RESEARCH ARTICLE
Production and purification of human Hsp90β in *Escherichia coli*

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Abstract
The molecular chaperone Hsp90 is an essential member of the cellular proteostasis system. It plays an important role in the stabilisation and activation of a large number of client proteins and is involved in fatal disease processes, e.g. Alzheimer disease, cancer and cystic fibrosis. This makes Hsp90 a crucial protein to study. Mechanistic studies require large amounts of protein but the production and purification of recombinant human Hsp90 in *Escherichia coli* is challenging and laborious. Here we identified conditions that influence Hsp90 production, and optimised a fast and efficient purification protocol. We found that the nutrient value of the culturing medium and the length of induction had significant effect on Hsp90 production in *Escherichia coli*. Our fast, single-day purification protocol resulted in a stable, well-folded and pure sample that was resistant to degradation in a reproducible manner. We anticipate that our results provide a useful tool to produce higher amount of pure, well-folded and stable recombinant human Hsp90β in *Escherichia coli* in an efficient way.

Introduction
The cellular proteostasis system evolved to maintain cellular health and stability and to protect cells from continuously occurring stress through tight control of protein production, quality control, folding, trafficking, aggregation and degradation [1,2]. Chaperones are crucial elements of the proteostasis system, they prevent protein misfolding and aggregation by various mechanisms and thereby contribute to cellular integrity [3].

Hsp90 interacts with up to 10% of the cellular proteome. Its main clients include transcription factors, kinases and hormone receptors (Didier Picard. Table of Hsp90 interactors. Available from: https://www.picard.ch/downloads/Hsp90interactors.pdf). But it also has unconventional partners, such as the disordered α-synuclein and Tau [5,10]. Hsp90 plays a crucial role in the progression of several diseases, for instance cancer, cystic fibrosis and Alzheimer’s disease [12–14].
The Hsp90 chaperone machinery is extensively studied to understand its complex working mechanism and involvement with multiple fatal diseases. Typically, in vitro biochemical and biophysical experiments require high amount of recombinant protein with excellent sample purity and stability. Significant overproduction of the recombinant human Hsp90β is challenging since it is a large, multi-domain protein. Moreover, Hsp90 is particularly sensitive to the proteolytic cleavage of the flexible, unfolded linkers between its domains, therefore a time-consuming purification protocol may compromise sample quality. Only very brief protocols for production and purification of human Hsp90 have been published before [15–20].

We systematically tested several parameters to find the best conditions for the overproduction of recombinant human Hsp90β. Furthermore, we developed a fast and efficient purification protocol that results in pure and stable sample with reproducible quality. We found that the nutrient value of the medium and the length of induction time had significant effect on Hsp90 overproduction, whereas the concentration of induction agent (isopropyl-β-D-1-thiogalactopyranoside (IPTG)), temperature and optical density (OD600) at induction (within the tested intervals) did not influence the process. The protein samples we purified with the optimised protocol were resistant to proteolysis upon incubation at physiological temperature up to one day. We showed that the folding, sedimentation and molecular weight of our Hsp90 sample corresponded to earlier results.

Materials and methods

Wild type full length Hsp90 construct

pet23a+ expression vector (Novagen) was used for His6-tagged wild type human Hsp90 production in Rosetta 2 E. coli strain (Novagen). The sequence of wild type Hsp90 with the non-cleavable His6-tag:

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HHHHHHMEKQRMETQKHTAEFAQVLDKLDSGKELKIIPNPQERTLTLVDGTIGMTKADLINLGTIACS
GTMKTFAKMEALQAOGADISMIQFCVGYPFAVLYVEVAKTVKADNEQAYEWAESSAGGGTVRADGG
FPIGRTKVLHLKDQTEYLEERKKEEVVKKHISEFIFGYTLPILNEKREKEISDEAEIERSKEKE
EDEKDSEEKPDVEKEDVSDEEDSGKDKKKTKKKEKDIQELNKTKIWRNFDITQEEYGEFYKSLT
NMDWHEVHHSVEGQLFRALLIFPRFAPDLFENKKNMKLKLKVRVIFMDSDELIPYFNLIRGVD
SEDMLHNSRMLQQQSKILKVRKIVKCLLEFSEALDKENYKFYEAFSNKCLGLICHEDSTNKRL
SELLRHHTSQGDEMTLSEYVSRMKTQKS1YYITGEKQVNADNFVIEKIFQKEKSGRLSVEVY
MTETFYKIDEVCGQKLKEFDKSLSVSTYKEELIPEEDEEKKMEEKMKFKNLCLKMILDKKVEK
TVISNRVSVPCCIWSTYGWTDNIMKQAQLRDNSTMGYMKKLEIFHDIPVETLQRKAEADKKND
KAVKDLVLLFETALISSGFLEDQTHSNRIYRMMKLGLIDEDVAEEEPAAVFDIPELEDDASRMEEVD.
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Protein overproduction test—Cell culturing

