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Production of milligram quantities of affinity tagged-proteins using automated multistep chromatographic purification

Rama Bhikhabhai*, Anna Sjöberg, Lotta Hedkvist, Markus Galin, Pia Liljedahl, Tuomo Frigård, Niklas Pettersson, Mats Nilsson, Jill A. Sigrell-Simon, Christine Markeland-Johansson

Department of Research and Development, GE Healthcare Company, Amersham Biosciences AB, SE-751 84 Uppsala, Sweden

Abstract

A new chromatography system, ÄKTAxpress (GE Healthcare, Amersham Biosciences, Uppsala, Sweden) has been designed to meet the demand for high-throughput purification of proteins in structural genomics and drug discovery. The system offers a number of automated multistep purification protocols for affinity-tagged proteins. All protocols start with affinity chromatography followed by combinations of desalting, ion exchange chromatography and gel filtration. As an option, tag removal can be included in the purification protocols. Up to 16 proteins can be purified per day and the yield can be as high as 50 mg of each protein at >90% purity. To highlight the versatility of the system, this paper presents several case studies; purifications of hexahistidine- and glutathione S-transferase-tagged proteins using different protocols, automated on-column tag cleavage and optimization studies for a hexahistidine-tagged kinase.

Keywords: ÄKTAxpress; Hexahistidine; GST; Affinity-tagged proteins; Automated multistep chromatography; On-column cleavage; Protein purification

1. Introduction

In the post era of Human Genome Project, the need for high-throughput in protein production has increased, for example, in drug discovery [1,2]. The task of producing many different pure proteins will be much easier if automation is available for all different steps such as; cloning, expression, screening of expression, cell lysis, sample preparation, chromatographic purification and analysis.

An automated system for high throughput cloning, expression and one-step affinity purification of tagged proteins using 96-well micro plate format has been reported [3], but the amount of protein purified extend to only about 90 μg. For structural determination of proteins using X-ray crystallography, milligram quantities of pure proteins are often needed, especially if extensive crystallization screening is required. Also, in most cases more than one chromatography step is necessary to achieve suitable protein quality.

To facilitate the production of milligram quantities of protein for research, standard chromatography instruments are available and can normally handle one chromatographic step at a time. When more than one chromatography step is involved in the purification process, tedious and time consuming manual handling such as pooling of eluted peaks, dialyzing or desalting and applying the sample to the next chromatography column are required. To automate these procedures, a standard chromatography system was modified with extra valves, tubing and updated software and protocols to perform multistep purification [4]. This semi-automated system can purify up to six proteins within a few hours by using affinity chromatography steps combined with either ion exchange or gel filtration/desalting. To increase the throughput even more, an automated multistep chromatography system, ÄKTAxpress has been designed [5]. The system is equipped with a peak detection algorithm allowing automation of four-step chromatographic purification. The user-friendly software, allows the choice of several purification protocols as well as an option to include affinity tag removal. ÄKTAxpress consists of four modules which

* Corresponding author. Tel.: +46 18 6120432; fax: +46 18 6121844.
E-mail address: rama.bhikhabhai@ge.com (R. Bhikhabhai).

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work in parallel and can purify up to 16 proteins in one day to yield up to 50 mg of each protein with a purity >90%. For the laboratories demanding even higher throughput protein purifications, up to 12 modules can be controlled by one computer and the throughput is then increased three times. The protocols in the system are optimized for the purification of recombinant proteins with tags such as histidine [6,7] and glutathione S-transferase (GST) [8].

To show the versatility of ÄKTAxpress system, this report presents several case studies for the purification of hexahistidine- and GST-tagged proteins using different multistep protocols. The possibilities to optimize protocols and to perform on-column cleavage of affinity tags are also exemplified.

2. Experimental

2.1. Material

All clones for the affinity tagged proteins except for P-450 (Crosslink Bt., Budapest, Hungary) were kind gifts from different research groups (see Acknowledgement). Other chemicals purchased were: lysozyme and EDTA from Sigma Chemical Co., St Louis, USA; glutathione from TANABE EUROPE N.V., Brussels, Belgium; Pefabloc SC from Roche Diagnostic GmbH, Penzberg, Germany; benzonase, Tris–HCl, imidazole, sodium chloride, glycerol and magnesium chloride from Merck KGaA, Darmstadt, Germany; dithiothreitol (DTT) from USB Corporation, Cleaveland, OH, USA; AcTEV Protease from Invitrogen, Carlsbad, CA, USA and Coomassie from ICI Plc, Macclesfield, Great Britain. The following products were from GE Healthcare, Amersham Biosciences, Uppsala, Sweden: PreScission Protease, homogeneous ExcelGel, all prepacked columns; HisTrap HP and GSTrap HP for affinity chromatography, Mono Q 5/50 GL and RESOURCE Q for ion exchange chromatography, HiPrep 26/10 Desalting for desalting, HiLoad 16/60 Superdex 75 prep grade and HiLoad 16/60 Superdex 200 prep grade for gel filtration, Ni Sepharose High Performance and Glutathione Sepharose High Performance as affinity media. All chemicals used were of high purity grade.

