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Preparative parallel protein purification (P4)

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Abstract

In state of the art drug discovery, it is essential to gain structural information of pharmacologically relevant proteins. Increasing the output of novel protein structures requires improved preparative methods for high throughput (HT) protein purification. Currently, most HT platforms are limited to small-scale and available technology for increasing throughput at larger scales is scarce. We have adapted a 10-channel parallel flash chromatography system for protein purification applications. The system enables us to perform 10 different purifications in parallel with individual gradients and UV monitoring. Typical protein purification applications were set up. Methods for ion exchange chromatography were developed for different sample proteins and columns. Affinity chromatography was optimized for His-tagged proteins using metal chelating media and buffer exchange by gel filtration was also tested. The results from the present system were comparable, with respect to resolution and reproducibility, with those from control experiments on an ÄKTA purifier system. Finally, lysates from 10 *E. coli* cultures expressing different His-tagged proteins were subjected to a three-step parallel purification procedure, combining the above-mentioned procedures. Nine proteins were successfully purified whereas one failed probably due to lack of expression.

Keywords: Automation; Protein purification; Affinity chromatography

1. Introduction

The structural analysis of pharmacologically relevant target proteins is one of the key elements in the drug discovery process [1]. Therefore, the demand to supply large numbers of different proteins at the right quantity and quality has dramatically increased. This can only be met by high throughput (HT) approaches. One prerequisite is the availability of complete genomic sequences of human and many other species, which set the molecular basis for recombinant expression of proteins [2]. Another important attribute is the possibility to fuse the recombinant protein to tags, thus enabling simplified and uniform affinity purification [3,4]. The process of protein expression and purification has been automated either in modules focusing on liquid handling robots [5–7] or as fully integrated platforms [8]. Currently, most HT platforms use the micro-titre plate (MTP) format and are therefore limited to small-scale not being able to deliver the amounts required for crystallography. Those approaches based on standard robots also suffer from drawbacks such as the absence of online UV monitoring and gradient forming capabilities. Often, negative pressure in form of vacuum as driving force is applied, thus deteriorating the flow characteristics and leading to poor resolution. Recently, some improvements of parallel protein purification have been described. In one case, a 24-channel manifold initially designed for solid phase extraction was applied for antibody purification [9]. In another example, gravity flow was driving a home-made 24-channel device [10]. The currently most integrated system, which is based on a programmable single channel LC system containing features like gradient forming and UV monitoring, is capable to perform multi-step purifications from up to six samples without user interference [11]. Further development of that device led to the integration of up to 12 modules to obtain a true parallel purification system. So far, multi-channel systems only existed for analytical purposes [12]. Here, we describe the modification of a 10-channel parallel flash chromatography system (CombiFlash OptiX 10, Isco Inc.) for automated parallel protein purification applications. This system enables us to

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perform 10 different purifications in parallel with individual gradients and UV monitoring. We present data from typical applications like capture steps using affinity chromatography, polishing steps with ion exchange chromatography and desalting/buffer exchange by gel filtration.

2. Experimental

2.1. System and modifications

All parallel purifications were performed with the CombiFlash OptiX 10 system (Isco Inc., Lincoln, USA) equipped with the X-Y fraction collector Foxy 200 and controlled by the PeakTrak software. This instrument features 10 separate channels for parallel flash chromatography with individual gradient formation and UV monitoring. The OptiX 10 instrument was modified in a number of ways to facilitate typical protein purification applications. The minimal flow rate was lowered from 5 to 1 mL min^{-1} by the Isco engineers and the UV flow cell was exchanged to one with a path length of 4 mm (standard is 0.2 mm). The metal plate covering the back unit of the OptiX 10 was replaced with Plexiglas to enable monitoring the air removed from the pump cylinders. The system was further stripped from sample loading parts, except for the manual four-way injection valves to which the MCP Standard multi-channel peristaltic pump (Ismatec, Glattbrugg, Switzerland) was added. The peristaltic pump was set up with a 12-cassette multi-channel pump head (CA 12) with 1.02 mm i.d. Tygon tubing capable of delivering flow rates between 0.13 and 31 mL min^{-1}. All external tubing fittings were replaced with Luer or M6 connectors and union adaptors.

2.2. Chromatographic columns and media

Five millilitres and 1 mL HiTrap CM FF, HiTrap DEAE FF, HiTrap Q FF, HiTrap SP FF, HiTrap SP HP ion exchange columns and HiPrep 26/10 Desalting columns were used (Amersham Biosciences, Uppsala, Sweden). Affinity chromatography was performed with Ni-NTA superflow resin (Qiagen, Hilden, Germany) either in pre-packed 1 and 5 mL Ni-NTA cartridges (IBA, Göttingen, Germany) or columns where 7 mL Ni-NTA was packed in solid sample cartridges (Isco Inc., Lincoln, USA). The latter were packed with approximately 3 mL of headspace above the settled bed and the columns were connected to the system using the sample load plungers from the OptiX system.