Rosetta 2 cells containing pet23a+ vector with wild type full length Hsp90 were inoculated into 100 ml 2x yeast tryptone extract (2x YT; 12.8 g Bacto Tryptone (Merck), 8 g Bacto Yeast Extract (Merck), 4 g NaCl (Merck) in 800 ml deml water (Millipore)) medium supplemented with 34 mg/l final concentration of chloramphenicol (Sigma-Aldrich) and 100 mg/l final concentration of ampicillin (Sigma-Aldrich). The cells were grown over night at 37˚C, shaking at 220 rpm. The next morning 100 (for low OD600) or 200 (for high OD600) μl culture was inoculated into 4 ml lysogeny broth medium (LB; 8 g Bacto Tryptone, 4 g Bacto Yeast Extract, 4 g NaCl, 20 g thymine (Sigma-Aldrich) in 800 ml deml water, 2x YT or terrific broth media (TB; 12 g Bacto Tryptone, 24 g Bacto Yeast Extract, 4 ml glycerol in 800 ml deml water)) supplemented with 34 mg/l final concentration of chloramphenicol and 100 mg/l final concentration of ampicillin. The cultures were grown at 37˚C, shaking at 180 rpm. The OD600 was monitored every 1.5 hours by using a 1:10 dilution of the culture in an Ultrospec 3000 pro UV/Visible...
Spectrophotometer (GE Healthcare). The cultures were induced at low (0.6–0.9) or high (1–1.3) OD$_{600}$ with 0.1/0.25/0.5 mM IPTG (Thermo Fisher Scientific). We did this study using the non-codon optimized wildtype gene, as our previous experiments with the codon-optimised version did not lead to significant Hsp90 overproduction. In fact, in our hands codon optimisation introduced a secondary ribosome-binding site close to the C-terminal part of Hsp90, which resulted in a small molecular weight side product that was not possible to remove from the sample. We decided to do our study at 16˚C and 18˚C as the previous expression studies at higher temperatures (25˚C to 37˚C) did not lead to significant Hsp90 overproduction. The cultures were incubated at 16˚C or 18˚C, the SDS-PAGE samples were taken before induction and 1/3/5 day(s) after induction.

**SDS-PAGE**

For the SDS-PAGE samples 0.5 ml of cell culture was taken and centrifuged for 1 minute by 13,300 rpm. The supernatant was discarded and the pellet was resuspended in 200 μl of 1x sample buffer (0.625 M Tris (Sigma-Aldrich), 12.5% glycerol (CARL ROTH), 1% SDS (Bio-Rad), 0.005% Bromophenol Blue (Bio-Rad), 5 mM freshly added β-mercaptoethanol (Sigma-Aldrich) 2x diluted with demi water (Millipore)) and homogenised with a syringe (BD Micro-Fine). This last step was essential for loading the samples on the gel.

15% SDS gels were prepared (Separation buffer: 0.38 M Tris pH 8.8, 15% acrylamide (National Diagnostics), 0.1% SDS, 0.1% APS (Sigma-Aldrich), 0.04% TEMED (Sigma-Aldrich), Stacking buffer: 0.125 M Tris pH 6.8, 4% acrylamide, 0.1% SDS, 0.075% APS, 0.1% TEMED) and run in 1x Laemmlie buffer (0.025 M Tris base, 0.152 M glycine (SERVA Electrophoresis GmbH), 0.1% SDS, diluted from 10x stock). The gels were stained with Coomassie staining solution (0.2% Coomassie Brilliant Blue (SERVA Electrophoresis GmbH), 45% methanol (Interchema Antonides-Interchema), 10% acetic acid (Biosolve) and 55% demi water) and destained using destaining solution (30% methanol, 10% acetic acid and 60% demi water).

