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Rapid screening of integral membrane proteins – detergent combinations suitable for structure determination

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Structural determination of integral membrane proteins (IMPs) requires protein samples of high purity and homogeneity. In the purification process, IMPs are extracted from their natural lipid environment into detergent-protein complexes. To find optimal conditions for solubilization and purification, different detergents need to be tested. In this study, seven transporters from the same protein family were recombinantly expressed in *E. coli*. Scouting of purification conditions was performed on a 3-ml Superdex™ 200 5/150 GL column to evaluate protein quality. Five of the proteins crystallized directly in initial sparse matrix screens, highlighting the strength of the screening strategy.

Introduction

More than 60% of all clinical drug targets are in fact membrane proteins and therefore a main focus of the pharmaceutical industry. These proteins are challenging to study due to their hydrophobic nature, which often leads to significant problems with expression, purification, and crystallization. Structural information on this group of proteins is, today, limited. The purification of integral membrane proteins (IMPs) requires that they are extracted from the lipid bilayer of the cell membrane, which can be achieved by detergent solubilization. The resulting water-soluble detergent-IMP complexes can then be purified by standard chromatography techniques. However, detergents represent a poor lipid bilayer mimetic and therefore often lower the stability and activity of IMPs.

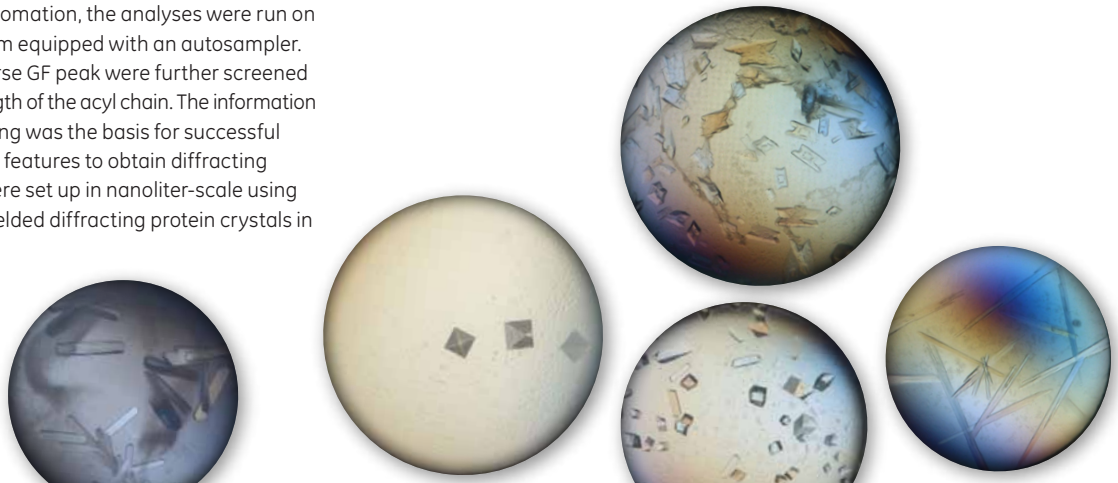
In this study, we describe a generic procedure for screening combinations of detergents and IMPs that are suitable for structure determination. Seven histidine-tagged transporters from the same protein family and with a prokaryotic origin were selected. The proteins were purified from solubilized membranes in four detergents: n-dodecyl-β-D-maltopyranoside (DDM), 5-cyclohexyl-1-hexyl-β-D-maltoside (CYMAL™-5), n-Dodecyl-N, N-dimethylamine-N-oxide (LDAO) and Fos-Choline™-12 (FC-12) using His MultiTrap™ FF 96-well plates. Directly after elution, protein purity and homogeneity were evaluated by gel filtration (GF) on a 3-ml Superdex 200 5/150 GL column. For increased automation, the analyses were run on an ÄKTAmicro™ chromatography system equipped with an autosampler. Target proteins eluting as a monodisperse GF peak were further screened in maltoside detergents with variable length of the acyl chain. The information obtained from the analytical GF screening was the basis for successful scale-up purification and one of the key features to obtain diffracting protein crystals. Crystallization trials were set up in nanoliter-scale using robotics. Five of the analyzed targets yielded diffracting protein crystals in initial sparse-matrix screens.