2.3. Chemicals and sample proteins

All chemicals were purchased from Sigma (St. Louis, Missouri, USA) with the exception of imidazole (Merck, Darmstadt, Germany). Aldolase, conalbumin, chymotrypsinogen, lysozyme and trypsin inhibitor from various suppliers were used as sample proteins.

2.4. Chromatographic procedures

2.4.1. System preparations

Prior to chromatography, the system was primed at different flow rates and stroke volumes, first with water for at least 10 min, and then buffer A (20 mM Tris pH 8.0) for 2 min followed by recirculating buffer A for at least 10 min. This was done in order to equilibrate the entire system with the proper buffer and to remove residual air from the pump cylinders and tubing. Air bubbles could also be avoided by degassing the solutions in vacuum and by using autoclaved water for buffer preparation.

2.4.2. Ion exchange chromatography

Ion exchange columns were connected to different channels of the OptiX 10 system. After equilibration of the columns with at least five-column volumes of buffer A (20 mM Tris pH 8.0), the different samples were loaded onto the corresponding column using the peristaltic pump at 5 mL min^{-1}. When the peristaltic pump is used, the system pump has to be run simultaneously in order to monitor UV absorbance and to activate the fraction collector. The subsequent washing step (five-column volumes) was also performed using the peristaltic pump at the same flow rate. After the wash step, the elution program was started. All channels were programmed with the same gradient, from 0 to 35% buffer B (20 mM Tris pH 8.0, 1 M NaCl) in 30 min with a hold at 35% buffer B for 3 min and a last step 90% buffer B for 4 min. Five millilitres fractions were collected in the fraction collector throughout the whole elution program. Both sample loading and elution steps were on-line monitored spectrophotometrically at 280 nm.

Control experiments were performed on an ÄKTA purifier (Amersham Biosciences, Uppsala, Sweden) using the same protein samples, buffers and gradient profile but 1 mL HiTrap columns.

2.4.3. Immobilized metal chelate affinity chromatography (IMAC)

Pre-packed 5 mL Ni-NTA superflow columns (IBA, Göttingen, Germany) and columns packed in solid sample cartridges with 7 mL Ni-NTA superflow (Qiagen, Hilden, Germany) were connected to the appropriate channels. After equilibration with buffer A (20 mM Tris pH 8.0, 200 mM NaCl), each column was loaded with *E. coli* cell lysate containing a recombinant 6× His-tagged protein using the peristaltic pump at a flow rate of 5 mL min^{-1}. Next, a wash step with at least 40 mL buffer A was performed, again using the peristaltic pump at 5 mL min^{-1}. Subsequently, the elution program was started, with two wash steps 5% buffer B (20 mM Tris pH 8.0, 200 mM, NaCl 200 mM imidazole) for 10 min, 15% buffer B for 13 min, prior to the protein elution step with 100% buffer B for 15 min. The flow rate during the whole elution program was 5 mL min^{-1}. Fractions of 5 mL each were collected and A_{280} was monitored during the entire chromatographic procedure.

2.4.4. Desalting

HiPrep 26/10 columns pre-packed with 50 mL Sephadex G-25 Fine were connected to the system and equilibrated with 1.5-column volumes of buffer. Equilibration, sample load and elution were run using the peristaltic pump, with the system pump running to waste in order to activate the UV monitoring and fraction collection. A flow rate of 5 mL min^{-1} for the peristaltic pump was used and 5 mL fractions were collected. A sample volume of 15 mL was applied and the desalted protein eluted in the next 20 mL. The conductivity of each fraction was measured off-line using a conductivity meter (CDM210 Radiometer, Copenhagen, Denmark) to confirm that the salt fraction elutes after the protein peak.

2.5. Analysis

Samples were analysed by SDS–PAGE using either NuPAGE 10% BisTris gels (Invitrogen, Carlsbad, CA, USA) or Criterion XT 4–12% BisTris gels (BioRad, Helcules, CA, USA) run with MES buffer according to instructions of the manufacturers and visualized with Coomassie staining [13]. Protein concentration was determined spectrophotometrically at 280 nm or with the BioRad protein assay (BioRad, Helcules, CA, USA) standardized with bovine serum albumin.