Despite utmost care to standardize loading of our gels, we noted minor fluctuations in some cases. To guide the eye of the reader for lane comparison within the same but also between different gels, we quantified the Hsp90 amounts in each lane and normalised on the EF-Tu band in the same lane. Therefore, gels were scanned in an Epson Perfection V700 Photo scanner. Quantification of the lane profiles was done using ImageJ.

**Overproduction and purification of wild type full length Hsp90**

Rosetta 2 cells containing pet23a+ vector with wild type full length Hsp90 were inoculated into 200 ml 2x YT medium supplemented with 34 mg/l final concentration of chloramphenicol and 100 mg/l final concentration of ampicillin. The cells were grown over night at 37˚C, shaking at 220 rpm. Next morning 200 ml culture was inoculated into 6x800 ml 2x YT supplemented with 34 mg/l final concentration of chloramphenicol and 100 mg/l final concentration of ampicillin. The cultures were grown at 37˚C, shaking at 180 rpm and induced with 0.5 mM IPTG at OD$_{600}$ = 1. After induction the cells were incubated at 18˚C for 5 days, shaking at 180 rpm.

The cells were harvested in an Avanti J-26 XP centrifuge (Beckman Coulter) using the JLA-8.1 rotor at 4˚C at 4500 rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in ice cold resuspension-buffer (50 mM Na-phosphate pH 7.2 (Sigma-Aldrich), 150 mM NaCl, 150 mM KCl (CARL ROTH)) and centrifuged in an MSE Harrier 18/80 Refrigerated Benchtop Centrifuge at 4˚C at 5000 rpm for 30 minutes. The supernatant was discarded and the pellet was stored at -20˚C until further usage.
The pellet was resuspended in ice cold lysis buffer (12.5 mM Na-phosphate pH 7.2, 75 mM NaCl, 5 mM β-mercaptoethanol, EDTA-free protease inhibitor (1 tablet/50 ml) (Roche)). The cells were disrupted by an EmulsiFlex-C5 (Avestin) cell disruptor. The lysate was centrifuged in Avanti J-26 XP centrifuge using JA-25.5 rotor at 21,000 rpm for 45 minutes at 4˚C. The lysate was filtered by 22 μm polypropylene filter (VWR) to remove the cell debris and insoluble aggregates. The purification was done using an AKTA Purifier (GE Healthcare).

Wild type full length Hsp90 was first purified on an IMAC POROS 20MC (Thermo Fischer Scientific) affinity purification column (solutions connected to pump A1 and A2: 50 mM Na-phosphate buffer pH 8.0 with 300 mM NaCl, B1: demi water with 10 mM β-mercaptoethanol, B2: 1 M imidazole (Sigma-Aldrich)). The eluted sample was diluted 4-fold with dilution buffer (25 mM Na-phosphate buffer pH 7.2, 5 mM DTT (Sigma-Aldrich), complete protease inhibitor (1 tablet/50 ml) (Roche)). Next, the sample was loaded on a POROS 20HQ anion exchange column (Thermo Fischer Scientific) (solutions connected to pump A1 and A2: 50 mM Na-phosphate pH 7.2, B1: demi water with 10 mM DTT, B2: 2 M KCl). The eluted sample was diluted 10-fold with dilution buffer (25 mM Na-phosphate buffer pH 7.2, 5 mM DTT, complete protease inhibitor (1 tablet/100 ml)). Finally, the sample was loaded on a HiTrap heparin affinity chromatography column (GE Healthcare) (solutions connected to pump A1 and A2: 50 mM Na-phosphate pH 7.2, B1: demi water with 10 mM DTT, B2: 2 M KCl) [21]. The eluate was concentrated and buffer exchanged to Hsp90 storage buffer (25 mM Na-phosphate pH 7.2, 150 mM NaCl, 150 mM KCl, 5 mM DTT, complete protease inhibitor (1 tablet/100 ml)) using a Vivaspin 20 column (50 kDa MWCO) (GE Healthcare) at 4˚C at 5000 rpm repeatedly for 15 minutes until above 100 μM protein concentration. The protein concentration was determined with ND-1000 program on an ND-1000 Spectrophotometer type NanoDrop using 57760 M⁻¹ cm⁻¹ extinction coefficient. The sample was frozen in liquid N₂ and stored at -80˚C.