Protein expression, solubilization, and small-scale affinity purification

Recombinant membrane proteins (with a cleavable C-terminal hexa-histidine tag) were overexpressed in *E. coli* C41(DE3) at low temperature (20°C) for 20 h in 100 ml Terrific Broth (TB) medium (0.2 mM IPTG induction). After cell lysis, the membrane fraction was collected by ultracentrifugation (100 000 × g at 4°C for 45 min). The membranes were resuspended in solubilization buffer (20 mM sodium phosphate buffer [pH 7.5], 300 mM NaCl, 20 mM imidazole, 100 × dilution of EDTA-free Complete Protease Inhibitor Cocktail [Roche], 5% glycerol). Small aliquots were then flash-frozen in liquid nitrogen and stored at -80°C. After gentle thawing in an ice-containing water bath, the membranes were solubilized by four different detergents (DDM, CYMAL-5, LDAO and FC-12, [Affymetrix]) and incubated with end-over-end rotation for 90 min at 4°C. Nonsolubilized material was removed by ultracentrifugation (100 000 × g at 4°C for 15 min).

Small-scale affinity protein purification was performed by immobilized metal ion affinity chromatography (IMAC) using His MultiTrap FF 96-well plates according to the manufacturer's specifications. The wells were equilibrated in binding buffer (20 mM sodium phosphate buffer [pH 7.5], 300 mM NaCl, 20 mM imidazole and the detergent used for solubilization). Unbound proteins were removed by washing with binding buffer containing 40 mM imidazole. Target proteins were eluted by addition of binding buffer containing 500 mM imidazole (Fig 1). All seven targets could be solubilized and purified by affinity purification in the four detergents tested.

In the standard protocol, target proteins were solubilized and purified by IMAC in four different detergents separately (solubilization/purification: 1%/0.03% DDM, 1%/0.2% CYMAL-5, 1%/0.1% LDAO, and 1%/0.1% FC-12). IMAC-purified proteins were subsequently analyzed by GF in the presence of corresponding detergents that were used in the IMAC step.



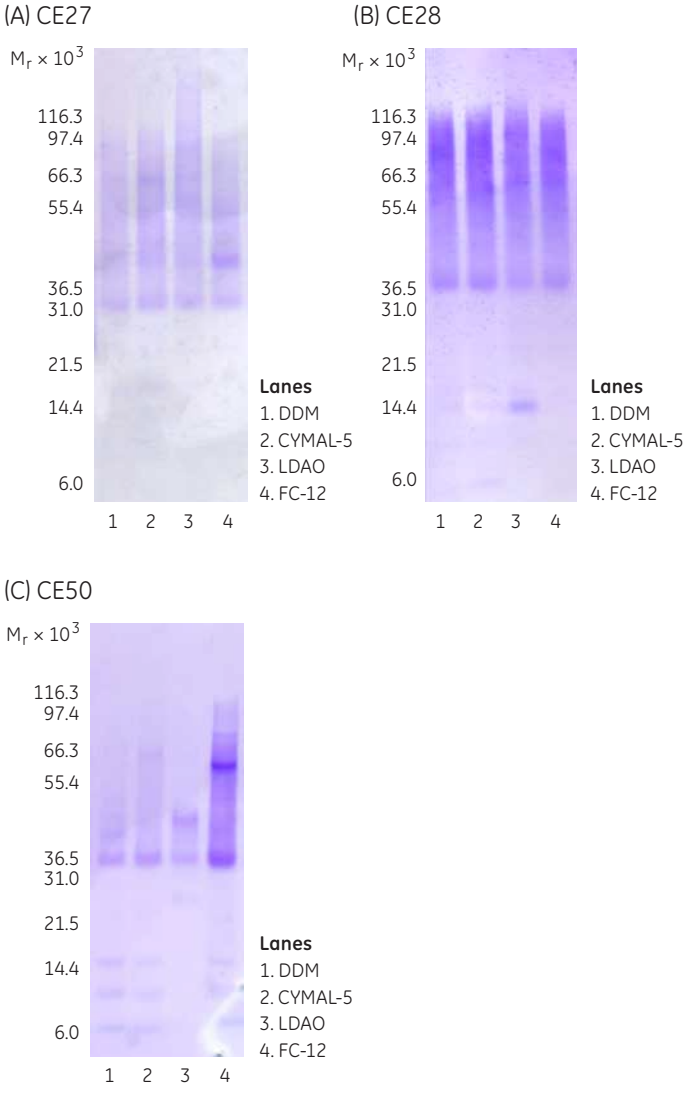


Fig 1. Parallel IMAC purification of integral membrane proteins using His MultiTrap FF. Membranes were solubilized and proteins purified in buffer supplemented with four detergents. (A) Target protein CE27 eluted with buffer containing 500 mM imidazole. (B) Target protein CE28. (C) Target protein CE50.