2.2. Sample preparation

All proteins were expressed as soluble proteins in *Escherichia coli*. Cells were sonicated in 5 ml of binding buffer for affinity chromatography/g wet cells. The sonication buffer also included 1 mM Pefabloc, 1 mg/ml lysozyme, 1 mM magnesium chloride and 50 U/ml benzonase. Each sample was clarified by centrifugation at 39,000 × *g* for 20 min prior to sample loading. The molecular weight, isoelectric point and code numbers of the proteins are listed in Table 1.

Table 2
List of buffers used for the different chromatography steps

Technique	Buffer composition
AC (His) binding	50 mM Tris–HCl, 0.5 M NaCl, 20 mM imidazole, pH 7.5
AC (His) elution	50 mM Tris–HCl, 0.5 M NaCl, 0.5 M imidazole, pH 7.5
AC (GST) binding	50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5
AC (GST) elution	50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0
DS/IEX binding	50 mM Tris–HCl, 25 mM NaCl, pH 8.0
IEX elution	50 mM Tris–HCl, 1 M NaCl, pH 8.0
GF	50 mM Tris–HCl, 150 mM NaCl, pH 7.5
AC (His) cleavage	50 mM Tris–HCl, 0.5 M NaCl, 50 mM imidazole, pH 7.5
AC (GST) cleavage	50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5

2.3. System and software

ÄKTAxpress (Fig. 1) together with the software, UNICORN version 5.01 (GE Healthcare, Amersham Biosciences, Uppsala, Sweden) was used for all the automated multistep protein purifications. Default settings were used in the methods unless otherwise stated. The proteins were detected at 280 nm wavelength. The peak fractions in the intermediate steps were automatically collected in internal capillary loops and directed to the next column used in the protocol. All purifications in this study were performed at room temperature, unless otherwise stated.

2.4. Automated multistep purification

All protein purification protocols start with affinity chromatography (AC) followed by combinations of desalting (DS), ion-exchange chromatography (IEX) and gel filtration (GF). A typical total chromatogram from an automated four-step protein purification protocol is schematically presented in Fig. 2. The tubing and columns are automatically washed and prepared during each run as part of the purification protocol. The buffers used in this study are listed in Table 2.

2.5. On-column cleavage

A two- and four-step purification protocols was used in combination with automated on-column tag cleavage. The sample was loaded onto an affinity column and then washed

Table 1
Molecular weight and isoelectric point for the proteins used in the case studies. All proteins except GST-Purα were hexahistidine-tagged

Protein	APC234	APC1040	Kinase	APB7	P-450	GST-Purα
$M_r \times 10^3$	32.5	39	42.5	28	121	61.6
Isoelectric point	5.8	5.7	5.8	6.0	5.3	5.9

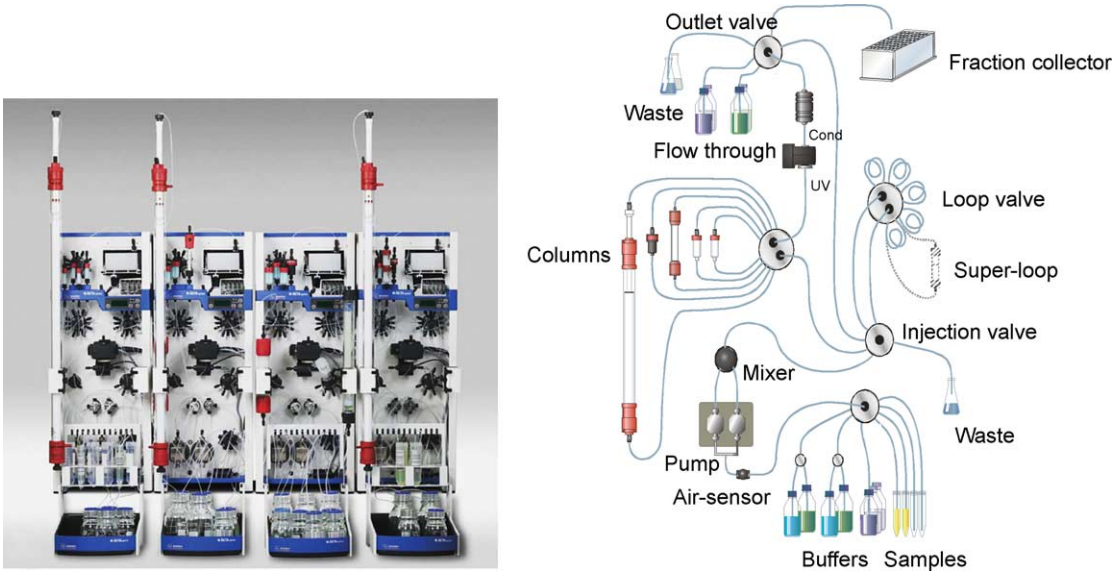

Fig. 1. Left: ÄKTAxpress system consisting of four separate protein purification modules. Right: the schematic flow diagram illustrating the system set up for each module.

with the appropriate cleavage buffer. Protease solution was loaded from a pre-filled sample reservoir and allowed to react for the optimized time and temperature. The cleaved protein was recovered by passing the cleavage buffer through the column and it was automatically purified further and collected in the fractionation plate. The affinity column was regenerated by AC elution buffer which desorbed the cleaved affinity tag, tagged protease and any remaining uncleaved protein. The eluate was collected in the vessel for unselected peaks. For the hexahistidine-tagged protein, a cleavage solution containing 200 units of AcTEV Protease, a hexahistidine-tagged protease from Tobacco Etch Virus, in AC (His) cleavage buffer was used. The run took place at room temperature with a cleavage time of 8 h. For the GST-tagged protein, a solution containing 20 units of PreScission Protease, a recombinant protein consisting of human rhinovirus 3C protease with GST-tag, in AC (GST) cleavage buffer was used. The run took place at 8 °C with a cleavage time of 8 h.