Throughout the purification procedure samples were taken from the steps of the purification that were run on SDS-PAGE to analyse sample purity. The innovative step in our protocol is the inclusion of fast running POROS columns which allows for a one day/three columns purification protocol.

**Protein stability**

10 μM Hsp90 was incubated in Hsp90-buffer ((25 mM Na-phosphate pH 7.2, 150 mM NaCl, 150 mM KCl, 5 mM DTT, complete protease inhibitor (1 tablet/100 ml)) for 24 hours at 4˚C, room temperature (~21˚C) and 37˚C. SDS-PAGE samples were taken and mixed with 2x sample buffer (1.25 M Tris, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 5 mM freshly added DTT) at 0, 3, 18 and 24 hours. The samples were analysed on SDS-PAGE.

**Silver staining**

The Coomassie-stained and destained gels were fixed for 30 minutes in fixation solution (30% ethanol (Interchema Antonides-Interchema), 10% acetic acid, 60% demi water). The gel was washed 3-times for 20 minutes in 50% ethanol, then pre-treated for 1 minute with 0.02% Na₂S₂O₃ (Scharlau Chemicals) [100x diluted from stock (stock: 2 g into Na₂S₂O₃ 100 ml water (Milli-Q))] and quickly rinsed 4 times with demi water. The gels were impregnated for 20 minutes with freshly prepared staining solution (2 g/l AgNO₃ (Merck) and 0.75 ml/liter formaldehyde (Calbiochem)). Next they were quickly rinsed 4 times with demi water, then developed until the desired result in developer solution (60 g/l Na₂CO₃ (Sigma-Aldrich), 0.5 ml/liter formaldehyde and 0.0004% Na₂S₂O₃ stock). The reaction was stopped in fixation solution (30% ethanol, 10% acetic acid, 60% demi water) for 10 minutes. The gel was stored in 1% acetic acid solution and scanned by Epson Perfection V700 Photo scanner.
CD spectroscopy

The protein was centrifuged on 4°C for 15 minutes at 13,300 rpm in Heraeus Pico 17 centrifuge (Thermo Scientific). The concentration was determined with ND-1000 program on ND-1000 Spectrophotometer type NanoDrop using 57760 M⁻¹cm⁻¹ extinction coefficient. The protein was diluted to 0.1 g/l concentration with CD-buffer (25 mM Na-phosphate pH 7.2 buffer, 150 mM NaF (Sigma-Aldrich)), loaded into a Teflon-sealed, polarimetrically checked quartz glass cuvette with an optical path length of 1 mm and a volume of 350 μl (Hellma Analytics). Far-UV CD spectrum was measured with the Spectra Manager (Jasco) program on a Jasco J-810 Spectropolarimeter instrument (Jasco). Experimental parameters included a wavelength increment of 1 nm, a scan speed of 20 nm/min, a temperature of 20°C. The data were analysed by MS Excel.

SEC-MALLS

The protein sample was centrifuged and its concentration was measured as described for CD spectroscopy. 10 μl of ~29 g/l sample was run with Shimadzu on a Superdex 200 column with 0.35 ml/min speed in running buffer (25 mM Na-phosphate pH 7.2, 150 mM NaCl, 5 mM freshly added DTT). The 220 nm absorption was detected by SPD-20A UV detector (Shimadzu), light scattering by Wyatt COMET™ light scattering detector (Wyatt Technology) and the refractive index by the RID-10A refractive index detector (Shimadzu). The data were analysed by program Astra 6.

Analytical Ultracentrifugation (AUC)

The protein sample was centrifuged and its concentration was measured as described for CD spectroscopy. The protein sample was diluted to 7.2 μM concentration with Hsp90-buffer (25 Na-phosphate pH 7.2, 150 mM NaCl, 150 mM KCl, complete protease inhibitor (1 tablet/50 ml), 5 mM freshly added DTT). The sample was centrifuged on 20°C for 16 hours at 42,000 rpm in a Beckman XL-I ultracentrifuge using An60Ti rotor. We used absorbance detection optics for our experiment. The data was analysed by SedFit.