Analytical gel filtration

GF analyses were performed on Superdex 200 5/150 GL column using ÄKTAmicro chromatography system equipped with an autosampler. The column was equilibrated and eluted in GF buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 5% glycerol, 0.5 mM TCEP and detergent.)

Using this procedure, it was easy to quickly evaluate and determine optimal detergents for the IMP targets. Figure 2 shows a complete set (12 runs) of GF chromatograms for three IMPs (CE27, CE28, and CE50) that were prepared using the standard protocol. On one hand, the results demonstrate that the IMP targets were efficiently solubilized and purified by IMAC (Fig 1) although on the other hand, the quality and homogeneity of the samples varied dramatically depending on which detergent had been used (Fig 2). In the presence of LDAO, all three target IMPs showed very broad and asymmetric GF peaks indicating multiple molecular forms and therefore low protein quality. CE27 showed a leading peak in DDM (Fig 2A), while its two homologs, CE28 (Fig 2B) and CE50 (Fig 2C), displayed sharper and more symmetrical GF peaks using the same detergent. The leading peak of CE27 may indicate that the sample contains two oligomeric species. CE28 was also stable in CYMAL-5 and in FC-12, leading to well-defined, symmetrical peaks in all tested detergents except LDAO.

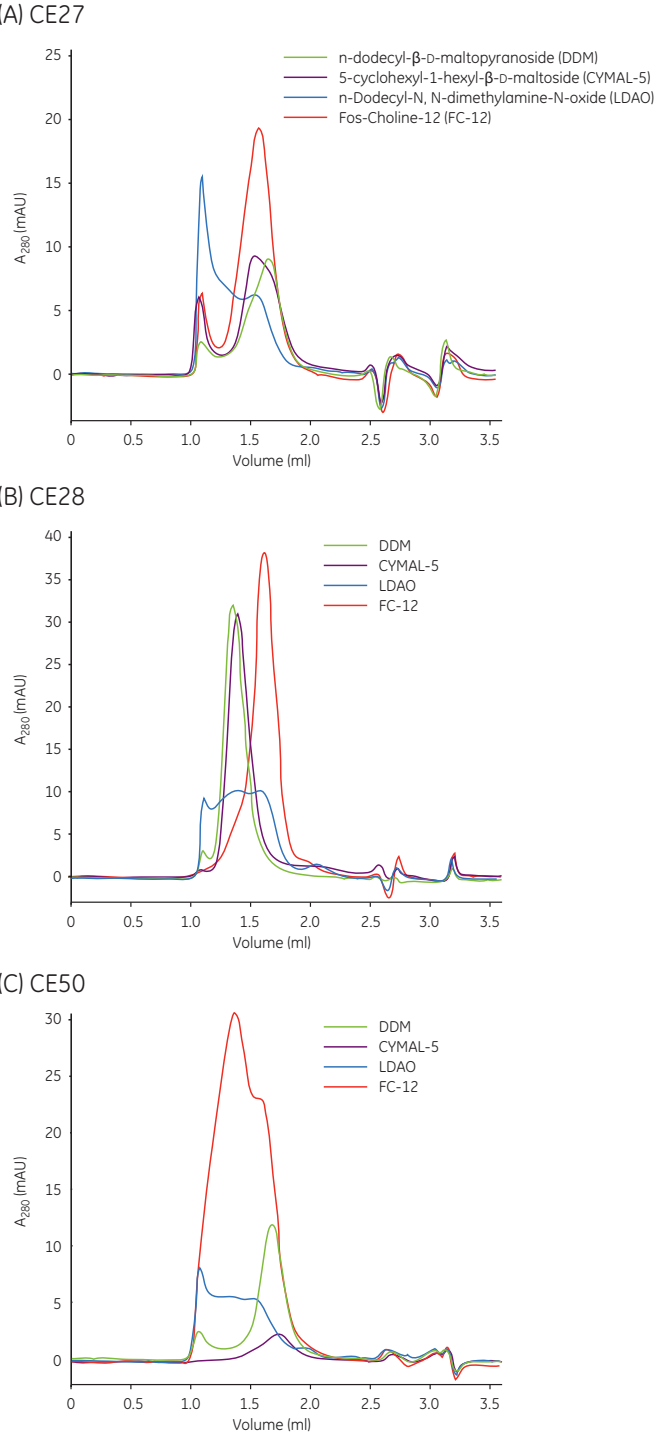


Fig 2. Generic buffer scouting of integral membrane proteins. (A) GF profiles of target protein CE27. GF buffer: 20 mM HEPES (pH 7.5), 300 mM NaCl, 5% glycerol, 0.5 mM TCEP and detergent (detergents used indicated on each chromatogram). (B) GF profiles of target protein CE28 (GF buffer as in 2A) and (C) GF profiles of target protein CE50 (GF buffer as in 2A).

