Purification and chromatographic characterisation of an integral membrane protein

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Application Note 18-1128-92 AB

Purification and chromatographic characterisation of an integral membrane protein

- One-step purification of an integral membrane protein
- Chromatography at low temperature and in the presence of non-ionic detergents
- Chromatographic characterisation of the purified protein on the same system

Summary

ÄKTA$_{FPLC}$™ controlled by UNICORN™ software was used at cold room temperature for one-step purification and for characterisation of an integral membrane protein in the presence of non-ionic detergents. The target protein was a recombinant histidine-tagged cytochrome bo_3 ubiquinol oxidase expressed in *Escherichia coli*. Detergent extracts of bacterial membranes were applied to HiTrap™ Chelating. Adsorbed proteins were eluted by an imidazol gradient in two well separated peaks, which both contained cytochrome bo_3.

The charge and size homogeneity of the purified protein was assessed under non-denaturing conditions, in the presence of non-ionic detergent by anion exchange chromatography on Mono Q™ HR 5/5 and by gel filtration on Superdex™ HR 10/30. A single sharp peak was observed for both fractions upon salt gradient elution of proteins adsorbed to the ion-exchanger, but minor peaks were detected for the low-imidazol fraction. Similarly, gel filtration gave essentially one single peak corresponding to monomeric protein. These results indicate that the preparations were homogenous in size and charge.

The application can be performed with the more recent ÄKTApurifier™ instead of the ÄKTA$_{FPLC}$. The Mono Q HR 5/5 and Superdex HR 10/30 are replaced by Mono Q 5/50 GL and Superdex 200 10/300 GL. HisTrap™ HP or HisTrap FF crude can be used instead of HiTrap Chelating.

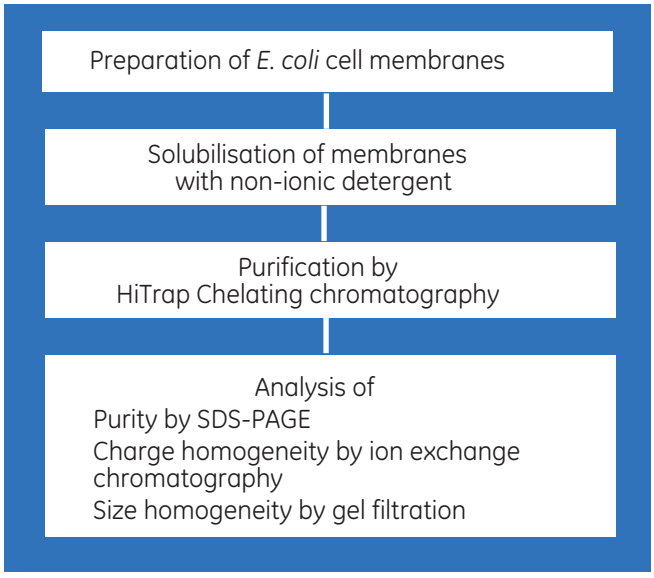


Fig. 1. Overview of the procedure.

Introduction

Detergent extracted membrane proteins can often be purified by the fractionation methods used for water-soluble proteins, although all steps of the purification must be performed in the presence of detergent. Membrane proteins in detergent solution frequently tend to aggregate or become denatured. Therefore, the concentration and type of detergent that is suitable for a particular fractionation must be tested for each situation. Due to the instability and tendency to associate there is often a need for fast purification protocols applied at low temperature.

Structure determination of proteins by crystallography and other high-resolution studies on membrane proteins usually demand that the protein can be obtained at high purity and homogeneity. Purity is usually taken to mean absence of contaminants, whereas homogeneity means similarity of the molecules in terms of conformation, association state, charge, etc. Purification of proteins should therefore not only be followed by purity analysis such as SDS-PAGE, but also by characterisation of the size and charge of the protein under non-denaturing conditions.