Results

Systematic testing of recombinant human Hsp90β overproduction

We set out to systematically test the effect of key parameters on recombinant human Hsp90β production in Rosetta 2 cells and to identify conditions resulting in high protein levels.

We outlined our study as follows: We cultured E. coli cells containing human Hsp90β open reading frame on a pET23a+ vector and tested media with different nutrient values and different temperatures of induction to modulate the metabolism of E. coli cells. We induced the cultures with different final concentrations of IPTG affecting the level of T7 polymerase. We also varied the length of induction and the OD₆₀₀ value at induction, because these may be essential parameters for successful overproduction.

At given time points we took samples from the cultures and analysed them by SDS-PAGE (Fig 1A). We loaded protein marker in the first, uninduced sample in the second and purified Hsp90 in the last lanes of the SDS-gels. These samples helped to identify the Hsp90 bands that appeared after induction. Lane 3–14 contained the induced samples of the different conditions tested. The most abundant intrinsic E. coli band that we later used for quantification (EF-Tu) and Hsp90 bands are marked on the right of the gels (Fig 1A) [22].

We determined the intensity profile of the uninduced (green) and induced lanes (orange) (Fig 1B, top panel) and normalised the induced lane profile to the uninduced lane profile using the EF-Tu peak of the uninduced lane as reference (100%). Subsequently, we aligned the
Fig 1. Experimental set up and quantification scheme for Hsp90 overproduction. (A) Schematic overview of the protein production experiment. E. coli cells containing human Hsp90β open reading frame were inoculated from glycerol stock and cultured overnight at 37°C. The culture was divided into small tubes and after induction the tubes were incubated in different conditions. Samples were taken...
induced lane profile (green) to the uninduced lane profile (orange) using the first two left peaks (stars) of the uninduced lane profile as reference points (Fig 1B, middle panel). We deducted the uninduced lane profile (orange) from the normalised and aligned induced lane profile (green) and calculated the sum of difference at the height of the Hsp90 peak (red area) (Fig 1B, lower panel). Finally, we divided the Hsp90 peak area by the area of the normalised and aligned EF-Tu peak area.

The yield of recombinant human Hsp90β protein in *E. coli* using LB medium was insufficient for preparative purposes

We tested Hsp90 overproduction in LB medium varying the length of induction, the temperature, the OD\textsubscript{600} at induction and the IPTG concentration to measure if any of these parameters affected the levels of the chaperone.

After one and three days of induction no Hsp90 band appeared in the induced samples, none of the conditions led to sufficient Hsp90 overproduction (Fig 2A and 2B, lane 3–14). When induced for five days an Hsp90 band was apparent in several conditions (Fig 2C, lane 4, 5, 11 and 12). However, the yield was still insufficient for preparative purposes and the production levels were variable.

**2x YT medium improved the yield of Hsp90 production**

Since protein production in LB resulted in poor yields we hypothesised that the nutrient value of the medium may be a critical parameter in case of Hsp90. Therefore, we repeated the overproduction experiment in the richer 2x YT medium. We varied the length of induction, the temperature, the OD\textsubscript{600} at induction and the IPTG concentration to determine if any these parameters affect Hsp90 production in 2x YT.

After one and three days of induction a new band appeared at the height of Hsp90 in the induced samples, suggesting that we could produce it (Fig 3A and 3B). The newly appearing Hsp90 band was stronger after three days of induction compared to one. Hsp90 production in 2x YT resulted in higher yields than in LB within the interval of the parameters we varied.

When induced for five days, we observed significant overproduction of Hsp90 (Fig 3C) in certain cases (lane 7–9), whereas other conditions led to similar results to those observed previously (lane 10, 12). Hsp90 production levels were variable in the different conditions. We concluded that Hsp90 overproduction resulted in higher yields in 2x YT after five days of induction compared to shorter induction times and culturing in LB medium, but the yields were still insufficient for preparative purposes.

**Recombinant human Hsp90β production had the best yield in the *E. coli* cells in TB medium**

Since the richer 2x YT medium had a positive effect on Hsp90 levels we decided to test the protein production in TB medium which is higher in nutrients than 2x YT. We varied the same parameters as described previously.
**Fig 2. Hsp90 overproduction in LB medium lead to insufficient protein yield.** Hsp90 overproduction after 1 day (A), 3 days (B), 5 days (C) of induction. Lane 1: protein marker, lane 2: uninduced sample, lane 3–8: protein production at 16˚C, lane 9–14: protein production at 18˚C, lane 3, 6, 9, 12: induction with 0.1 mM IPTG, lane 4, 7, 10, 13: induction with 0.25 mM IPTG, lane 5, 8, 11, 14: induction with 0.5 mM IPTG, lane 15: purified Hsp90 sample.