Preparative protein purification

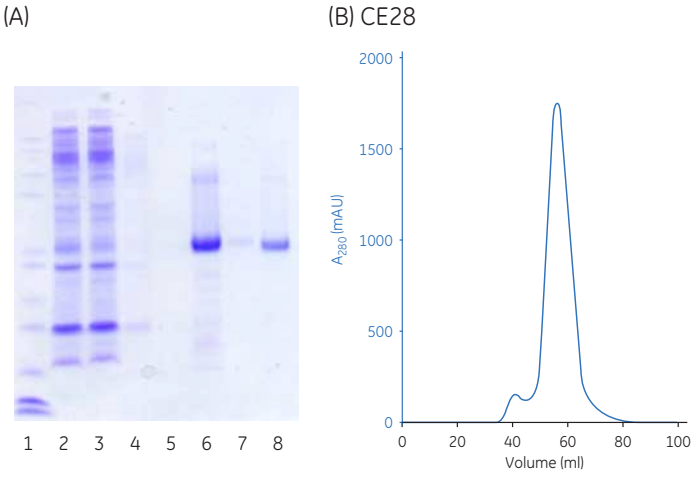
Proteins were overexpressed in 2 × 1 l TB medium and membrane fractions were isolated as described above. Subsequently, membranes were solubilized using the appropriate detergent as determined by the analytical GF strategy outlined above and incubated for 90 min at 4°C with intensive stirring. Nonsolubilized material was removed by ultracentrifugation (100 000 × g at 4°C for 15 min).

Preparative IMAC purification was performed by batch-adsorption of 140 ml of solubilized membranes by end-over-end rotation with 6 ml of Ni Sepharose™ 6 Fast Flow chromatography medium for 30 min. After binding of proteins to the medium, the Ni Sepharose 6 Fast Flow was packed in a 10-mm (inner diameter, i.d.) open gravity-flow column and unbound proteins were removed by washing with 10 bed volumes of binding buffer (*cf.* above). Thereafter, the IMAC medium was further washed by 10 bed volumes of binding buffer containing 30 mM imidazole. Target proteins were eluted by addition of 5 bed volumes of binding buffer containing 500 mM imidazole. Directly after elution, the target protein was transferred to a dialysis bag (molecular-weight cutoff [MWCO] 25 000) and recombinant TEV protease was added to a final concentration of 0.7 µM. Dialysis against GF buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 0.03% DDM) was performed overnight in the cold room. Subsequently, the TEV protease (containing uncleavable histidine tag) and uncleaved protein were removed from the sample by passing it over 2 ml of IMAC medium (reverse IMAC). Small aliquots from each IMAC (washing and elution) step as well as from the tag cleavage were analyzed on SDS-PAGE gel (Fig 3A). SDS-PAGE demonstrated an efficient binding (Fig 3; lane 3) to the medium and a high purity of the target protein after elution (Fig 3; lane 6). After TEV cleavage, a small shift could be observed on the gel, showing that the histidine tag had been efficiently cleaved (Fig 3; lane 8).

The cleaved target protein was concentrated to 5 ml using a Vivaspin™ 20 (MWCO 100 000) sample concentrator and thereafter loaded onto a HiLoad™ 16/600 Superdex 200 pg column using ÄKTAexplorer™ 10 chromatography system. The column was equilibrated and eluted in GF buffer (*cf.* above) containing 0.03% DDM. Figure 3B shows a sharp and symmetrical GF peak for CE28, indicative of a homogeneous protein sample. Peak fractions were pooled and concentrated to between 6 and 10 mg/ml protein.

Large micelle-forming detergents are more likely to maintain IMPs in solution. However, the large size of the micelle masks the protein and less of the protein is thus accessible to form protein-protein interactions that are essential for crystal-lattice formation. In contrast, small micelle detergents expose more of the surface and thus increase the chance of obtaining a solid crystal-lattice arrangement. Although detergent with short alkyl chains may be favorable for crystallization, it should be noted that they also tend to be more destabilizing than longer chain detergents. Therefore it is necessary to have a screening method such as analytical GF to validate protein stability and homogeneity.