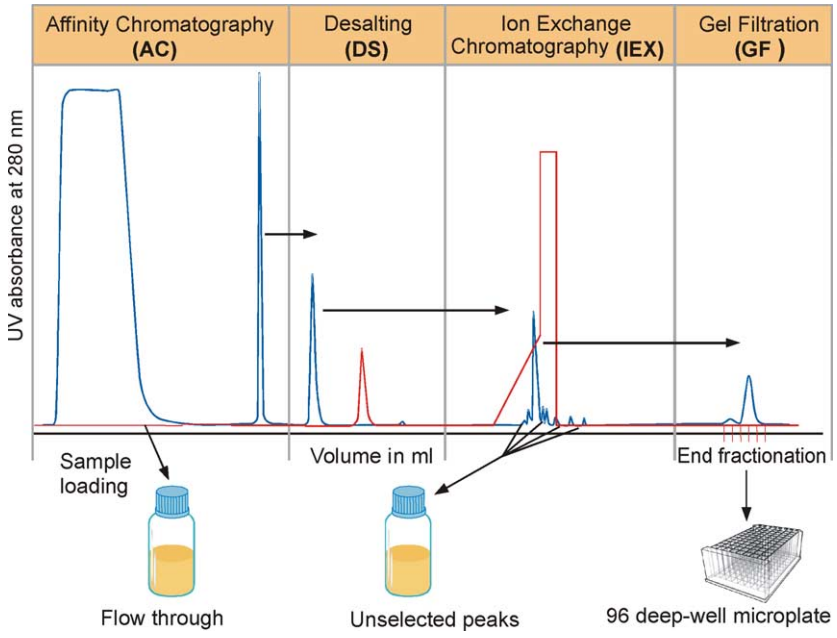

Fig. 2. A schematic total chromatogram of an automated four-step protocol AC–DS–IEX–GF intermediate peaks are stored in internal capillary loops and the largest peak is automatically injected onto the next column. Fractionation after the last chromatographic step takes place in a deep-well microplate. Flow through and unselected peaks are saved in two separate vessel.

2.6. Analysis

Each protein sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [9]. The reduced samples were applied to 8–18% gradient or 12.5% homogeneous SDS Excel gels under reducing conditions as described by the supplier. Approximately, 7.5 μg of total protein was loaded per lane. The gels were stained with Coomassie. Purity was assessed by visual inspection of the gels and profiles of the chromatograms. Yields were calculated by integrating the chromatographic peaks using UNICORN software.

3. Results and discussion

ÄKTAxpress offers a number of automated, multistep protein purification protocols for tagged proteins. All protocols start with affinity chromatography (AC) followed by combinations of desalting (DS), ion exchange chromatography (IEX) and gel filtration (GF). The available protocols are named as; AC–DS, AC–DS–IEX, AC–GF, AC–DS–IEX–DS and AC–DS–IEX–GF. Depending on the amount of protein required, different sizes of prepacked columns can be chosen. The number of samples to be purified and the time taken depends on the choice of the protocol. For example, 16 samples can be purified using AC–DS protocol in about 5 h whereas for AC–GF protocol, purification of 16 samples will take about 11 h.

To perform automated high throughput purification successfully, it is important to plan the experiment based on the properties of proteins and the knowledge of the system configuration. Information about isoelectric point and stability of the proteins is necessary when deciding the type of ion exchanger (anion or cation) to use and pH of the running buffers. For gel filtration, information regarding the size of the protein is of help when choosing a column with suitable separation range. To calculate the concentration of purified protein, knowing the extinction coefficient of the target protein will be of great help. ÄKTAxpress system has four modules which run in parallel. Each module carries out the multistep purification in series for up to four different proteins. For each module only one set of column combination can be made, in other words, proteins with similar p*I* and molecular weight should be purified on the same module.

Experienced protein biochemists, as a routine, determine yield and recovery of protein after each step in a non-automated multi-step chromatographic process. When using ÄKTAxpress, recovery of protein in between the steps can only be estimated by using integrated peak area calculations and extinction coefficient of the protein. Most of the time, the concentration of target protein is so high in the start sample that the peak height in the chromatogram is more than two absorbance units, thus limiting the use of peak integration. The expression level of each tagged protein, if known, is of great help in deciding the size of the column, but often in routine high throughput laboratories, the clarified lysates are applied to a standard set up of columns and a chosen protocol without checking the exact expression level. In those cases, where the results are not satisfactory, e.g. when the protein was not pure enough or final yield was low, the chromatograms from each step have to be carefully inspected. The most common explanation for low recovery is either the low expression level of the protein or instability of the protein in the conditions used leading to proteolysis. For such protein one needs to optimize the purification conditions separately.