EF-Tu intrinsic E. coli protein band was used for quantification of Hsp90 overproduction.

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Fig 3. Hsp90 overproduction was improved but still insufficient in 2x YT compared to LB. Hsp90 overproduction after 1 day (A), 3 days (B), 5 days (C) of induction. Lane 1: protein marker, lane 2: uninduced sample, lane 3–8: protein production at 16˚C, lane 9–14: protein production at 18˚C, lane 3, 6, 9, 12: induction with 0.1 mM IPTG, lane 4, 7, 10, 13: induction with 0.25 mM IPTG, lane 5, 8, 11, 14: induction with 0.5 mM IPTG, lane 15:
After one day of induction, a strong band appeared at the height of Hsp90 in several conditions (Fig 4A, lane 3 and 11) indicating that overproduction in TB was notable. The conditions that resulted in high protein amount, however, were variable. Already after three days of induction in TB medium all the conditions tested showed a strong Hsp90 band (Fig 4B). We observed similar Hsp90 levels with five days of induction (Fig 4C). Thus, TB medium resulted in higher yields than both LB and 2x YT media, and after a shorter period of time.

Also in case of other proteins, some studies report higher protein production levels when changing media composition to richer [23–26]. The impact of medium composition depends, however, on the protein of interest, and must thus be tested for each protein [24]. Therefore, the trends observed here for Hsp90 production may not apply to unrelated proteins.

Quantification of overproduction gels revealed that the richness of the medium and the length of induction are the most significant parameters for Hsp90

To compare the protein production results of the different gels we estimated Hsp90 overproduction by gel densitometry as described in Fig 1B. In LB medium we observed low levels of Hsp90 overproduction in each condition (Fig 5A). The tendency improved with the length of induction but even after five days the yields were insufficient for preparative purposes in all conditions.

We noted similar trends in case of 2x YT medium, the level of protein overproduction was insufficient and had similar yields after one and three days of induction (Fig 5B). After five days of induction in 2x YT, however, Hsp90 overproduction was still insignificant and the other varied conditions did not improve Hsp90 levels in this medium either. Although, the yield was higher in every condition in comparison to LB.

Hsp90 overproduction in TB was significantly higher than in the other two media. The length of induction had a significant effect on the Hsp90 yields, and here we reached high yields after just three days. The other parameters tested did not influence the Hsp90 yield in TB.

Overall we concluded that the quantification of the gels in Figs 2, 3 and 4 confirmed our observations about the overproduction (Fig 5A, 5B and 5C, respectively). We found that of the parameters tested, medium nutrient content and the length of induction were the only two that had a significant effect on Hsp90 overproduction, and should be considered in the future. Temperature, OD$_{600}$ at induction and IPTG concentration used for induction did not significantly changed the yields of the chaperone within the tested intervals.

Recombinant human Hsp90β purified in one day

After successful protein overproduction, we set out to optimise a fast, efficient and trustworthy purification protocol to ensure excellent sample quality. This was necessary because like other multi-domain proteins, human Hsp90β is also prone to the degradation. Its accessible, flexible regions, especially the charged linker between the N-terminal domain and the middle domain is often targeted by proteases, causing N-terminal degradation. Widely used purification protocols include time-consuming purification steps (such as dialysis, size exclusion chromatography or both) that slow down the procedure which may have detrimental effects on protein quality [20,27–29].
**Fig 4. Hsp90 overproduction lead to the highest yields in TB medium.** Hsp90 overproduction after 1 day (A), 3 days (B), 5 days (C) of induction. Lane 1: protein marker, lane 2: uninduced sample, lane 3–8: protein production at 16°C, lane 9–14: protein production at 18°C; lane 3–5 and 9–11: induction at low OD
600, lane 6–8 and 12–14: induction at high OD
600; lane 3, 6, 9, 12: induction with 0.1 mM IPTG, lane 4, 7, 10, 13: induction with 0.25 mM IPTG, lane 5, 8, 11, 14: induction with 0.5 mM IPTG, lane 15: purified Hsp90 sample. EF-Tu intrinsic *E. coli* protein band was used for quantification of Hsp90 overproduction.