3. Results and discussion

Automation of protein expression and purification with the aims of selecting high expressing constructs, finding soluble protein conformations and screening for potential candidates for structural determination have been widely described [1,3,5–8]. The majority of these platforms utilize the MTP format and therefore, culture volumes and subsequent yields are relatively small. Up-scaling is then traditionally performed using laborious sequential methods. We are currently developing a platform for preparative parallel protein purification (P4) and in this report, we describe typical protein purification applications, which we have set up on a modified 10-channel flash chromatography system. This instrument is driven by a 10-channel piston pump, which can deliver pump strokes from 5 to 18 mL per channel before it has to be refilled. The gradients, which can be different for each channel, are formed within the individual pump cylinders and the gradient steps are hence dependent on the stroke volumes. Ten fast-switching valves control the individual proportions of A and B buffers introduced into each cylinder during pump refill. We also use a multi-channel peristaltic pump for sample loading, as well as for certain isocratic steps. The two separate pumps connect via 10 manual four-way valves onto the system flow path, directing the flow either onto the columns or to waste. The flow from each column then passes a UV detector where a single deuterium lamp and monochromator deliver the light via optic fibres to each chamber of the flow cell. Ultimately, the flow is directed either to the 10-channel parallel fraction collector or through a 10-port manifold to a single waste line (Fig. 1). Tubing connectors have been adapted to allow columns to be conveniently connected to the system via FPLC or Luer standard fittings.

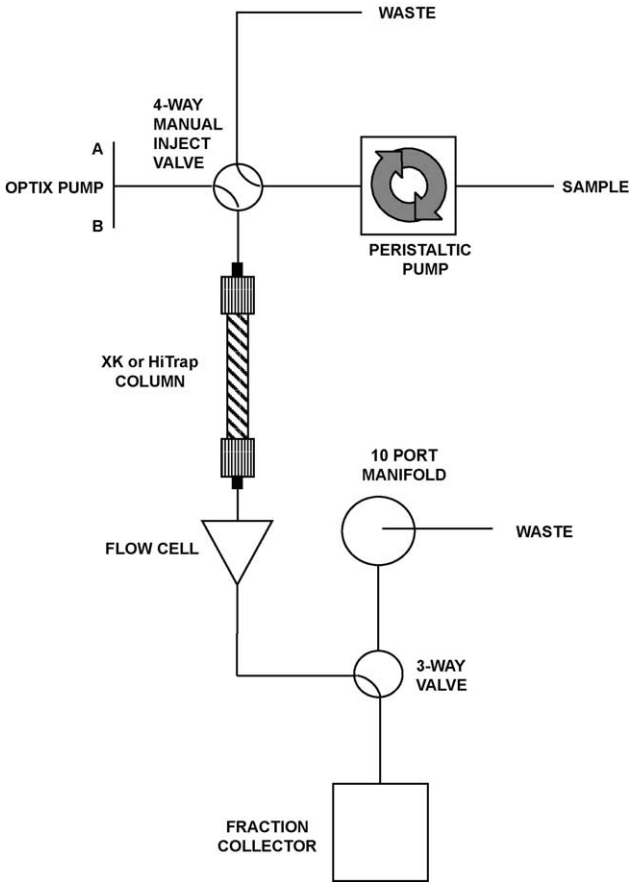

Fig. 1. Flow chart of the Optix 10 after modifications. An additional peristaltic pump was connected to the four-way injection valve and the flow cell was exchanged to a more sensitive one.

Before testing this platform with protein purification applications, we investigated the gradient formation capacity using water containing 1% (v/v) acetone to enable UV monitoring (data not shown). Since the pump is not continuous and the gradients are formed in the pump cylinders, the stepwise nature of gradient becomes apparent. In addition, we have decreased the refill rate of the cylinders to minimize air bubble formation during pump refill, which further makes the gradient mixing less efficient. However, when introducing a column or additional dead-volume allowing further mixing, the gradients appear more linearly. It is therefore recommended to add a mixing vesicle prior to the columns, or alternatively to pack columns with some headspace above the settled gel.

Ion exchange experiments were performed with samples containing commercially available sample proteins and prepacked columns. Since the current system only has one inlet each for buffers A and B, the proteins and buffer were chosen so that both anion- and cation exchange could be performed at the same pH. An experiment testing duplicate

