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High-throughput protein purification using an automated set-up for high-yield affinity chromatography

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Abstract

One of the key steps in high-throughput protein production is protein purification. A newly developed high-yield protein purification and isolation method for laboratory scale use is presented. This procedure allows fully automated purification of up to 60 cell lysates with milligram yields of pure recombinant protein in 18.5 h. The method is based on affinity chromatography and has been set up on an instrument that utilizes positive pressure for liquid transfer through columns. A protocol is presented that includes all steps of equilibration of the chromatography resin, load of sample, wash, and elution without any manual handling steps. In contrast to most existing high-throughput protein purification procedures, positive pressure is used for liquid transfer rather than vacuum. Positive pressure and individual pumps for each liquid channel contribute to controlled flow rates and eliminate the risk of introducing air in the chromatography resin and therefore ensure stable chromatography conditions. The procedure is highly reproducible and allows for high protein yield and purity.

Keywords: High-throughput; Protein purification; IMAC; Recombinant protein; Proteomics

Characterization and functional analysis of proteins have become an important research focus in life sciences after the completion of genome sequencing projects and the advent of the field of proteomics in the last decade. One of the major bottlenecks in proteomic studies is protein purification of large number of samples. As the need for high-throughput characterization and production of pure proteins increase, parallel and automated protein purification methods are required. Consequently, protein purification applications have been developed for several different molecular biology workstations with the capacity of multi-sample handling [1–5]. All these applications lack detector systems for sample monitoring, but have the capacity for parallel processing to achieve a high sample throughput. However, these robotic workstations are expensive and often spacious. There are also chromatography systems with multi-sample handling as well as detection systems, but these are limited to a smaller amount of samples [6].

Affinity chromatography is frequently used for projects handling large numbers of samples because of the high selectivity of the technique. The most commonly used method is immobilized metal affinity chromatography (IMAC),[1] where poly-histidine tags are used as purification handles [7]. Protein purification using magnetic beads coated with an IMAC-matrix are efficient and easy to automate but best suited for small-scale screening as the total yield of protein is low, approximately 15 μg [1,3]. For studies that require more protein, i.e., crystallization

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[1] *Abbreviations used:* IMAC, immobilized metal affinity chromatography; His_6ABP, Hexa-histidine albumin binding protein; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

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experiments or antibody generation, protein purification procedures with higher capacity levels are necessary. Several high-throughput protein purification systems with higher yields are reported where the chromatography resin is packed in individual columns or in wells in a microtiter-format and where buffers are extracted either by vacuum [5] or centrifugation [4,8]. For these systems the yield is mainly limited by the amount of chromatography resin the well or column can hold. The use of vacuum or centrifugation for liquid transfer enables efficient handling of large sample sets, but gives low control of the flow rate in each sample and this might affect the purification result and reproducibility.

Here, the challenge to purify nearly two hundred proteins from lysates of different cell cultures per week, and to acquire milligram amounts of pure recombinant protein, was overcome with the development of a fully automated protein purification protocol. This protocol has been set up using a commercially available instrument based on positive pressure over columns packed with an IMAC chromatography resin. The advantage over existing high capacity and throughput methods is the combination of positive pressure extraction and a relatively inexpensive laboratory scale instrument with high yield and reproducibility.

Materials and methods

Protein expression

Hexa-histidine albumin binding protein (His_6ABP) and different target proteins with His_6ABP as an N-terminal fusion partner were expressed in *Escherichia coli* BL21(DE3) cells [9]. The target proteins were 100–150 amino acid long fragments without transmembrane regions from different human proteins. One milliliter of overnight culture in tryptic soy broth (Merck), 30 g/L, supplemented with 5 g/L yeast extract (Merck) and 50 μg/mL kanamycin (Sigma–Aldrich), were used to inoculate 100 mL of identical culture media in 1 L shake Erlenmeyer-flasks. Cultures were incubated at 37 °C until A_{600} exceeded 0.5. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Appollo Scientific Ltd.) to a final concentration of 1.0 mM. After induction, the cultures were incubated at 25 °C over night. The cells were harvested by centrifugation at 2400*g* and the cell pellet was resuspended in 5 mL denaturing lysis buffer (7 M guanidiumchloride, 47 mM Na_2HPO_4, 2.65 mM NaH_2PO_4, 10 mM Tris–HCl, 100 mM NaCl, and 20 mM β-mercaptoethanol, pH 8.0) and incubated at 37 °C for 2 h. Cells lysed under non-denaturing conditions were freezed at −20 °C and thawed before addition of 5 mL native buffer (47 mM Na_2HPO_4, 2.65 mM NaH_2PO_4, and 300 mM NaCl, 0.1% Tween-20, pH 8.0) supplemented with 0.75 mg/mL lysozyme (Sigma) and incubated for 30 min at room temperature. Cell debris was removed through centrifugation of the lysates at 35,300*g*.