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Fig 5. Quantification of Hsp90 overproduction in different media. (A) Summary of the quantification of Hsp90 overproduction in LB, after 1 day (white), 3 days (grey) and 5 days (black) of induction. Average of
To avoid degradation that might occur as a consequence of long purification procedures and eventual freeze-thaw steps, we set out to develop a condensed protocol for purification of Hsp90 in one day that results in highly pure and stable sample. We purified N-terminally His\textsubscript{6}-tagged Hsp90 protein by Ni-affinity chromatography, anion exchange and heparin affinity chromatography (Fig 6A). To ensure sample quality and avoid degradation we carried out the experiment in the presence of protease inhibitors at low temperature (0–4˚C). To check for protein quality throughout the purification process analysed samples of the peak fractions of each column using SDS-PAGE (Fig 6B). We observed a gradual gain in purity and loss in degradation products and impurities. After the last step the new Hsp90 purification protocol resulted in a protein sample free of any significant contaminations detectable by Coomassie staining. The combination of the three columns was necessary for high sample purity and reproducibility.

Certain biochemical and biophysical experiments are carried out at a higher, physiological temperature (37˚C) and for elevated time intervals (days or months). In case of proteins that are sensitive to proteolysis, incubation at high temperature for longer time can be detrimental for sample quality. To test the stability of the Hsp90 sample purified by the new protocol we incubated the protein at 4, 21 and 37˚C and examined samples taken at 3, 18 and 24 hours by SDS-PAGE. We visualised Hsp90 in the gels first with Coomassie (Fig 7A) and subsequently, with more sensitive silver staining (Fig 7B).

No additional degradation products appeared in the incubated samples compared to the starting sample within the time frame of the experiment on the Coomassie-stained gels (Fig 7A). Therefore, we concluded that Hsp90 purified using the new purification protocol was stable if incubated at 37˚C up to 24 hours. Since Coomassie staining has limited sensitivity especially in the range of low molecular weight, we further examined the stability of the sample by silver staining (Fig 7B). Here, we observed impurities and/or degradation products in every sample but we did not see a systematic increase of any bands upon incubation at 37˚C up to 24 hours.

This newly optimised, reproducible purification protocol enabled us to prepare a highly pure, stable and homogeneous sample within one day.

**Hsp90 purified by our method was properly folded, had the correct molecular weight and sedimentation coefficient**

To reveal the folding status of proteins, we to analysed our Hsp90 samples with circular dichroism spectroscopy (CD) that can potentially reveal the secondary structure composition and folding status of the chaperone. The CD spectrum revealed that Hsp90 is mainly composed of α-helices (Fig 8A). We observed two minima at ~209 nm and at ~222 nm and a maximum at ~194 nm in the spectrum which is in agreement with the previous findings [28].

To ensure that our Hsp90 sample had the correct molecular weight and did not degrade during the purification process we measured its molecular weight using size exclusion...
We observed a homogeneous peak at 10.07 ml that was fitted to 167.5 ± 13.3 kDa. This result corroborates with the expected size of two Hsp90 molecules (168.2 kDa), confirming that our sample is in a dimeric state at the concentration used in the experiment and also in agreement with data published earlier [30,31]. Taken together with the outcome of the stability experiments SEC-MALLS results suggested that our sample contained the intact full length Hsp90 dimer without degradation.

We measured the sedimentation coefficient of our Hsp90 sample to analyse sedimentation properties of the Hsp90 dimer. In the sedimentation profile of the chaperone we observed a
peak at 5.6 S that corresponded to the dimer and a smaller peak at 10.6 S that was probably the tetramer molecule (dimer of dimers) (Fig 8C) [32]. The increasing fraction above 14 S suggested that the sample aggregated to some extent (but this is a typical phenomenon in case of analytical ultracentrifugation (AUC) samples). Below 1 S we observed a small peak, that could have originated from impurities or degradation products.