This application note shows the use of ÄKTAFPLC with UNICORN control for chromatographic preparation and characterisation of a recombinant histidine-tagged integral membrane protein on prepacked 1 ml and 5 ml HiTrap Chelating columns at low temperature and in the presence of non-ionic detergent. The homogeneity of the preparations was characterised by anion exchange and gel filtration on Mono Q HR 5/5 (now replaced by Mono Q 5/50 GL) and Superdex 200 HR 10/30 (now replaced by Superdex 200 10/300 GL) columns.

Results and discussion

One-step purification

Membrane protein extract in dodecyl-β-Dmaltoside solution was applied to a 1 ml prepacked HiTrap Chelating column loaded with nickel ions (Fig 2a). Elution was done in the presence of detergent by applying a gradient of imidazol concentration. Two distinct peaks were obtained and the material in fractions corresponding to both peaks had the characteristic colour of cytochrome bo_3. SDS-PAGE showed that both fractions contained the four subunits expected for cytochrome bo_3 (Fig 3). Fraction 1 gave additional bands indicating contaminations. Fraction 2 showed only traces of contaminants. Scaling-up five times using 5 ml HiTrap Chelating gave the same result, consiting of 25 mg and 18 mg protein in Fraction 1 and 2, respectively (Fig 2b). Both cytochrome bo_3 preparations were kept in the refrigerator.

Sample: Detergent extracts of Escherichia coli membranes (see Materials and methods)
Binding buffer: 20 mM Tris-HCl, 5 mM imidazol, 0.03% dodecyl-β-D-maltoside and 300 mM NaCl, pH 7.5
Elution buffer: 20 mM Tris-HCl, 500 mM imidazol, 0.03% dodecyl-β-D-maltoside and 300 mM NaCl, pH 7.5
Gradient: 0 to 60% in 20 column volumes
Temperature: 5 °C
System: ÄKTAFPLC with UNICORN control
Sample loop: Superloop 10 ml or 50 ml

a)
Sample volume: 8.5 ml
Column: HiTrap Chelating 1 ml, Ni^{2+}-loaded
Flow: 1 ml/min

b)
Sample volume: 48.5 ml
Column: HiTrap Chelating 5 ml, Ni^{2+}-loaded
Flow: 5 ml/min

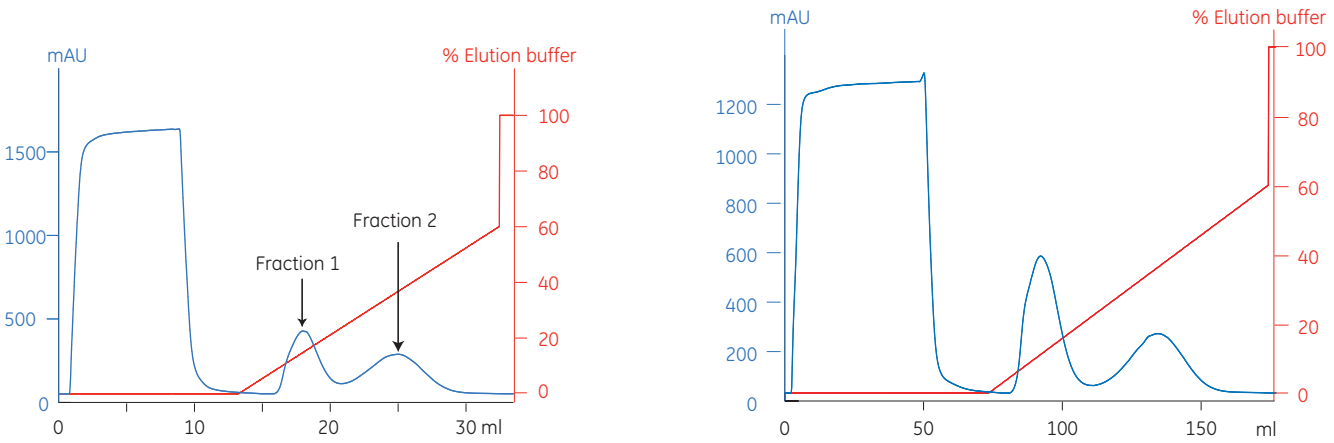
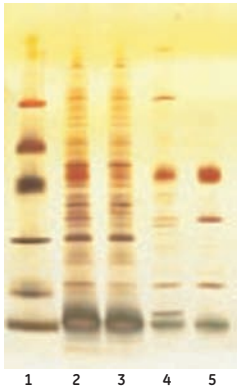


Fig. 2. Purification on HiTrap Chelating.