In the current report, tagged proteins (Table 1) were purified using different automated multistep protocols. In all cases, the purified proteins were analyzed by SDS-PAGE to estimate purity. The emphasis was made on the purity rather than yield of tagged-protein. The following case studies are reported: (i) one hexahistidine-tagged protein was purified using three different protocols; (ii) four different hexahistidine-tagged proteins were purified using the same protocol; (iii) optimization of a purification protocol of a hexahistidine-tagged kinase and (iv) on-column cleavage of a hexahistidine- and GST-tagged protein with appropriate proteases followed by purification.

3.1. One protein and three different protocols

This experiment shows purification of a hexahistidine-tagged protein, APB7, using three different protocols (Fig. 3). The degree of purity of the purified target proteins was examined by visual inspection of the SDS-PAGE gels and was in the following order: AC–DS < AC–GF < AC–DS–IEX–DS. The four-step protocol had higher purity than the two-step protocols. The amount of pure APB7 produced was between 15 and 22 mg, sufficient enough to carry out further analysis. In general when more than one chromatographic step is used in a process, the protein yield is lower and purity is higher in the final step. When gel filtration is performed in ÄKTAxpress, the column size limits the volume of sample application to 5 ml. In those cases where the volume of the collected peak from the preceding step is greater than 5 ml, a slight loss in the protein yield may be expected, but in most cases the required amount of protein will still be produced. In the ion exchange chromatography, an anion exchanger is used. Proteins with high isoelectric point can be grouped together and purified on a cation exchanger using a buffer with appropriate pH.

The choice of which purification protocol to use when purifying the target protein often depends on the application that follows. For biological studies and enzymatic assays, affinity chromatography followed by desalting can be sufficient, as small molecular weight substances from the elution buffers are removed in the desalting step and the target protein is transferred to an appropriate stabilizing environment. Gel filtration instead of desalting as the last step is usually appropriate for crystallographers, as undesired aggregates and small sized contaminants are removed in the gel filtration step, thus resulting in a size homogeneous protein.

