


Fig. 3. Supplementation of LB medium with GlcNAc increases the cell density and replication rate.

Hsp70-transformed *E. coli* were grown in MBR bioreactor (3.5 l culture) either in LB or in LB supplemented with GlcNAc (45 mM final concentration) for 6 h. Each 20 min, a 2-ml sample was aseptically taken and the OD_{600} was measured on a spectrophotometer against LB medium or LB plus GlcNAc. The OD values shown represent the arithmetical mean of two measurements. The nonsupplemented culture was saturated within 4–5 h whereas the GlcNAc-supplemented one did not reach saturation even after 6 h.

was set to control optimal growth conditions (pH ~ 7.2, temperature ~ 37°C, continual aeration, and agitation ~ 500 rpm).

In the final step, the optimal volume of Ni–NTA agarose column per gram of the bacterium pellet obtained 4 h after IPTG induction in the bioreactor was tested. Increasing volume of Ni–NTA agarose increased the column binding capacity, but the amount of impurities increased as well. Thus, two important factors needed to be compared to find the optimal Ni–NTA agarose volume: (a) the Ni–NTA agarose volume that binds the total hsp70 produced and (b) the level of impurities in dependence on the volume of Ni–NTA column used. Both parameters were evaluated directly from Coomassie Blue-stained SDS–PAGE gels (data not shown) and by quantification of the total protein yield (Bradford method). By simultaneous Ni–NTA agarose isolations (with various ratios of Ni–NTA agarose per 1 g of bacterium pellet obtained from the bioreactor 4 h after IPTG induction), the optimal volume of Ni–NTA agarose was set to 0.5 ml of 50% Ni–NTA agarose slurry (see Material and Methods) per 1 g of bacterium pellet. Under such conditions, the impurities were almost undetectable on Coomassie blue-stained SDS–PAGE gels (Fig. 4A). Quantitative densitometry analyses of the gels confirmed 95% purity of the isolated recombinant hsp70 protein. Western blot analysis using monoclonal Anti-V5 antibody identified only one band with molecular mass corresponding to recombinant hsp70 (Fig. 4B).

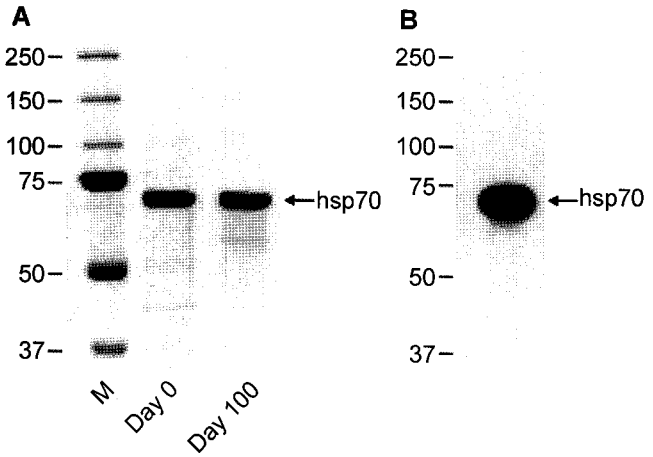


Fig. 4. Hsp70 expressed and purified under optimized conditions is free of contamination and stable at 4°C.
Under the optimal growth and purification protocol, the purity of recombinant mouse hsp70 expressed in *E. coli* reached over 95% as determined by densitometric analysis. The transformed *E. coli* were grown in 3.5 l LB supplemented with 45 mM GlcNAc in a MBR bioreactor until OD_{600} was 0.8. The protein expression was induced by 1 mM IPTG for 4 h. The culture was pelleted and protein was purified on Ni–NTA agarose [0.5 ml of 50% Ni–NTA agarose slurry (see Material and Methods) per 1 g of bacterium pellet] under native conditions. Eluted fractions were collected, concentrated by ultrafiltration, and subjected to the endotoxin removal by Triton X-114. Protein was analyzed on 10% SDS–PAGE and stained by Coomassie blue R-250 (**A**). Under the above conditions, no detectable GFAT contamination was present (Day 0). Stability of protein at 4°C was determined 100 days after purification on 10% SDS–PAGE stained by Coomassie blue R-250 (Day 100). In parallel, protein preparation from Day 0 was separated on 10% SDS–PAGE, blotted on PVDF, and detected using Anti-V5 antibody (**B**).