Lane 1: Low Molecular Weight Calibration kit (LMW) 14 400, 20 100, 30 000, 43 000, 67 000, 94 000

Lane 2: Detergent extract of Escherichia coli membranes

Lane 3: Flow-through material

Lane 4: Fraction 1 from HiTrap Chelating 1 ml

Lane 5: Fraction 2 from HiTrap Chelating 1 ml

Fig. 3. SDS electrophoresis on PhastSystem using PhastGel 8–25%, silver staining.

Charge characterisation

The cytochrome *bo*$_3$ preparations were assessed for purity and charge homogeneity under nondenaturing conditions by anion exchange chromatography on Mono Q HR 5/5 in the presence of the non-ionic detergent octyl β-D-glucoside. Fraction 1 eluted in one main peak (Fig 4a), but several small peaks were also observed that may be caused by trace contaminants. This agrees with the SDS-PAGE analysis (Fig 3). Fraction 2 gave a single sharp peak on Mono Q HR 5/5 (Fig 4b). These results confirm the high purity observed by SDS-PAGE and show that the material in Fraction 2 was highly homogenous by charge.

Size characterisation

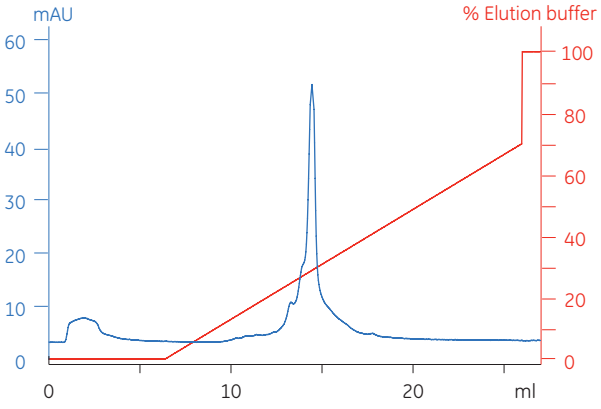
The cytochrome *bo*$_3$ preparations were analysed under non-denaturing conditions for size characterisation by gel filtration on Superdex HR 10/30 in the presence of octyl-β-D-glucoside (Fig 5). The main peak positions for both fractions were identical. Fraction 2 eluted in a narrower zone than Fraction 1, but there was no indication of large aggregates in the samples. The origin of the small unresolved peaks forming shoulders on the main peaks is unknown, but may correspond to optical effects caused by protein-free detergent micelles.

The column was calibrated by application of standard proteins from the LMW and HMW Gel filtration kits. Apparent molecular weights of the detergent-protein complexes were calculated from their elution positions by the Molecular weight calibration function. A molecular weight of 160 000±5 000 was obtained for the detergent-protein complexes eluted in the main peak. This value is probably under estimated due to the dynamic structure and flexibility of the detergent moiety of the complexes, but indicated that cytochrome bo$_3$ is inserted in the detergent micelles as monomer. Chromatography in 1% octyl-β-Dglucoside revealed detergent-protein complexes of a molecular weight of 410 000±3 000 (data not shown), indicating that cytochrome *bo*$_3$ formed dimers under these conditions.

