Stability of membrane proteins analyzed by gel filtration

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Well-ordered three-dimensional crystals for structure determination require that the protein preparation is size-homogeneous and stable over a long period of time. Purification of integral membrane proteins requires extraction from their natural lipid environment into detergent-protein complexes. Detergents represent a poor lipid bilayer mimetic and therefore the stability and activity of IMPs is often low. This article describes the use of gel filtration for size-homogeneity analysis to study protein stability and influence of freezing-thawing on integral membrane protein quality.

Introduction

After years of research, there is still no golden bullet for receiving well-diffracting membrane protein crystals for structure determination. It is generally accepted that success can only be obtained by extensive screening and optimization of conditions for their preparation. A key requisite for well-diffracting membrane protein crystals is a protein preparation of high purity and size-homogeneity.

In most research laboratories, histidine-tagged membrane proteins are purified in two steps. In the first step, detergent solubilized protein is captured by immobilized metal ion affinity chromatography (IMAC), removing most impurities. In the second step, gel filtration (GF) is used to remove remaining impurities as well as unwanted oligomers and aggregates of the target protein. Earlier, we described the use of GF for screening a wide range of detergents and buffer conditions (pH, salts, and additives) to obtain size-homogeneous integral membrane proteins (IMP) preparations (1). A high enough purity and size-homogeneity can often be obtained, but stability of the purified protein is often limited.

In an ideal workflow for IMP purification, all steps from cell-harvesting over protein purification to start-up of crystallization trials are performed within one day. In reality, an isolated protein often needs to be stored either in the cold room, on ice, or frozen to be thawed later. This handling is often destructive to the protein. During the freeze-thaw process, ice crystals are formed causing buffer salts and proteins to be concentrated. This can influence the stability of the protein significantly (2). To minimize the risk of damaging the protein, process precautions can be made by, for example, addition of glycerol (5% to 10%) and snap-freezing in small aliquots, preferably using liquid nitrogen. Freezing in phosphate buffer sometimes causes decrease in pH due to precipitation of the basic component. Therefore, addition of Tris buffer is often advantageous.

Quality control should preferably be performed before the stored protein is used for further experiments to investigate whether storage at 0°C to 4°C or freeze-thawing has affected the protein. Analytical GF is a powerful tool for determining the homogeneity of proteins. A symmetrical and sharp elution peak in the chromatogram indicates a correctly folded and homogeneous protein preparation.

Here we demonstrate the use of GF for studying the influence of long-term storage and freeze-thaw processes using minimal amounts of purified IMPs. Analytical GF runs were performed on Superdex™ 200 5/150 GL (column volume of 3 ml) using an ÄKTAmicro™ chromatography system. Samples of 25 µl were injected using an autosampler, and separation was performed at 0.25 ml/min with cycle times of 12 min.

Protein expression and purification

IMPs carrying N-terminal hexa-histidine tag were expressed in *E. coli* C41 (DE3) cells at 20°C for 20 h. Cells were disrupted using a high-pressure homogenizer (Avestin), and membranes were harvested by ultracentrifugation at 100 000 × g for 1 h. Membranes were resuspended in solubilization buffer (S-buffer; 20 mM sodium phosphate buffer, 300 mM NaCl, 20 mM imidazole, 5 % glycerol, pH 7.5), and subsequently solubilized in the presence of 1% n-dodecyl β-D-maltoside (DDM) with stirring for 1 h at 4°C. Nonsolubilized material was removed by ultracentrifugation at 100 000 × g at 4°C for 15 min. Purification was performed using IMAC by batch adsorption on Ni Sepharose™ 6 Fast Flow medium for 30 min with end-over-end rotation in a cold room. The chromatography medium was packed in a 10-mm (i.d.) open column for gravity flow.

The column was washed with 20 mM imidazole (10 column volumes, CV), 45 mM imidazole (20 CV), and eluted with 500 mM imidazole (5 CV), all in S-buffer supplemented with 0.03% DDM. Eluted material (5 ml) was applied on HiLoad™ 16/60 Superdex 200 pg gel filtration column at 1 ml/min in GF buffer without imidazole and supplemented with 0.03% DDM.

Long-term stability test

An aliquot of 50 µl from a preparation (1 mg/ml) was stored in a cold room for a period of 10 days. Aliquots of 5 µl were taken out at selected times and diluted with 45 µl of 20 mM HEPES, 150 mM NaCl, 5% glycerol and 0.03% DDM, pH 7.5, and 25 µl of this sample was immediately analyzed by analytical GF. To remove precipitated material in the stored sample, the test tube was centrifuged in a benchtop centrifuge at 20 000 × g for 5 min in the cold room before sample was taken.