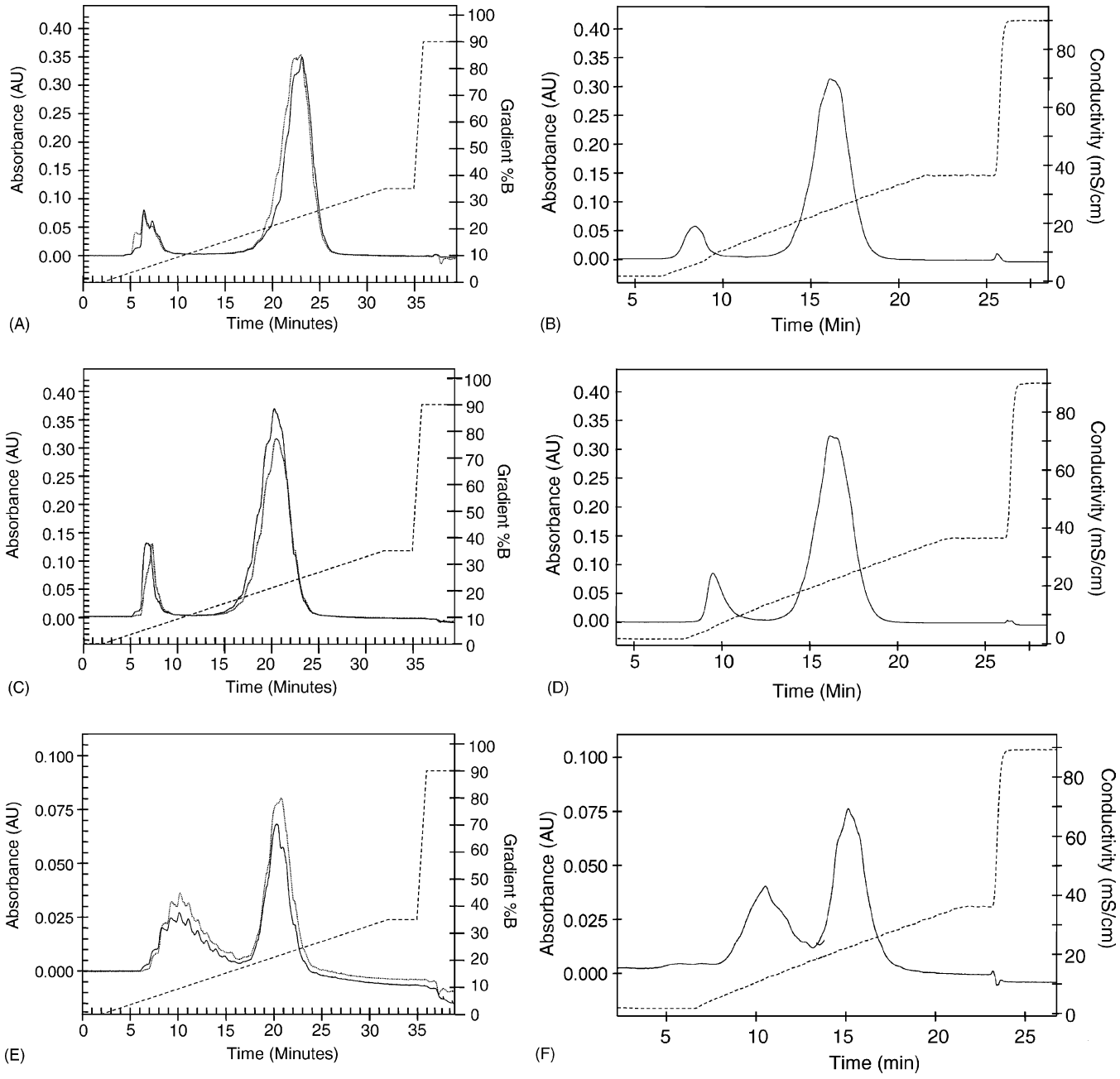

Fig. 2. Ion exchange chromatography experiment showing chromatograms from the modified CombiFlash OptiX 10 system and from control experiments on an ÄKTA purifier system. Sample volumes of 25 and 5 mL were applied to 5-mL columns connected to the OptiX system and 1-mL columns on the ÄKTA system, respectively. Proteins were eluted using a linear gradient from 0 to 350 mM NaCl over 30-column volumes at pH 8.0 (20 mM Tris/HCl). (A) An overlay of the UV trace from channels 1 and 2 of the OptiX system. HiTrap SP FF columns were loaded with sample containing aldolase 0.25 mg mL^{-1} and lysozyme 0.50 mg mL^{-1}; (B) the ÄKTA control experiment using the same sample and column media; (C) channels 3 and 4 with HiTrap CM FF columns and sample containing aldolase 0.25 mg mL^{-1} and lysozyme 0.50 mg mL^{-1}; (D) the control experiment of (C); (E) channels 5 and 6 with HiTrap Q FF columns and sample containing trypsin inhibitor 0.40 mg mL^{-1} and conalbumin 0.20 mg mL^{-1}; (F) the control experiment of (E); (G) channels 7 and 8 with HiTrap DEAE FF columns and sample containing trypsin inhibitor 0.40 mg mL^{-1} and conalbumin 0.20 mg mL^{-1}; (H) the control experiment of (G); (I) channels 9 and 10 with HiTrap SP HP columns with sample containing aldolase 0.25 mg mL^{-1}, lysozyme 0.50 mg mL^{-1} and chymotrypsinogen 0.23 mg mL^{-1}; (J) the control experiment of (I).

columns with five different chromatographic media is depicted in Fig. 2. The sample proteins were dissolved in buffer A and loaded onto the columns via the peristaltic pump while monitoring A_{280} on each channel and collecting flow-through fractions. Proteins were then eluted from the columns using a separate experiment file with a linear gradient of 0–350 mM NaCl over 30-column volumes. Control experiments at five times lower scale using the same protein mixes, gradients and