Column:	Mono Q HR 5/*5*
Binding buffer:	20 mM Tris-HCl, 1% octyl-β-D-glucoside, pH 7.5
Elution buffer:	20 mM Tris-HCl, 1% octyl-β-D-glucoside, 1 M NaCl, pH 7.5
Gradient:	0 to 50%, in 20 column volumes
Flow:	1 ml/min
Temperature:	5 °C
System:	ÄKTA$_{FPLC}$ with UNICORN control
Sample loop:	Superloop 10 ml

a)

Sample: 2.5 ml fraction 1-material diluted 10 times with binding buffer



b)

Sample: 3 ml fraction 2-material diluted 10 times with **binding buffer**

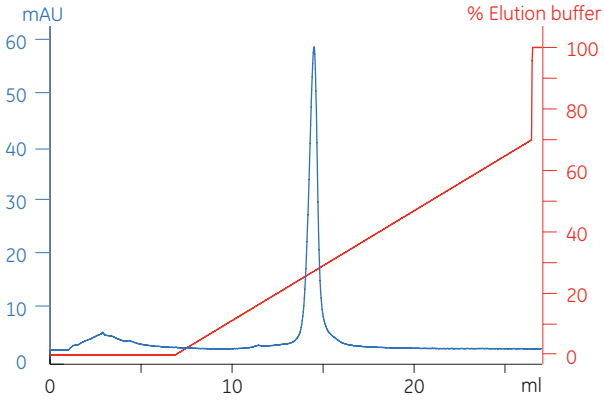
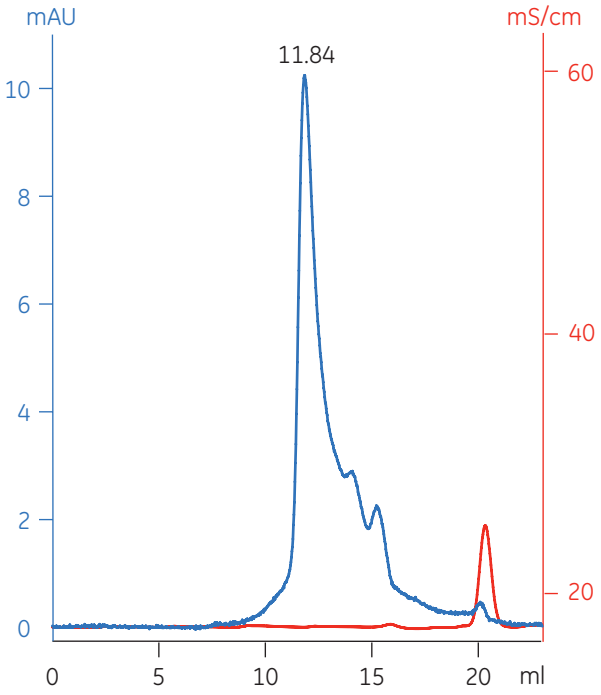


Fig. 4. Chromatographic characterisation on Mono Q HR 5/5.

Column: Superdex 200 HR 10/30
Sample application: 300 µl applied via 0.1 ml sample loop
Buffer: 20 mM Tris-HCl, 1.5 % octyl-β-D-glucoside, *150 mM NaCl, pH 7.5*
Flow: 0.25 ml/min
Temperature: 5 °C
System: ÄKTA$_{FPLC}$ with UNICORN control

a)
Sample: Fraction 1 from HiTrap Chelating 1 ml



b)
Sample: Fraction 2 from HiTrap Chelating 1 ml

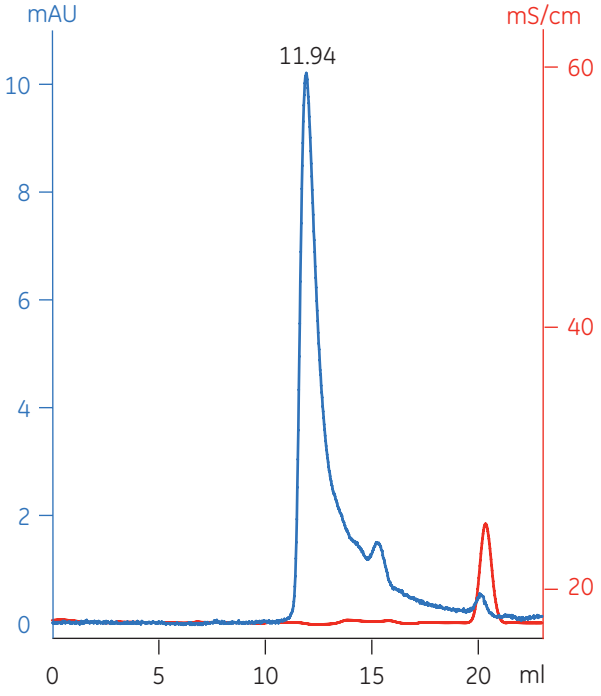


Fig. 5. Chromatographic characterisation on Superdex 200 HR 10/30.