Automated protein purification

The automated protein purification procedure was set up on an ASPEC XL4 four-probe SPE system controlled by 735 Sampler software 5.2 (Gilson Inc.). This instrument consists of three parts, a pump unit, a robotic arm with tubing connected to the pump unit, and a working table. It can handle transfer of liquid samples on the working table, as well as the addition of up to nine different solutions with its four parallel syringe pumps (Fig. 1A). A range of racks are available for configuration of the working table for the particular task at hand. A schematic overview of the sample working table used here is shown in Fig. 1B. To allow for cold keeping of cell lysates and eluates during runs, the working table was equipped with two racks with thermostated blocks that can hold up to 60 tubes at temperatures from 4 to 40 °C. The thermostated blocks were custom-made from standard blocks for 2 mL vials by drilling the vial-holes deeper and wider to instead

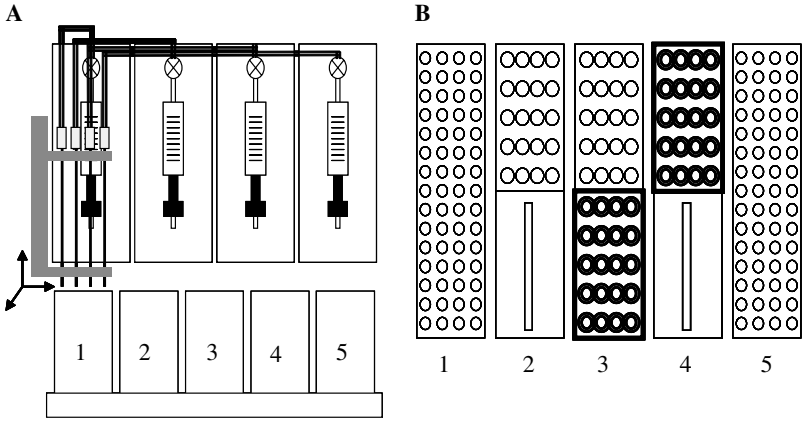

Fig. 1. The instrument set-up. (A) Front view of pump system and working table with sample racks 1–5. (B) Top view of working table. Each of the four syringe pumps are connected to the system buffer and a steel-probe mounted on a robotic arm above the working table. The different buffers are collected by the probes from reservoirs behind the racks (not shown in figure). Racks 1 and 5 holds thermostated blocks for 60 tubes for sample (1) and eluate (5). Sliding column trays above a waste and eluate collect zone constitutes the middle racks on which the trays are moved into position with the robotic arm. Rack 2 is displayed without the column tray, 3 with the column tray in waste position, and 4 in eluate collect position.