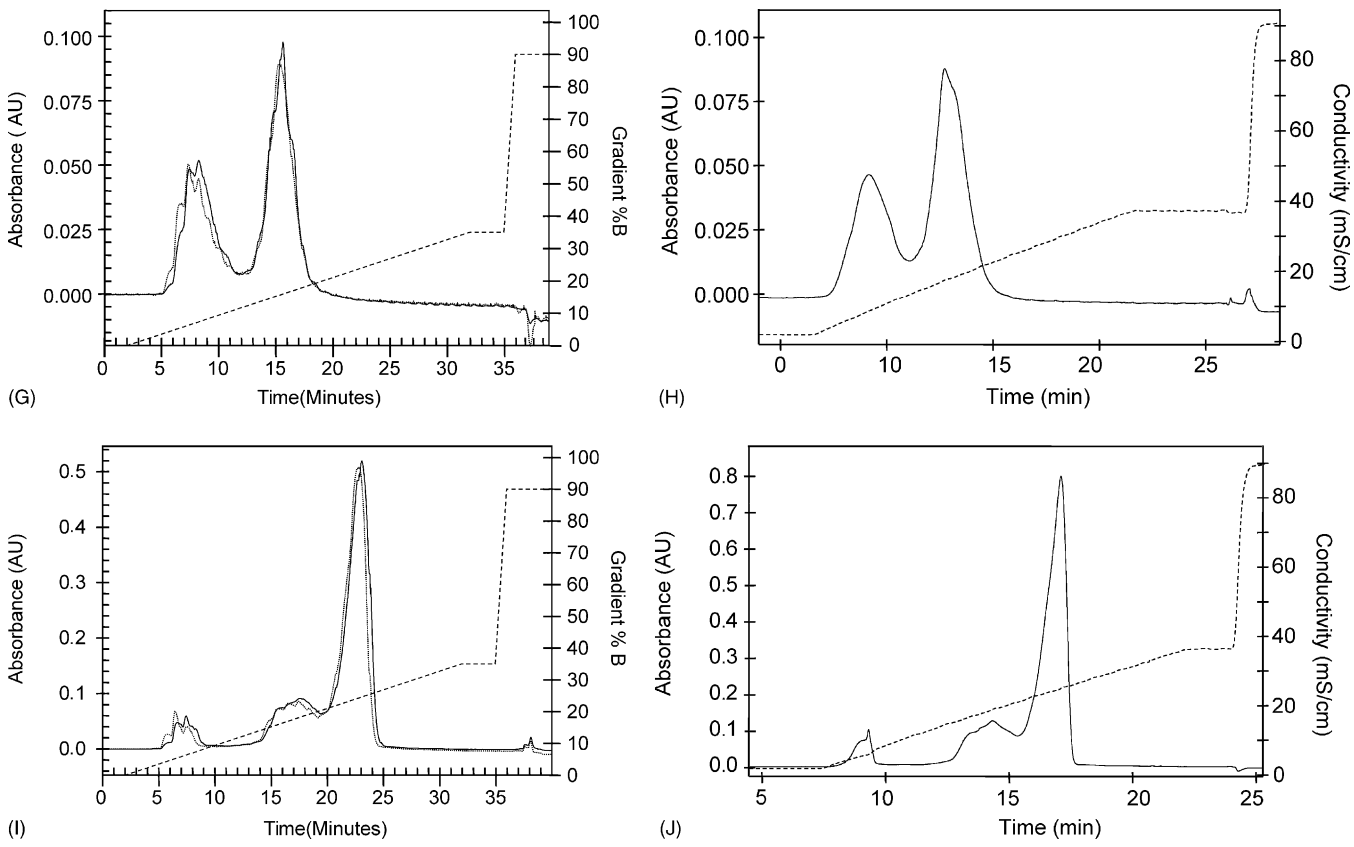

Fig. 2. (*Continued*).