Endotoxin Removal by Triton X-114

The hsp70 preparation obtained after Ni–NTA affinity chromatography was heavily contaminated by *E. coli* endotoxin originated from the bacterium cell wall. The endotoxin level reached at least 17,900 EU/mg of hsp70. Two methods of endotoxin removal were tested: affinity chromatography and a two-phase separation with Triton X-114. Chromatographic methods were ineffective owing to inherent hsp70 protein binding onto the matrix. Losses of protein reached about 18% per one purification cycle when EndoTrap Red was used, and 8% when EndoTrap Blue was used as detected by Bradford quantification. The remaining endotoxin level was still between 170 and 1,700 EU/mg of hsp70 even after five cycles of the column chromatography. The second approach utilizing Triton X-114 provided much better results. After four cycles of phase separation, the endotoxin content was lower than 1.79 EU/mg of hsp70 protein in solution (Table 1). This means that the endotoxin level in the final solution was <2.5 EU/ml, assuming the final protein concentration of 1.4 mg/ml of 100 mM Tris-Cl, pH 7.4. Thus, when this hsp70 protein preparation will be used for experimental vaccination, 10 or 50 μg of recombinant protein dose corresponds to 0.018 or 0.09 EU of endotoxin respectively. To eliminate the endotoxin contaminations, alternative endotoxin removal approaches were published such as DEAE–anion-exchange chromatography [10] or treatment by deoxycholate [24]. Both are effective but associated with considerable loss of hsp70. In our experiment, the total yield of endotoxin-free hsp70 from one batch preparation (using a 3.5 l bioreactor culture supplemented with GlcNAc) was about 20 mg of total protein as determined by the Bradford method. Such yield is about three times higher in comparison with the Menoret approach (2–3 mg of recombinant endotoxin-free hsp70 per liter of bacteria culture) [10].

ATPase Activity of Hsp70

The functional evaluation of the purified recombinant hsp70 was performed by ATPase assay. The measured

Table 1. The endotoxin level after successive Triton X-114 phase extractions.

Triton X-114 extraction step	Before	3	4
Endotoxin level (LAL) [EU/mg]	17,900	17,9	1,79

Endotoxin contamination from recombinant hsp70 was removed by successive Triton X-114 extraction. Endotoxin levels before extraction and after consecutive extraction steps 3 and 4 were determined by Pyrotell Limulus Amebocyte Lysate and expressed as EU/mg of recombinant protein.

phosphatase activity was characterized by a k_{cat} value of 1.1 min^{-1} [3, 5]. Using the same assay procedure, a reference sample of hsp90 from *Candida albicans* [14, 15] showed a k_{cat} value of 33.5 min^{-1}. This is in accordance with previously published results: Eukaryotic hsp70 and *E. coli* DnaK have been shown to possess moderate ATPase activities (k_{cat} values 1 min^{-1}), whereas hsp90 proteins usually show much higher k_{cat} values of 10–150 min^{-1} [11, 13].

Storage Stability of Hsp70

Finally, the low-temperature storage stability of isolated protein was tested. When stored on ice, the only negligible signs of degradation were observed within 100 days of storage. Quantitative densitometry analysis of the Coomassie blue-stained SDS–PAGE gel confirmed that ~ 90% of the isolated recombinant hsp70 protein remains intact (Fig. 4A). For a long-term storage, freezing at –70°C was tested without signs of protein precipitation. As an alternative, the hsp70 protein was lyophilized and stored in the refrigerator for 14 days. Surprisingly, subsequent reconstitution in water was incomplete, although the volume of water used for the reconstitution was expanded by 100 mM Tris-Cl, pH 7.4, to achieve a final concentration of 0.7 mg protein per 1 ml.

Hsp for Immunization Experiment

hsp70 proteins were already tested in several *in vivo* and *in vitro* experiments. The nonspecific and/or antigen-specific activation of immune system is one of the most important effects of hsp70. The purity of hsp tested was described to be critical, because the endotoxin could activate antigen-presenting cells nonspecifically [2]. The critical endotoxin concentration was determined to be >10 EU/mg, which is higher in comparison with our preparation. Alternatively, a eukaryote expression system, naturally endotoxin free, could be used [4, 20], but eukaryotic expression systems are very expensive in comparison with *E. coli* and upscaling to reach the milligrams of hsp70 is difficult to perform.

Some *E. coli*-origin proteins, for example flagellin (FliC), could aberrantly activate the host immune system [24], but such contaminations were not detected in our preparation after the optimized hsp70 purification protocol (GlcNAc supplementation, Fig. 4).

In conclusion, a simple two-step purification method for the isolation of recombinant hsp70 from an *E. coli* expression system was described. One contaminant protein originating from host cells, GFAT, was suppressed by supplementation of the growth medium with GlcNAc, which moreover increased the protein yield. Using affinity Ni–NTA chromatography followed by endotoxin removal by Triton X-114, the recombinant hsp70 was obtained in purity generally acceptable for subsequent utilization of the protein as a recombinant vaccine component. This protocol may be useful for production of other His-tagged proteins in *E. coli* for immunological applications.

Acknowledgments

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