accommodate 5 mL tubes. Furthermore, the working table includes three chromatography racks with sliding column trays for disposable columns above a waste and eluate collecting zone. Four individual pumps connected to steel probes on the robotic arm work in parallel to pump the different buffers to the columns and transfer samples onto the columns and from the eluate collecting tubes. To ensure positive pressure, a cap (Argonaut Technologies) was put onto the columns to seal the probe during liquid transfer. Three milliliter columns (Argonaut Technologies) were used filled with 1 mL Talon metal affinity resin (BD Biosciences) with a 20 μm bottom filter and a 90 μm top filter. The top filter ensures that the resin remains fully saturated with buffer even as the probes are disconnected from the columns during sample and buffer transfer. In the final protein purification protocol, the IMAC columns were first equilibrated with 20 mL denaturing washing buffer (6 M guanidiumchloride, 46.6 mM Na_2HPO_4, 3.4 mM NaH_2PO_4, and 300 mM NaCl, pH 8.0–8.2) or native buffer, with a flow rate of 2 mL/min. Clarified cell lysates were then added to the columns at a flow rate of 0.35 mL/min and the resins were washed with denaturing or native washing buffer with an initial step at a lower flow rate (0.25 mL/min for 1.5 mL, 2 mL/min for 30 mL). Elution of purified protein was achieved with 2.5 mL denaturing elution buffer (6 M urea, 50 mM NaH_2PO_4, 100 mM NaCl, 30 mM acetic acid, and 70 mM Na-acetate, pH 5.0) or native elution buffer (47 mM Na_2HPO_4, 2.65 mM NaH_2PO_4, 300 mM NaCl, 0.1% Tween-20, and 250 mM imidazole, pH 8.0) at 2 mL/min and the eluates were transferred from the collect zone to the pre-cooled rack by the four probes. Prior to starting over with the next set of four samples the probes were thoroughly cleaned with 10 mL phosphate-buffered saline (PBS) with 0.1% Tween-20 (1.9 mM NaH_2PO_4, 8.1 mM $NaHPO_4$, and 154 mM NaCl, 0.1% Tween-20) and thereafter 20 mL deionized water. When analyzing the amount of sample-to-sample carry-over through the probes, an extra 5 mL fraction of system water was eluted by the probes and analyzed by Western blot.

Analytical methods

Protein samples were analyzed by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS–PAGE). The whole cell extracts and purified proteins were separated on 12.5 or 10–20% Criterion Precast Gel (Bio-Rad Laboratories) and stained with GelCode Blue Stain Reagent (Pierce) according to the manufacturer's recommendations.

After SDS–PAGE separation, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Criterion Gel Blotting Sandwiches, Bio-Rad Laboratories) according to the manufacturer's recommendations. The membrane was incubated over night at 4 °C in a blocking solution of 5% skimmed milk (Semper) in PBS containing 0.05% Tween-20 (PBST). After blocking, the membrane was incubated 1 h in room temperature in blocking buffer containing 0.38 μg/mL rabbit-α-His_6ABP [10]. Following wash with PBST, 8 ng/mL horseradish peroxidase-conjugated goat-α-rabbit IgG (Pierce) in PBST was added for one hour. After incubation the membrane was washed four times for 5 min in PBST. Super Signal West-Dura extended duration substrate system (Pierce) was used and the blotted proteins were detected in a ChemiDoc CCD camera (Bio-Rad Laboratories).

Protein concentrations were determined using the bicinchoninic acid (BCA) kit (Pierce), micro assay protocol, which utilizes a bovine serum albumin standard. The purity of the samples was analyzed by Bioanalyzer protein 50 chip (Agilent Technologies).

Results

Development of high-throughput protein purification systems has become a necessity to realize the goals of many proteomics projects. Here, the challenge has been countered by automation on a bench-top instrument developed for solid phase extraction (Fig. 1). With this instrument and the developed protocol, the whole procedure from clarified *E. coli* cell lysates to isolated pure proteins, is fully automated with no manual handling steps or surveillance. Equipping the working table with two cooling blocks, modified to accomodate 5 mL tubes, renders it possible to keep the whole cell extract and the purified proteins at low temperatures. The four-probes of the instrument allows for parallel processing of four samples in individual columns.

In Table 1, the general outline of the developed purification protocol is presented along with the time consumption for each step. In the first step the IMAC resin was equilibrated with IMAC wash buffer. Including the resin equilibration in the automated procedure allows the columns to be packed in its storage buffer, thus simplifying the manual handling of the columns. However, if time on the instrument is a limiting factor, equilibration of the matrix can be done beforehand and the automated purification time can thus be reduced by 19%. The following step of sample loading of clarified cell lysates, as well as the initial part of the subsequent column wash, was done at a reduced flow rate to make optimal protein binding to the resin possible. Loading the samples to the column is the second most time consuming step of the protocol, 28% of total time, but increasing the load rate significantly decreases the efficiency of target protein binding (data not shown). The most time