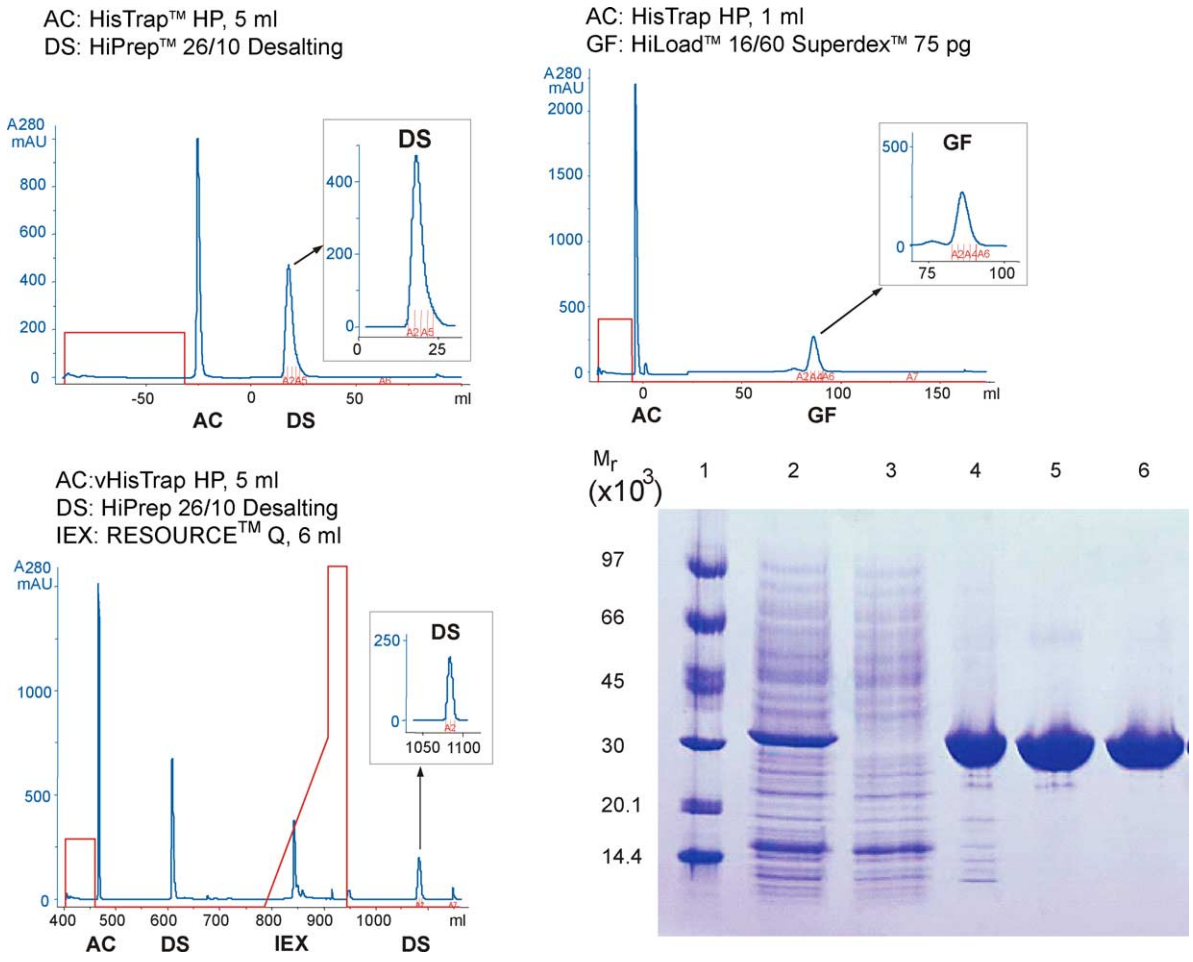

Fig. 3. Purification of APB7 (M_r 28 × 10^3, p*I* 6.0) using three different protocols. The total chromatogram for each protocol is presented and the prepacked columns are listed above each chromatogram. The chromatograms obtained for the final step are shown as insets in the framed boxes to the right. The amount of pure APB7 produced in this study was: AC–DS, 22 mg; AC–GF, 15 mg and AC–DS–IEX–DS, 17 mg. The samples analyzed by SDS-PAGE are: lanes 1 and 8, low molecular weight markers; lane 2, start sample; lane 3, flow through; lanes 4–6, pooled fractions from the final step in the protocol; lane 4, AC–DS; lane 5, AC–GF; lane 6, AC–DS–IEX–DS.

3.2. Four proteins and one protocol

Four hexahistidine-tagged proteins (P-450, APC234, APC1040 and APB7) were purified using the AC–DS–IEX–GF protocol (Fig. 4), to emphasize that this system can handle different proteins using the same pre-programmed protocol. For APC234, both from the IEX and GF step, one symmetrical peak was observed indicating that a one- or two-step protocol could be sufficient for purification of this particular protein. For the other three proteins, besides the main peak, some more peaks were observed in IEX as well as GF. SDS-PAGE of the fractions from the GF steps shows that high purity was achieved for all proteins.

For protein APC1040, faint protein bands just below the main protein and a stronger protein band at M_r 14.4 × 10^3 were observed indicating that the protein may not be stable at room temperature or in the buffer conditions used. To minimize degradation, purifications can be performed at lower temperature or the buffers used can be altered to stabilize the target protein. In general, a four-step protocol is preferable when separation of isomers and aggregates is required. Heterogeneously charged molecules due to post translational modifications will be separated in the ion exchange chromatography step and the dimers or multimer forms of proteins will be separated in the gel filtration step. Depending on the protein, the final yield in a four-step protocol is comparatively lower.

3.3. Optimization of the purification protocol for a hexahistidine-tagged kinase

The purification of a hexahistidine-tagged kinase expressed in *E. coli* was performed using the default settings of the protocol AC–DS–IEX (Fig. 5a). To optimize the protocol for purification of this protein, the default settings were