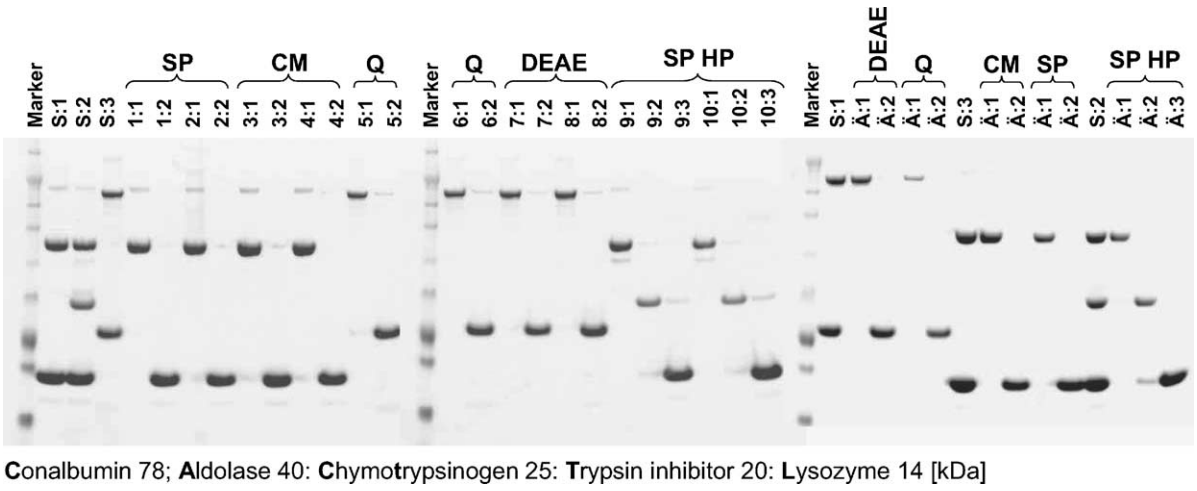

Fig. 3. SDS–PAGE analysis of the ion exchange experiment depicted in Fig. 2. Electrophoresis was performed using NuPAGE 10% BisTris gels run in MES buffer and the gels were subsequently stained using Coomassie Brilliant Blue. The SeeBlue Plus2 (Invitrogen) marker containing proteins of 188, 98, 62, 49, 38, 28, 17, 14, 6, 3 kDa was used as a reference. S:1 is the sample loaded on the columns in channels 1, 2, 3 and 4, and contains aldolase and lysozyme; S:2 was loaded on the columns in channels 9 and 10, and contains aldolase, lysozyme and chymotrypsinogen; and S:3 was loaded on the columns in channels 5, 6, 7 and 8, and contains trypsin inhibitor and conalbumin. The numbers above the lanes represent the different channels and the peak numbers (pooled fractions), respectively; and Ä is the pooled fractions from the ÄKTA control experiments. Ten microlitres of samples and 5 μL marker were loaded onto the gel.

columns were carried out on an ÄKTA purifier system. For all columns that were loaded with two sample proteins, good separation was achieved (Fig. 2). For the columns packed with SP sepharose high performance media, we tried to resolve three different proteins. Here, two proteins partially co-eluted, which was also seen to a similar extent in the ÄKTA control experiment (Fig. 2I and J). The UV trace in the OptiX chromatograms sometimes displayed a jagged appearance, which is probably due to insufficient mixing within the pump cylinders and the discontinuous flow during pump refill, but generally the channel-to-channel variation was low between duplicate columns. Moreover, peak fractions from both the OptiX and ÄKTA runs were pooled and analysed by SDS–PAGE (Fig. 3), showing that the OptiX instrument results in IEX resolution of almost the same quality as the ÄKTA system.

IMAC purification of 6× His-tagged proteins was optimized using an *E. coli* lysate containing a 35 kDa recombinant test protein (Fig. 4). Four columns with Ni-NTA superflow resin were used, two 5 mL pre-packed cartridges and two 7-mL columns with approximately 3 mL headspace. The same lysate was loaded onto all four columns using the peristaltic pump and proteins were eluted using a three-step gradient (two wash steps and a final elution step with 200 mM imidazole). A control experiment using the same lysate, gradient and a 1 mL Ni-NTA cartridge was performed on an ÄKTA purifier system (Fig. 2C). Again, similar results were obtained from both the OptiX and ÄKTA systems. The jagged shape of the OptiX UV trace is also seen in this experiment, especially for the pre-packed cartridges where protein elution occurs in multiple peaks (Fig. 4B). Adding column headspace smoothes the curves but these columns showed larger column-to-column variability (Fig. 2A). Pooled fractions from the elution peaks were then subjected to gel filtration using pre-packed 50 mL Sephadex G-25 columns (data not shown) in order to remove salt and imidazole. Fig. 5 shows samples from the IMAC purification separated using SDS–PAGE.

In order to validate the methods we had set up, we expressed proteins from 10 different plasmids in 1-L cultures of *E. coli*. The protein products were His-tagged variants of seven different nuclear receptor ligand binding domains with molecular weights from approximately 25–40 kDa and p*I* values ranging from 5.5 to 9. The lysates of the harvested bacterial cells were applied onto ten 7-mL Ni-NTA columns (packed in sample cartridges and with headspace, as above) via the peristaltic pump. Elution was conducted as in the previous experiment and peak fractions were analysed by