Table 1
Protocol for protein purification on ASPEC XL4 workstation

Steps	Time/cycle
1. Equilibration of the affinity resin with wash buffer	14 min (19%)
2. Slow addition of clarified lysate to the resin	21 min (28%)
3. Column wash to remove unspecific proteins	28 min (38%)
4. Elution of the target protein through addition of elution buffer	3 min (4%)
5. Transfer of the recombinant proteins to pre-cooled tubes from the collect zone	1 min (1%)
6. System wash	7 min (10%)
7. Repetition of next four samples	
Time consumption for four samples	74 min

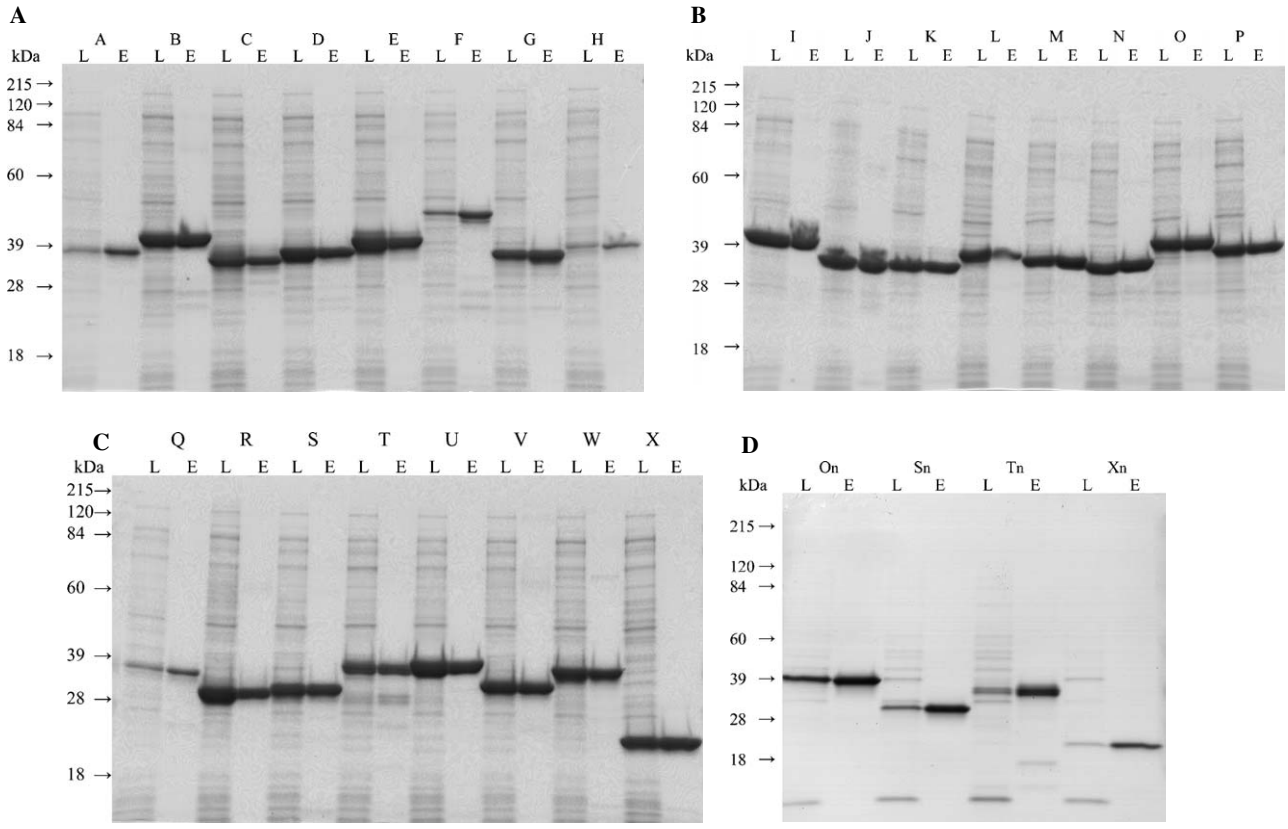

Fig. 2. High-yield protein purification of His-tag proteins under denaturing (24 samples) and native (4 samples) conditions. Clarified lysate (L) and eluate (E) pair-wise. 15 μL of each sample is analyzed and the lysate is a 1:20 dilution and the eluate a 1:10 dilution to give comparable data. (A–C) Denaturing conditions; (D) native conditions. The A-X naming of the proteins correspond to the accession numbers in Table 2.