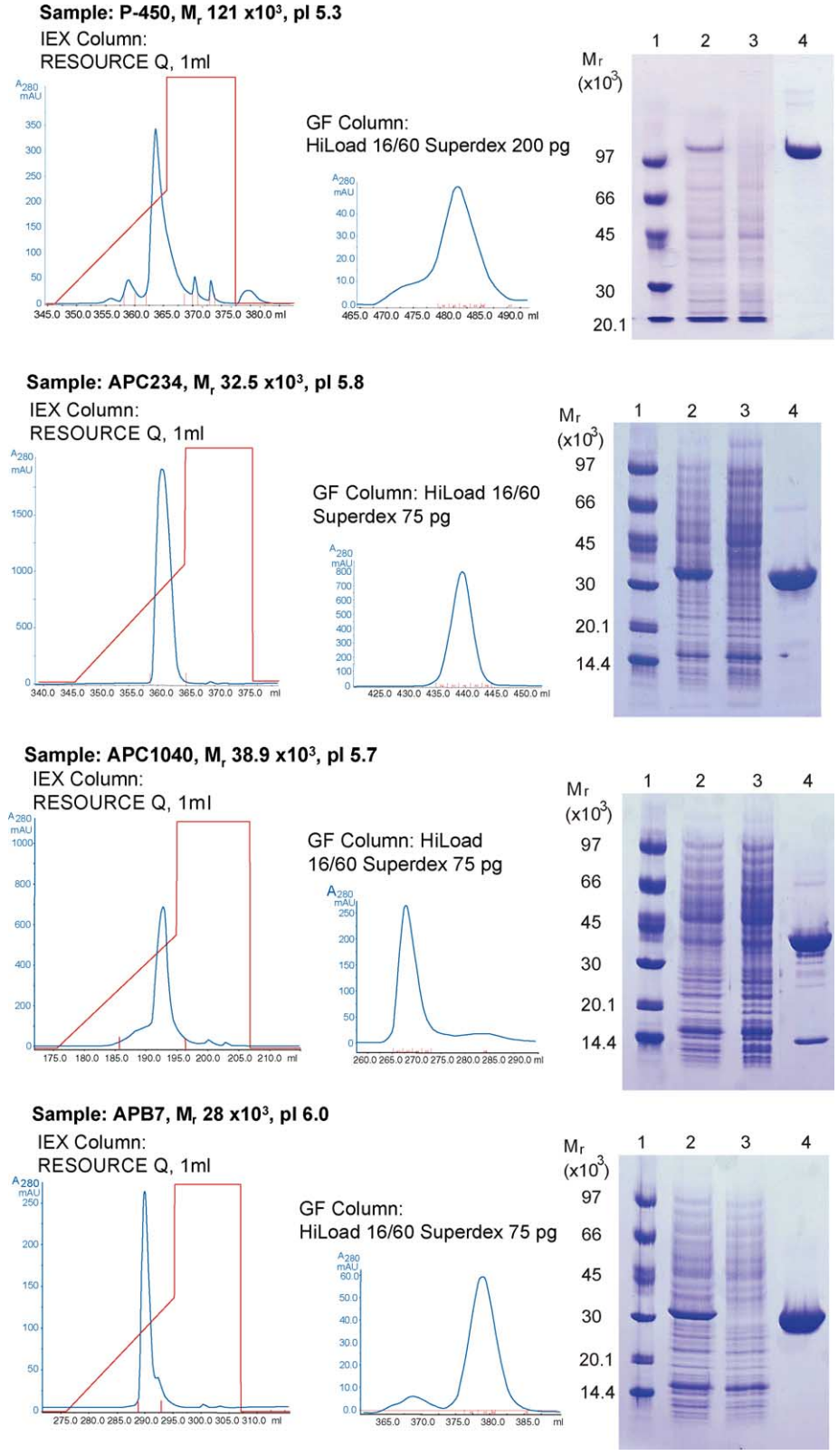

Fig. 4. Purification of four hexahistidine-tagged proteins using the protocol, AC–DS–IEX–GF. The chromatographic profiles for the ion exchange and gel filtration step are presented. The name, size and isoelectric point of protein, and prepacked columns used are mentioned on the top of each chromatogram. For SDS-PAGE analysis, samples in the lanes are: lane 1, low molecular weight markers; lane 2, start sample; lane 3, flow through; and lane 4, pooled protein after AC–DS–IEX–GF.