Proteins displaying homogeneous GF profiles in the presence of DDM were further analyzed using several maltosides of variable acyl chain length. Proteins were solubilized and purified in DDM as described above, thereafter the detergent was exchanged on a Superdex 200 5/150 GL column to either n-undecyl-β-D-maltopyranoside (UDM) or decyl maltose neopentyl glycol (DM-NG). Proteins were also both solubilized and purified in UDM and DM-NG, respectively. DM-NG is an example of a novel detergent that has emerged as a new tool for stabilizing and crystallizing IMPs (1). CE28 (Fig 4A) is most stable in DDM. Analytical GF revealed a dramatic drop in peak height when DDM was exchanged to DM-NG or UDM. A possible explanation for the reduced peak height is a less stable protein resulting in protein aggregation. CE96 (Fig 4B), however, is stable in all detergents tested. Due to different sizes of micelles, a shift in the retention time of the eluted proteins is observed. The volume of the column is small (3 ml) and the cycle time only 12 min. Still, it is sufficient time to exchange detergents as shown by identical retention time, independent of whether proteins are solubilized and purified in the same detergent or purified in one detergent that is thereafter changed on the column (Fig 4B).



Lanes

1. Molecular weight marker
2. Total membranes
3. Material not bound to IMAC medium
4. Protein washed out from IMAC with buffer containing 20 mM imidazole
5. Proteins washed out from IMAC with buffer containing 30 mM imidazole
6. Target protein eluted with 5 bed volumes buffer containing 500 mM imidazole
7. Second elution, addition of a further 5 bed volumes buffer containing 500 mM imidazole
8. TEV protease cleaved target purified on negative IMAC

Fig 3. Preparative purification of CE28. (A) IMAC (Ni Sepharose 6 Fast Flow) batch purification of 140 ml of solubilized membrane proteins. (B) GF chromatogram (Superdex 200 16/600 GL) of TEV cleaved CE28.

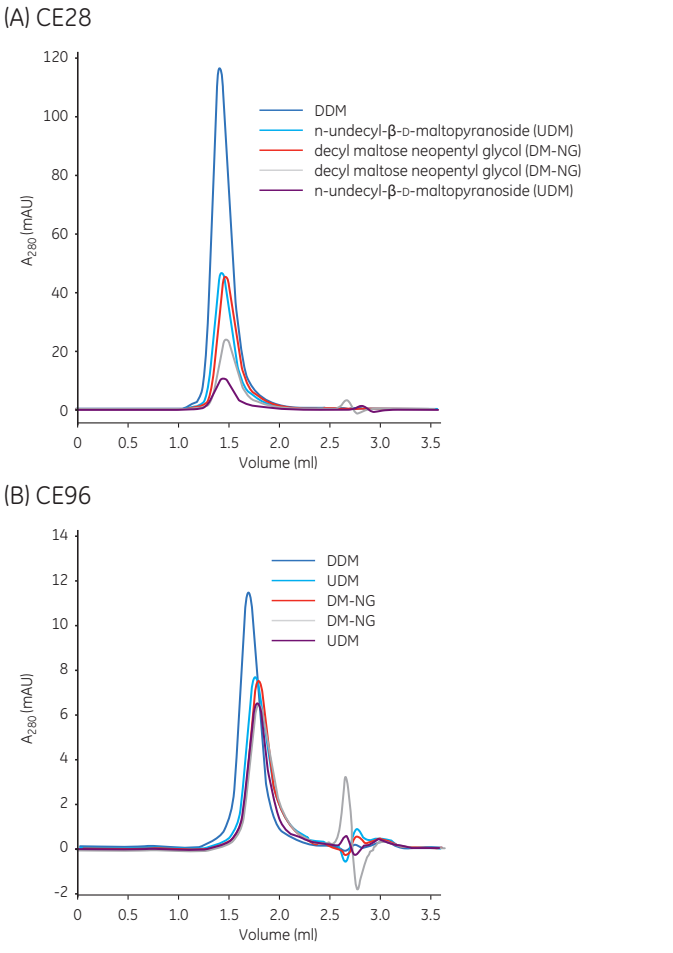


Fig 4. Detergent optimization for purification of CE28 and CE96 IMPs. Five maltosides of variable acyl chain length were tested (detergents used for solubilization and purification indicated on each chromatogram).