consuming step of the purification, 38% of the total time, was the washing of columns to remove unwanted cellular proteins. Elution of target protein and the transfer of the purified protein samples to tubes in the second temperature controlled block on the working table is a relatively quick final step before a thorough system wash of the steel probes ending the first cycle of four samples. Going through the whole procedure of column equilibration, sample loading, column washing and elution in one cycle minimizes the required washing steps and the time the samples are exposed to room temperature. During the purification process the dispense speed through the columns can be limiting as it may result in an overpressure exceeding the recommended maximum pressure for the resin or the limit of the column cap. In the presented configuration the instrument can handle up to 60 individual samples and columns with elution in one fraction or up to 15 different elution fractions for a set of four samples (Fig. 1). The number of samples or elution fractions is easily set when building the method for a particular application in the software. With the current protocol one purification cycle of four samples takes 1 h and 15 min, and for example a purification of 28 samples in two fractions takes about nine hours and is thus ideal for over night purification. The total time can be reduced by using pressure-durable resins and by equilibrating the resin before packing the column.

In Fig. 2 and Table 2, the results from a purification under denaturing conditions of a set of 24 different proteins of varying expression yields are shown. Four of the proteins were also lysed and purified with native buffers. Purification gave protein of high purity and yields up to 13 mg, which agrees well with the 5–10 mg/mL capacity of the chromatography resin stated by the manufacturer. Furthermore, the purification protocol was shown to be highly reproducible through the purification of five identical cell lysates for four different proteins (Table 2).

A concern with any technique using the same probes and tubing for multiple samples is the risk of sample-to-sample carry-over. A thorough wash of the probes between different samples is therefore of greatest importance. To investigate the amount of protein left in the tubes after the system wash, an additional fraction of water was collected from each of the steel probes and examined on western blot (Fig. 3). By comparison with a protein dilution series of known concentrations it was concluded that the protein carry-over was less than 5 μg/mL.

Discussion

Generation of large sets of pure protein samples in sufficient quantities for study has become an important issue in many proteomic research projects. For instance,

Table 2
Results from automated IMAC protein purification under denaturing and native conditions of His-tag proteins

Protein	Description[a]	MW[b] (kDa)	Amount (mg)	Purity (%)
A	Q6ICM1	31.3	4.7	95.0
B	Q99538	34.4	13	96.8
C	P15090	32.0	7.3	96.5
D	Q14624	33.6	9.3	99.2
E	P36776	35.0	7.3	100
F	P12259	32.1	5.8	92.6
G	Q9HB21	28.7	8.2	98.5
H	Q9BX97	32.5	2.7	100
I	Q12794	34.2	10	100
J	P00352	30.6	8.6	93.9
K	P01106	31.3	8.6	100
L	P16109	32.6	3.6	100
M	Q5VYQ9	32.8	8.5	100
N	P48643	30.6	7.9	100
O	P27797	32.8	8.1	98.9
P	Q86W92	31.0	7.2	100
Q	Q8ND30	28.4	3.7	94.4
R	P78543	29.2	8.0	100
S	Q9Y681	26.6	7.2	93.7
T	Q9UGK3	28.4	6.6	79.4
U[c]	P08133	30.7	6.1 ± 0.21	87.2 ± 1.64
V[c]	P62324	29.9	3.0 ± 0.25	97.9 ± 3.08
W[c]	Q8N9L9	33.9	2.3 ± 0.10	98.7 ± 1.85
X[c]	His_6ABP	19.3	5.6 ± 0.25	87.9 ± 4.55
O_n	P27797	32.8	7.9	100
S_n	Q9Y681	26.6	3.7	100
T_n	Q9UGK3	28.4	4.3	81.1
X_n	His_6ABP	19.3	2.6	77.8

The amount of protein was determined by using the bicinchoninic acid (BCA) kit (Pierce), micro assay protocol, which utilizes a bovine serum albumin standard. The purity of the samples was analyzed by Bioanalyzer protein 50 chip (Agilent Technologies). Subscript 'n' for proteins O, S, T, and X denote purification under native conditions.