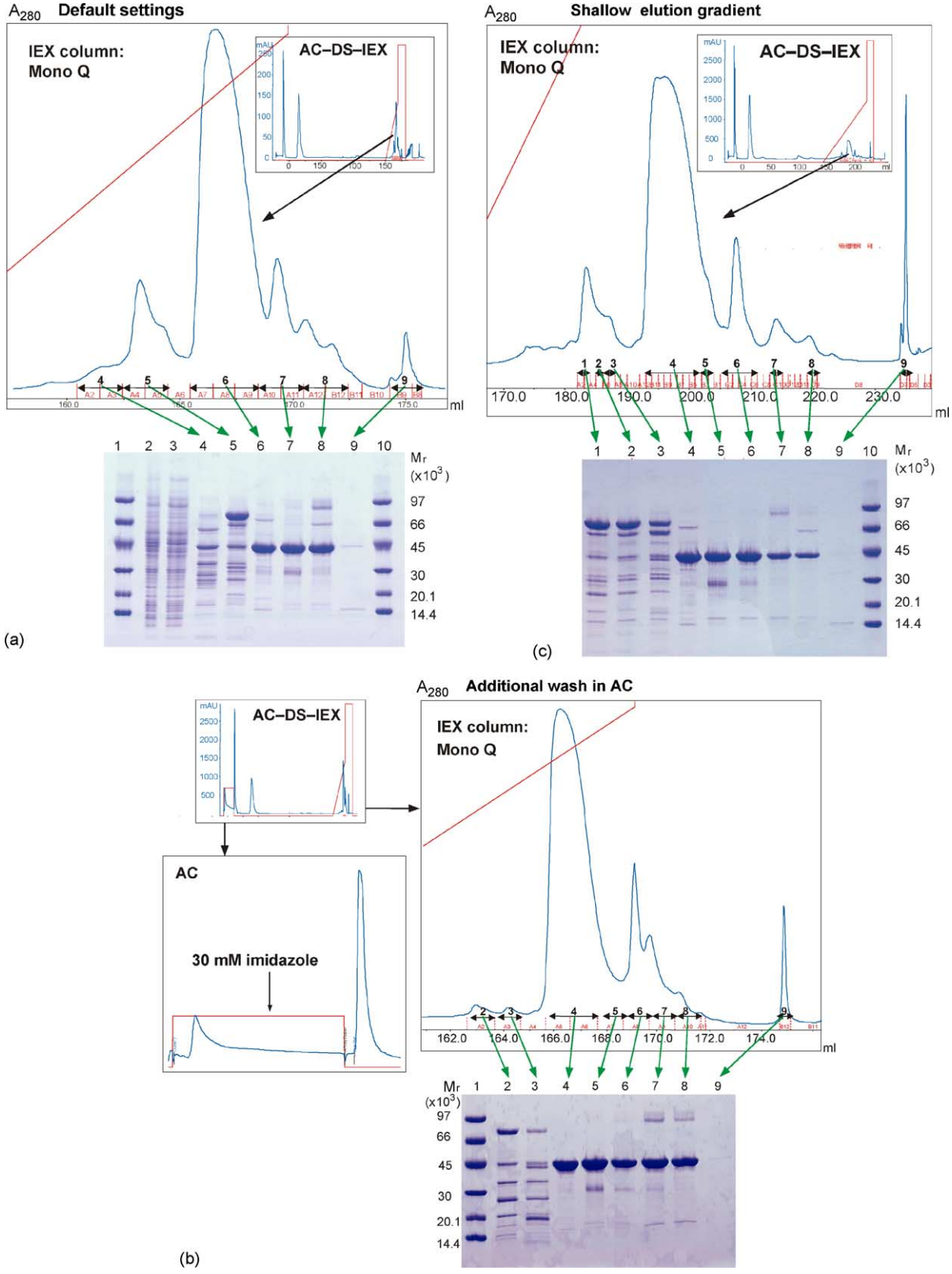

Fig. 5. Purification and optimization of a hexahistidine-tagged kinase. (a) Purification using default settings. (b) Increased imidazole concentration in the wash step prior to affinity elution. (c) Shallow gradient (80 CV) in ion exchange step. A high resolution, anion exchanger (Mono Q, 5/50 GL 1 ml) was used in the IEX step. To the standard buffers, 1 mM DTT and 10% glycerol were added. The chromatographic profiles of Mono Q and total chromatograms (top right for a and c, top left for b) are presented. The arrows indicate fractions from the chromatograms that were analyzed by SDS-PAGE. Low molecular weight markers are labeled.

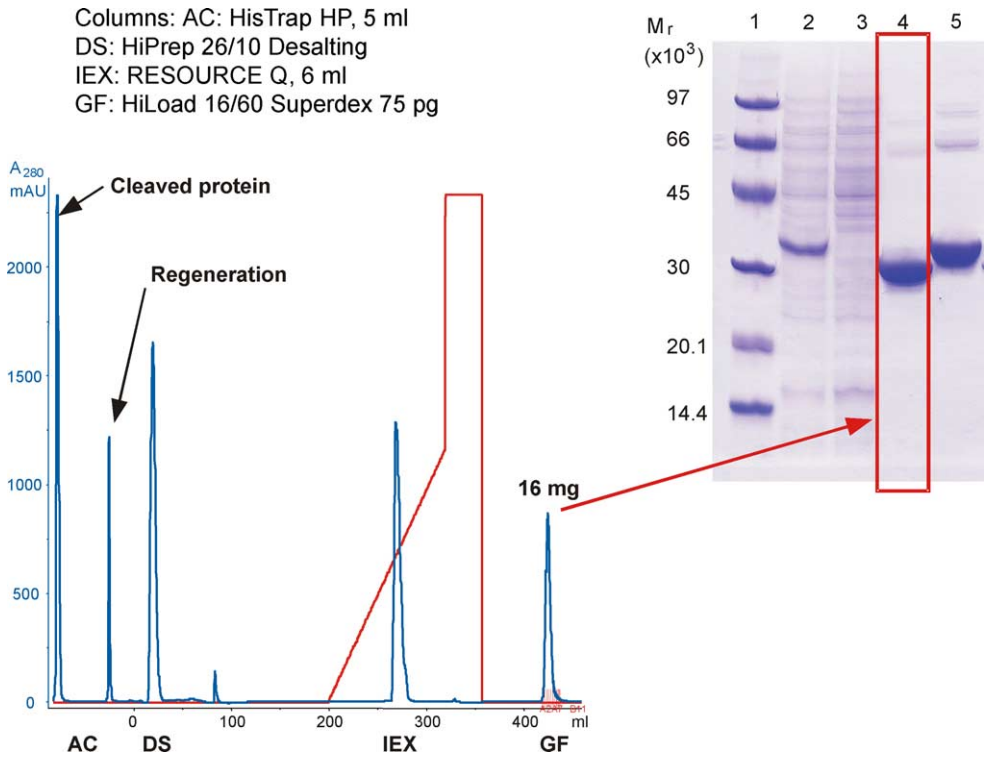

Fig. 6. A total chromatogram of on-column cleavage of hexahistidine-tagged APC234 cleaved with AcTEV protease, combined with AC–DS–IEX–GF purification. For SDS-PAGE, samples are: lane 1, low molecular weight marker; lane 2, start sample; lane 3, flow through; lane 4, purified cleaved APC234 (M_r 30.1 × 10^3) and lane 5, as a reference, uncleaved APC234 (M_r 32.5 × 10^3).

easily changed in the method wizard of UNICORN. In one case, the concentration of imidazole was increased from 5 to 30 mM imidazole in the wash step prior to affinity elution (Fig. 5b). In a second case, the length of the elution gradient in the anion exchange step was increased from 20 to 80 column volumes, to investigate eventual effect on the resolution of the separation (Fig. 5c). SDS-PAGE showed that an increase in the imidazole concentration in the wash step, prior to affinity elution, removed the weakly bound protein of M_r 66 × 10^3 and low molecular weight contaminants (compare the first peak in the chromatograms of Fig. 5a and b and SDS-PAGE lanes 4 and 5 in Fig. 5a with lanes 2 and 3 in Fig. 5b). The resolution was slightly increased using a shallow elution gradient in the IEX step (Fig. 5c) compared with the default gradient setting. By adding a GF column as the fourth step (results not shown), size homogeneity of the protein and buffer exchange was achieved. However, the purity of the kinase after both three- and four-step purifications was very similar. In all experiments, the main peak contained about 50 mg of protein.

All the fractions were analyzed by western immunoblotting (data not shown) using antibodies against phosphothreonine and phosphotyrosine residues. The results showed that one of the small peaks which eluted after the major kinase peak was more phosphorylated than the major peak. These results indicate that to separate the isoforms due to heterogeneity in phosphorylated proteins or glycoproteins, ion exchange chromatography can be used as a third step.