Materials and methods

Materials

All columns and sample loops were from GE Healthcare.

Sample preparation

Recombinant histidine-tagged cytochrome bo_3 from *Escherichia coli* was expressed in *E. coli* (1). This 130 000 Dalton enzyme is composed by four subunits (molecular weights 58 000, 33 000, 22 000, 17 000) of which the subunit II contain the histidine-tag on the carboxy-terminus. Bacterial membranes were prepared essentially as described by Rumbley *et al.* (1) and were solubilised by addition of 10 ml 1% dodecyl-β-D-maltoside, 300 mM NaCl in 20 mM Tris-HCl buffer, pH 7.5 per 1 g sedimented membranes followed by stirring on ice for 30 min. Non-solubilised material was removed by centrifugation at 40 000 × g for 20 min.

Chromatography

ÄKTA$_{FPLC}$ with UNICORN was used for chromatography. The system was equipped with Pump P-920, Monitor UPC 900 with a 2 mm pathlength and 280 nm filter, Monitor pH/C-900, Fraction Collector Frac-900, mixer M-900 with 0.6-ml mixing chamber, Buffer A inlet valve (IV-908), Column position valves (PV-908), and an outlet valve (PV-908). Buffers were prepared at 22 °C to given pH. Chromatography was performed at 5 °C. Experimental details are given in the figures.

Acknowledgments

The purification protocol was developed in corporation with Dr. So Iwata and Mr. Jeff Abramson at Department of Biochemistry, Uppsala University, Sweden. This work was supported by EU grant BIO4-CT97-2357 (DG12-SSMI) "New approaches in the crystallisation and crystallography of membrane proteins".

References

1. Rumbley, J. N., Furlong Nickels, E. and Gennis, R. B. (1997) One-step purification of histidine-tagged cytochrome bo_3 from *Escherichia coli* and demonstration that associated quinone is not required for the structural integrity of the oxidase. *Biochim. Biophys. Acta 1340*, 131–142.

Ordering information

Products	Quantity	Code no.
ÄKTA$_{FPLC}$		18-1118-67
HiTrap Chelating 1 ml	5	17-0408-01
HiTrap Chelating 5 ml	1	17-0409-01
Mono Q 5/50 GL	1	17-5166-01
Superdex 200 10/300 GL	1	17-5175-01
Superloop 10 ml	1	19-7585-01
Superloop 5 ml	1	19-7850-01

Related products

ÄKTApurifier 10		28-4062-64
Superdex 200 5/150 GL	1	28-9065-61
HisTrap HP 1 ml	5	17-5247-01
HisTrap HP 5 ml	1	17-5248-01
HiTrap IMAC HP 1 ml	5	17-0920-03
HiTrap IMAC HP 5 ml	5	17-0920-05

Accessories

Gel Filtration LMW Calibration Kit	1	28-4038-41
Gel Filtration HMW Calibration Kit	1	28-4038-42

Related product literature

Literature	Code no.
Application note: Purification of a labile, oxygen-sensitive enzyme for crystallisation and 3D structure determination	18-1128-91
Rapid optimisation and development of an automated two-step purification procedure for monoclonal IgG antibodies	18-1128-93
Data File: ÄKTAexplorer™ System Series	18-1124-09
Data File: ÄKTApurifier	18-1119-48
Data File: Monitor UPC-900	18-1128-40
Data File: UNICORN control system	18-1111-20
Purifying Challenging Proteins Handbook	28-9095-31
Gel Filtration Handbook	18-1022-18
Recombinant Protein Purification Handbook	18-1142-75

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