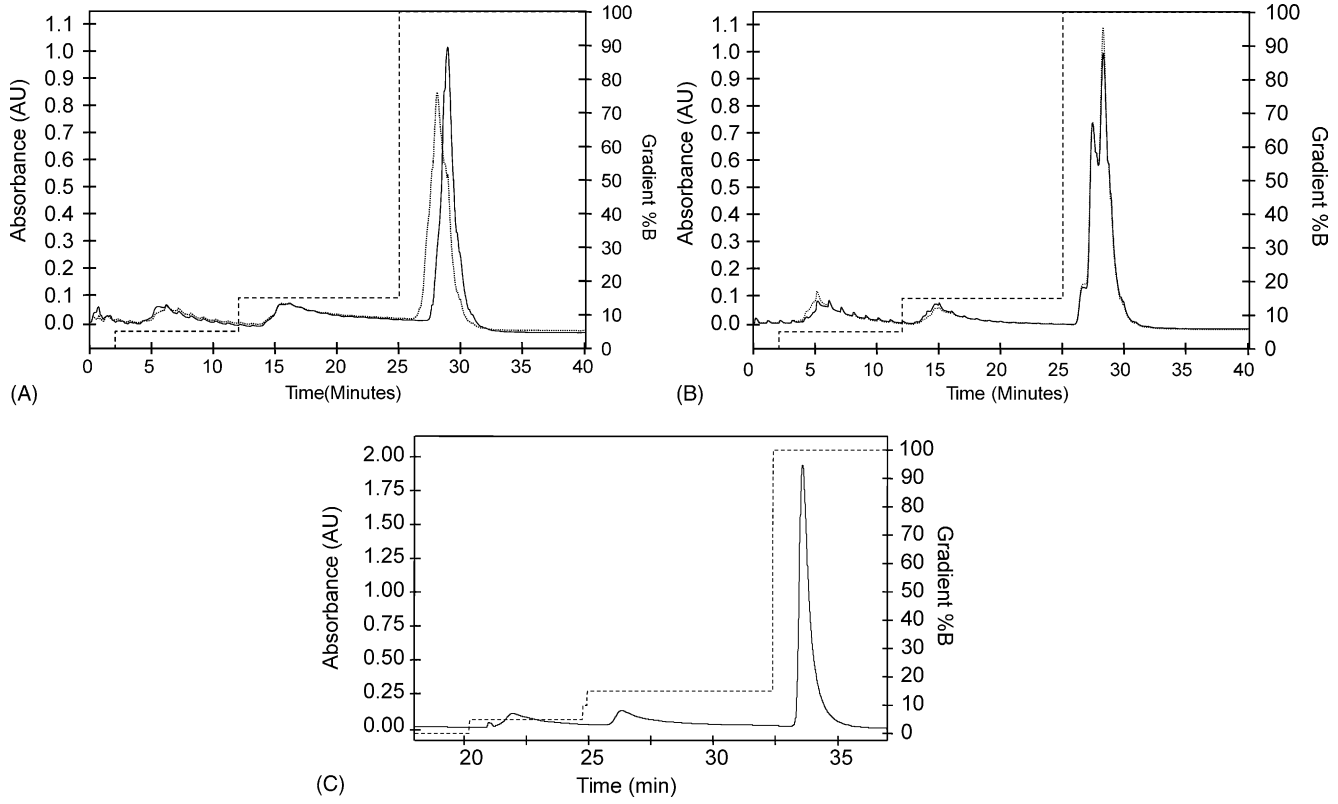

Fig. 4. IMAC experiment showing chromatograms from the modified CombiFlash OptiX 10 system and from a control experiment on an ÄKTA purifier system. Four columns containing Ni-NTA superflow (Qiagen) were connected to different channels of the OptiX10 system. Fifty millilitres *E. coli* cell lysate containing a recombinant His-tagged protein was loaded on each column using the peristaltic pump. The elution method file contains two wash steps with low concentrations of imidazole (10 and 30 mM) before eluting with an imidazole concentration of 200 mM. (A) The results from the two 7-mL Ni-NTA superflow columns packed in solid sample cartridges; (B) the results from the two pre-packed 5-mL Ni-NTA superflow columns (IBA); (C) a control experiment performed on an ÄKTA system, using a pre-packed 1-mL Ni-NTA superflow column (IBA), 10 mL sample volume and comparable elution conditions.