Freeze-thaw test

Aliquots of 50 µl of pure protein were subjected to different freeze-thaw cycles: Sample 1: no freezing; Sample 2; freezing in -20°C overnight; Sample 3: freezing in liquid nitrogen and stored at -80°C for 24 h. Samples were thawed by incubation at 4°C for 1 h, centrifuged at 20 000 × g for 5 min, and subsequently analyzed by GF as described earlier.

Results

The prokaryotic T2 channel 2 was stored at 4°C and GF analyses were performed at different times (Fig 1). The fresh protein was monodisperse as judged from the single peak after GF. After one day, a small void peak appeared. Increased storage time led to a dramatic quality loss of channel T2 due to aggregation, as shown by lower and broadened GF peaks compared with the fresh material.

Column:	Superdex 200 5/150 GL
Buffer:	20 mM HEPES, 150 mM NaCl, 5% glycerol, 0.03% DDM, pH 7.5
Flow rate:	1 ml/min
System:	ÄKTAmicro

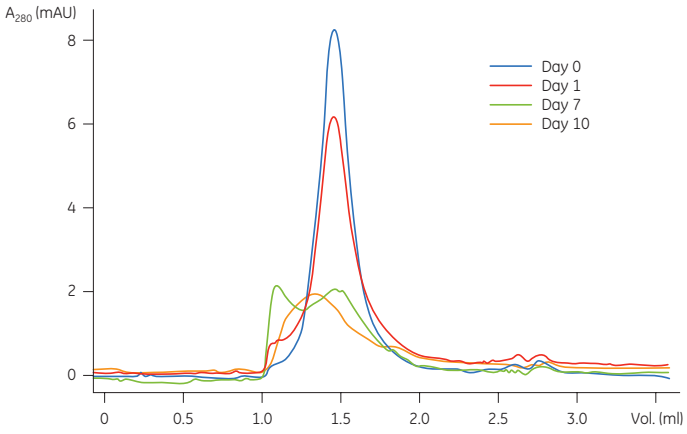

Fig 1. Long-term stability of the prokaryotic T2 channel 2-$(His)_6$. GF analyses were performed after storage for 0, 1, 7, and 10 days. A broader and lower peak indicates loss of protein integrity. Early peaks indicate protein aggregates.

The comparison of GF profiles after a slow freezing process (-20°C, red curve) or flash freezing in liquid nitrogen (green curve) with the nonfrozen sample (blue curve) revealed the importance of fast freezing (Fig 2). Freezing slowly at -20°C in the presence of 5% glycerol and thawing completely precipitated the sample.

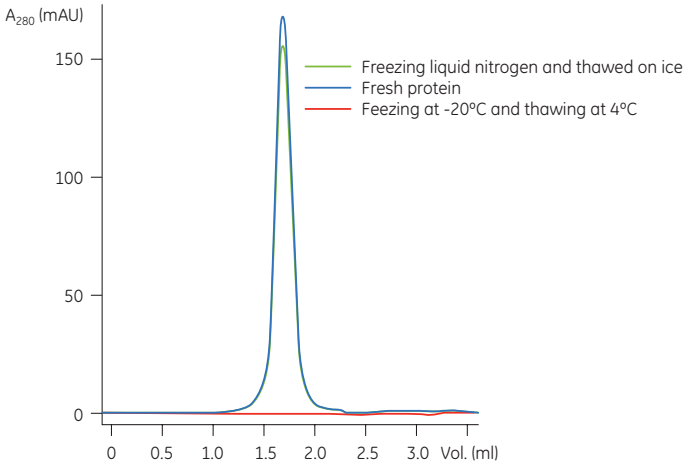

Fig 2. Effects of freezing and thawing of a purified prokaryotic and enzymatically active IMP in detergent solution. GF analyses were performed as in Fig. 1. Fresh protein (blue), protein snap-frozen in liquid nitrogen and thawed on ice (green), and protein frozen at -20°C and thawed at 4°C (red, no peak).

Conclusions

Analytical GF demonstrates how storage time and/or freezing process affect the stability and homogeneity of IMPs. The short analysis time obtained with a Superdex 200 5/150 GL column enables rapid quality control of IMPs with limited consumption of sample, buffer, and detergent.

References

1. C. Löw, *et al.* Rapid buffer scouting and quality control of integral membrane proteins. *Discovery Matters* **12**, 10–11 (2010).
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Ordering information

Product	Code number
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