Crystallization of IMPs

Prior to crystallization trials, IMPs were concentrated to between 6 and 10 mg/ml using Vivaspin 20 concentrators. In order to avoid concentrating empty detergent micelles, the largest possible pore size was chosen. Analytical GF has turned out to be a powerful tool to perform quality control (QC) prior to initiating crystallization trials. A symmetrical and sharp elution peak in the chromatogram is indicative of a monodisperse protein-detergent complex preparation. Samples of only 2 µl were injected using the micro-pickup setting on the autosampler. Figure 5 shows GF chromatograms of three homologous IMPs (in 0.03% DDM) at a protein concentration of 6 to 8 mg/ml. All protein samples are monodisperse and homogeneous according to the GF profile.

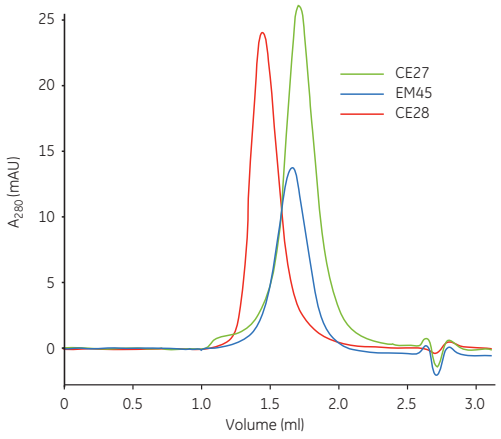


Fig 5. Quality control of concentrated protein samples. Pooled fractions from preparative GF were concentrated to between 6 to 8 mg/ml using Vivaspin 100 000 MWCO cutoff concentrators. Two microliters of concentrated sample were injected onto a Superdex 200 5/150 GL. GF buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, and 0.03% DDM.

For the crystallization screening, three sitting-drop 96-well plates (Corning™) were used. The protein was dispensed onto the plate using a Mosquito™ (TTP LabTech) nanodrop crystallization robot. Each drop contained protein and precipitant in a ratio of 1:1, 2:1, and 1:2 (150:150 nl). Commercially available sparse matrices, which are based on successful published crystallization conditions, were used to screen for the best crystal condition. Three sparse matrices were tested: MemGold™ (Molecular Dimensions), NeXtal™ JCSG, and NeXtal MbClass II (Qiagen™). Plates were incubated at room temperature and in cold-room conditions.

Protein crystals appeared after 4 to 10 days and grew to full size within two weeks (Fig 6). Crystals were flash-frozen in liquid nitrogen. Five (C27, CE28, CE50, CE95, and CE96) of seven IMP targets resulted in diffracting protein crystals already in sparse-matrix screens. Currently, the initially identified crystallization conditions are being optimized to increase the resolution of the crystals.

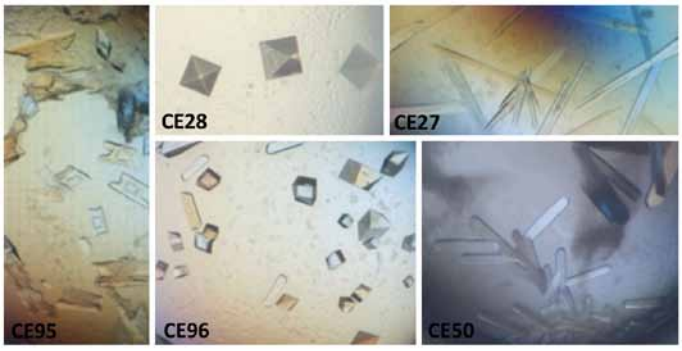


Fig 6. Integral membrane protein crystals diffracting to 4 to 14 Å maximum resolution. Protein crystals appeared after 4 to 10 days and grew to full size within two weeks.

References

1. Gellman, S. H. *et al.* Maltose-neopentyl glycol (MNG) amphiphiles for solubilization, stabilization and crystallization of membrane proteins. *Nat Methods* **7**, 1003–1008 (2010).

Acknowledgment

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Ordering information

Product	Code number
Superdex 200 5/150 GL, 3 ml	28-9065-61
HiLoad 16/600 Superdex 200 pg, 120 ml	28-9893-35
His MultiTrap FF, 4 × 96-well filter plates	28-4009-90
ÄKTAmicro system	28-9483-03

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