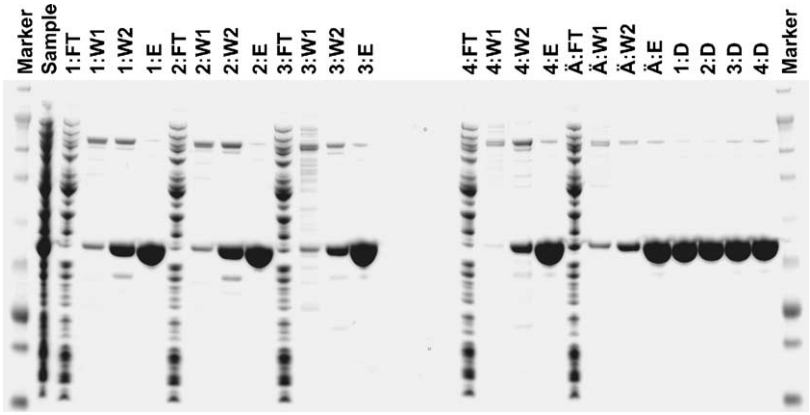

Fig. 5. SDS–PAGE analysis of the IMAC experiment depicted in Fig. 4. The pooled peak fractions were analysed using NuPAGE 10% BisTris gels run in MES buffer and the proteins were subsequently stained using Coomassie Brilliant Blue. The marker is SeeBlue Plus2 (Invitrogen) containing proteins of 188, 98, 62, 49, 38, 28, 17, 14, 6, 3 kDa in size. Sample is the cell lysate loaded on the columns, FT is the flow-through, W1 the first wash step with 10 mM imidazole, W2 the second wash step with 30 mM imidazole, E the eluted protein at 200 mM imidazole and D is the elute from the desalting step using HiPrep 26/10 desalting columns (Amersham Biosciences). '1' and '2' represent the channels packed with 7-mL Ni-NTA columns packed in solid sample cartridges, '3' and '4' the channels with pre-packed 5-mL Ni-NTA columns and Ä the control experiment on the ÄKTA.

SDS–PAGE (data not shown). After subsequent desalting, the Ni-NTA pools were loaded onto 5-mL Q sepharose FF columns and elution was achieved with a linear gradient of increasing concentration of NaCl. Using this protocol, nine of the cultures yielded purified His-tagged protein. One protein seemed not to be expressed at all, since only contaminant proteins eluted from the Ni-NTA step. The protein content in the final ion exchange pools was estimated with the Bio-Rad protein assay and yields from the different cultures varied from 6 to170 mg L^{-1}. Purity was assessed with SDS–PAGE (Fig. 6).

The experiments described above demonstrate that the modified OptiX 10 is a suitable instrument for a preparative parallel protein purification platform. However, there are still some flaws that have to be improved. For example, solvent exposed material should be replaced with inert metal-free parts to tolerate long-term usage of aqueous solutions and salts. Some optimization of the gradient mixing is needed to improve gradient formation. Moreover, it would be convenient to be able to collect fractions in MTP deep-well blocks instead of tubes. The software should be adapted for the present applications as well and, since the peristaltic pump is equipped with the RS232 serial interface, this pump should also be amenable to software control.

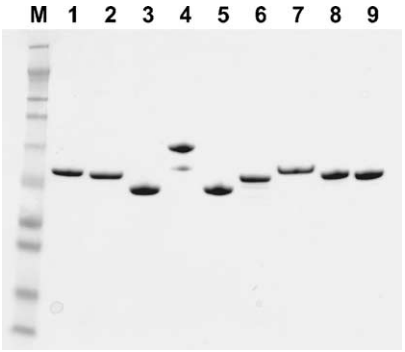

Fig. 6. SDS–PAGE analysis of purified His-tagged sample proteins. Ten *E. coli* lysates were subjected to a three-step parallel purification procedure using the modified OptiX system. Samples were loaded onto Ni-NTA columns and eluted with imidazole (as above). The Ni-NTA pools were subsequently desalted using HiPrep 26/10 desalting columns, and loaded onto HiTrap Q FF columns. The final elution was achieved by applying a linear gradient from 0 to 500 mM NaCl over 30-column volumes. pH was 8 throughout the entire purification protocol. Purified proteins were obtained from nine out of 10 lysates and 3.5 μg of each final pool was separated on a NuPAGE 10% BisTris gel and stained with Coomassie Brilliant Blue.

In order to produce proteins of sufficient purity for crystallographic studies, multi-step purification procedures often have to be performed [11]. Ideally, HT procedures should be automated and generic. Generating generic protocols for capture purification steps utilizing specific affinity tags are readily achievable [3–7]. However, for applications such as ion exchange, which is dependent on variable protein characteristics like charge and p*I* values, creating standard protocols is much harder. The appropriate chromatographic media and buffer pH often have to be selected for each individual protein. By extending the tubing from the 10-port manifold of the existing buffer inlets, the present system could quite easily be modified to provide individual buffer inlet tubing for each channel. This would enable simultaneous usage of multiple column media with different buffer systems and elution strategies. As a result, three-step procedures (affinity, buffer exchange, ion exchange) could be performed in an entirely parallel fashion despite targeting proteins with different physico-chemical characteristics.

4. Conclusions

A 10-channel flash chromatography instrument was successfully adapted for preparative parallel protein purification purposes. We conclude that using the platform described in this study, results of similar quality could be obtained, for

both ion exchange and affinity chromatography applications, as in control experiments on an ÄKTA system. The system displays low channel-to-channel variation and offers flexibility toward up-scaling. In a sample experiment, proteins of high purity were obtained from 9 out of 10 cultures expressing different His-tagged proteins, using a three-step parallel purification procedure. When finally customized, this system can be used to prepare milligram amounts of tenths of different proteins on a daily basis.

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