[a] Accession numbers from Uniprot.

[b] Molecular weight of the protein fragments including the His_6ABP-tag (17.7 kDa).

[c] Mean values ± SD from five purifications with identical cell lysate samples.

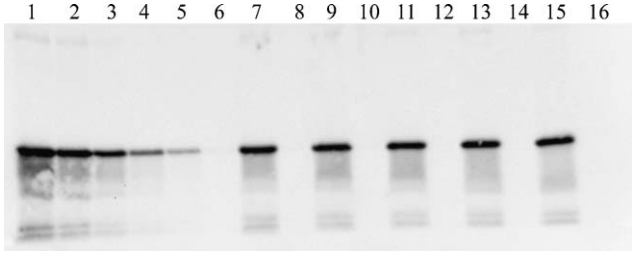

Fig. 3. Western blot analysis of sample-to-sample contamination carry-over. Five samples of His_6ABP were purified according to the protocol, with an additional water fraction sampled from the steel probes after the system wash. 10 μL of the additional fraction and the purified protein were analyzed on western blot and compared to a His_6ABP standard of known concentration. Lane 1, 80 μg/mL; lane 2, 40 μg/mL; lane 3, 20 μg/mL; lane 4, 10 μg/mL; lane 5, 5 μg/mL; lane 6, 1 μg/mL. Lanes 7–16, pairwise eluate sample (diluted 1:100) and corresponding test sample (undiluted). No sample carry-over could be detected.

milligram amounts of pure recombinant protein are required to generate specific polyclonal antibodies to human proteins. The developed method is used for protein purification within the Swedish Human Proteome Resource (HPR) project. The HPR-project generates a large number of antigens for the generation of monospecific polyclonal antibodies, directed against human proteins [11]. Fragments of human proteins with low homology to other proteins within the human proteome, and without membrane-spanning parts are chosen as antigens to induce immunogenic response. The protein fragments are produced in fusion with a His_6-tag used for purification. In this antibody proteomics project, one to two hundred protein purifications per week are performed to acquire the protein needed for antibody generation, and as affinity ligands for antibody purification. Therefore, the need for a high-throughput protein purification system is obvious.

The main advantage of the purification protocol and method demonstrated here is the combination of a conveniently sized bench-top instrument and the high yield and reproducibility afforded by the precise and accurate control of flow rates achieved by the positive pressure. In contrast to many other protein purification strategies which utilize vacuum or centrifugation, this set-up eliminates the risk of introducing air into the chromatography media that may result in poor chromatography as well as foaming. The precise control of the flow rate is achieved by the individual pumps connected to each probe, which furthermore ensures that blockage of one column will not interfere with chromatography of any other sample. Purification methods utilizing positive pressure have previously been presented using a microplate format [2]. However, the microtiter format gives a high throughput, but sets limitations in sample and resin volumes and is thus better suited for moderate yield applications of the order of hundreds of micrograms of protein, or for screening purposes. In the presented method cell lysate volumes of 5 mL has been used, but with minor reconfiguration of the instrument set-up 25 mL cell lysate samples can be handled. Another advantage is the larger volumes of chromatography matrix that can be used. In this protocol 1 mL resin has been used, but up to 3 mL can be packed in the columns used here, which would allow even higher yields. Adjusting the configuration of racks on the working table to hold 6 mL columns is also possible.

Within the HPR-project around 4500 cell lysates was purified during 2005 using this set-up. The method as well as the instrument has proven to be very robust and reliable. The instrument can be run over night without attendance, and as the lysate and the eluate samples are kept cooled there is no risk of thermal degradation. Moreover, the use of positive pressure allows high-chromatographic performance for each sample. Hence reproducibility, milligram yield and purity of the samples make this set-up ideal for high-throughput IMAC-chromatography. However, it should also be applicable to any other chromatographic techniques for which a common buffer protocol can be set for all samples.

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