**Fig 7.** Purified Hsp90 sample incubated at physiological temperature for one day remained free of degradation products. (A) SDS-PAGE about the stability of the purified Hsp90 sample. Protein marker (lane 1), Hsp90 incubated for 0 hours (lane 2), Hsp90 incubated at 4°C (lane 3–5), 21°C (lane 6–8), 37°C (lane 9–11), Hsp90 incubated for 3 hours (lane 3, 6, 9), 18 hours (lane 4, 7, 10), 24 hours (lane 5, 8, 11). (B) Silver staining about the stability of the purified Hsp90 sample. Protein marker (lane 1), Hsp90 incubated for 0 hours (lane 2), Hsp90 incubated at 4°C (lane 3–5), 21°C (lane 6–8), 37°C (lane 9–11), Hsp90 incubated for 3 hours (lane 3, 6, 9), 18 hours (lane 4, 7, 10), 24 hours (lane 5, 8, 11).
Fig 8. Purified Hsp90 sample folded, sedimented correctly and its molecular mass was appropriate. (A) CD spectrum of Hsp90 at 1.2 μM monomer concentration. The overall structure of the protein typically
Discussion

Human Hsp90β is often produced in baculovirus system in insect Sf9 cells that is expensive, requires long preparation steps and is often difficult to scale-up [33–35]. Our optimised recombinant human Hsp90β production protocol in \textit{E. coli} provides an attractive alternative to the baculovirus system.

First, we systematically tested the production of recombinant human Hsp90β in \textit{E. coli} cells to identify parameters that modulate the yield of the chaperone. We showed that the length of induction and the nutrient level of the medium had significant effect on Hsp90 overproduction, whereas temperature, IPTG concentration and OD$_{600}$ at induction (within the tested intervals) did not significantly affect the yields of Hsp90. Induction for three days in TB medium resulted in good yield of the chaperone.

Moreover, we purified Hsp90 using a protocol that allowed us to finish within one day and resulted in well-folded, stable and pure sample. Since Hsp90 is extremely sensitive to proteolysis because of its flexible linkers, fast preparation increases sample quality, in addition to being cheaper and time-efficient. Throughout the purification procedure impurities and degradation products disappeared from the sample and final product resisted further degradation upon incubation on elevated temperature for long time intervals.

Here we used an Hsp90 construct with a non-cleavable N-terminal His$_6$-tag. The His$_6$-tag is small and is connected via a very short linker. It did not affect the secondary structure (Fig 8A) and C-terminal dimerisation (Fig 8B and 8C). Earlier studies showed that such a short tag does not change functional or biophysical properties of Hsp90 [16,36]. Therefore, we anticipate that the presence of the His$_6$-tag does not cause a difference in most biochemical and biophysical experiments.

Previous Hsp90 purification protocols often contained time consuming preparation steps such as dialysis, size exclusion chromatography or both that may affect protein quality [20,27–29]. Using the combination of Ni-affinity chromatography, anion exchange and the Hsp90-specific heparin column allowed us to get rid of degradation products and impurities in only three purification steps. The resulting sample was sufficiently pure and resistant to proteolytic degradation upon incubation at physiological temperature for up to one day, and showed expected biophysical properties (CD, SEC-MALLS).

In our AUC experiments human Hsp90 sedimented predominantly as a dimer with S = 5.6 and a small fraction as a tetramer with S = 10.6. It is notable that human Hsp90 is known to be in extended conformation (D$_{max}$ = 26 nm) in the absence of nucleotides and co-chaperone p23 [36]. In account with our results, earlier findings showed that in the absence of nucleotides, and thus in its extended conformation, yeast Hsp90 also had a sedimentation coefficient of 5.6 [32]. These results indicate that these two proteins sediment similarly when in extended conformation. However, in the presence of ATP that stabilises compact conformation, yeast Hsp90 sedimented with the coefficient of 6.8, suggesting that structural rearrangements altered its sedimentation properties [32]. In similar experiments Hsp90 sedimented with the coefficient of 6.1 [37]. The variations between these results may be explained by the differences between the origin of the samples (porcine brain Hsp90 that has different post-translational
modification pattern and was a mixture of α and β isoform vs human Hsp90β), or, by buffer, salt and reducing agent conditions.

In summary, we optimised Hsp90 overproduction and developed a fast and efficient purification protocol for the protein. The resulting sample was pure, properly folded and resistant to degradation, and therefore it is suitable for biochemical and biophysical experiments.

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