3.4. On-column cleavage

Tag removal is sometimes necessary to obtain better crystals for structural analysis or to restore the “native protein”. The availability of tagged proteases on the market eases on-column cleavage, as the cleaved proteins are recovered easily without additional chromatography steps. Parameters such as time, temperature, and concentration of imidazole and the proteases were optimized. The development protocol was initially performed in batch or using a traditional chromatography system. The optimized conditions were then applied to each purification protocol using ÄKTAxpress system.

On-column tag cleavage of two proteins, each purified using different protocols and proteases were performed in this study. For APC234, AcTEV protease was used to cleave off the histidine-tag and on-column cleavage was combined with a four-step purification protocol (Fig. 6), resulting in 16 mg of highly pure APC234, without the hexahistidine-tag (Fig. 6, SDS-PAGE, lanes 3 and 4).

To cleave off the GST-tag from GST-Purα and purify the Purα protein, a two-step protocol was chosen (Fig. 7).

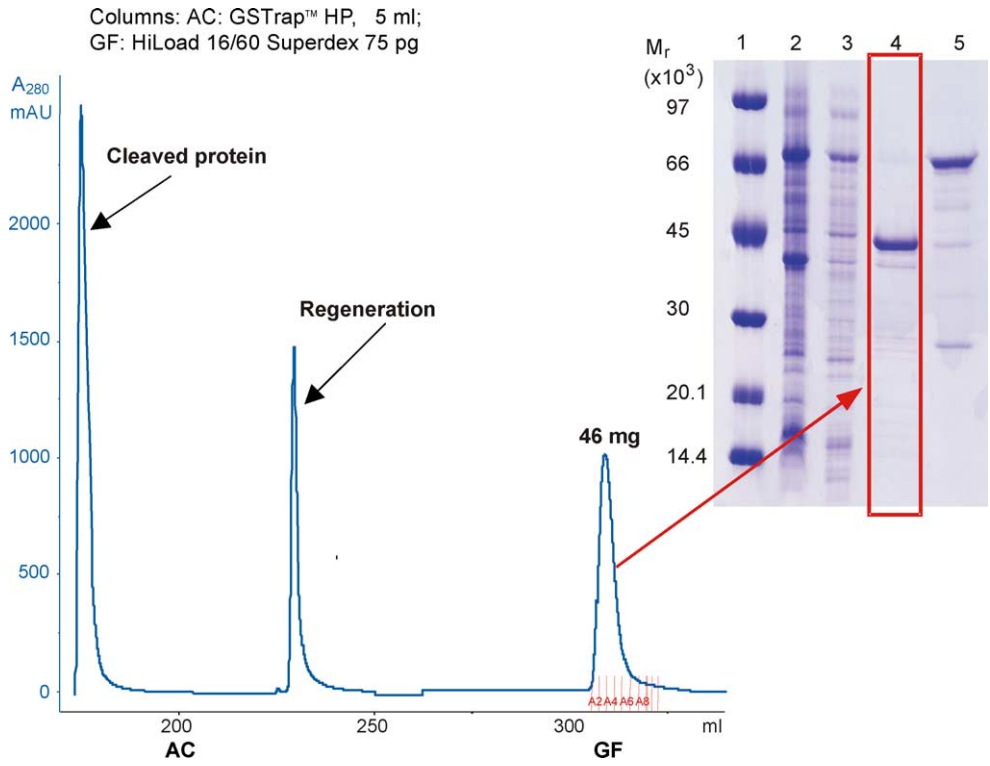

Fig. 7. A total chromatogram of on-column cleavage of GST-tagged protein cleaved with PreScission Protease combined with AC–GF purification. For SDS-PAGE, the samples were: lane 1, low molecular weight markers; lane 2, start sample; lane 3, flow through; lane 4, purified, cleaved GST-Purα (M_r 35.2×10^3) after AC–GF; and lane 5, as a reference, uncleaved GST-Purα (M_r 61.6×10^3).

The affinity column was subjected to a cleavage solution containing 20 units of PreScission Protease. After the two-step purification, 46 mg of highly pure Purα, without the GST-tag was obtained (Fig. 7, SDS-PAGE, lanes 3 and 4). The results suggest that on-column cleavage can be successfully performed and extended to various proteases as long as the cleavage conditions are optimized. The incubation times can be shortened if a protease with faster hydrolysis rate is used.

4. Conclusion

We have shown that the automated and multistep purification protocols in ÄKTAxpress system can be used to produce milligrams of high purity of hexahistidine- and GST-tagged proteins. The chromatographic parameters can be conveniently altered and optimized with UNICORN software when the default settings do not give desirable results. Although the protocols are dedicated to hexahistidine- and GST-tagged proteins, any affinity tag-ligand system can be used as long as binding and elution conditions are optimized. Removal of the affinity tag and the purification of the cleaved protein can be easily and efficiently performed automatically and this method can also be extended to other proteases. A protease with fast kinetics is beneficial since it shortens the incubation times. Automation during the purification saves time and minimizes the human error. To handle all the proteins that need detailed analysis in the post-genomic era is an enormous challenge. ÄKTAxpress can help by removing some of the tedious tasks and by saving time.

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