

Membrane protein production in *Pichia pastoris* for structural biology

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Recently an exciting increase in the number of structures of eukaryotic heterologously expressed membrane proteins have been observed (*i.e.* Long *et al.* 2006, Molina *et al.* 2007 and Tornroth-Horsefield *et al.* 2006). *Pichia pastoris* provides an eukaryotic expression host that is simple to use and can reach high cell density. Here we show production and purification of ion channels and GPCRs using *Pichia pastoris*. Moreover we show a set off biophysical methods used for protein characterization and optimization of protein stability.

Pichia pastoris expression screening

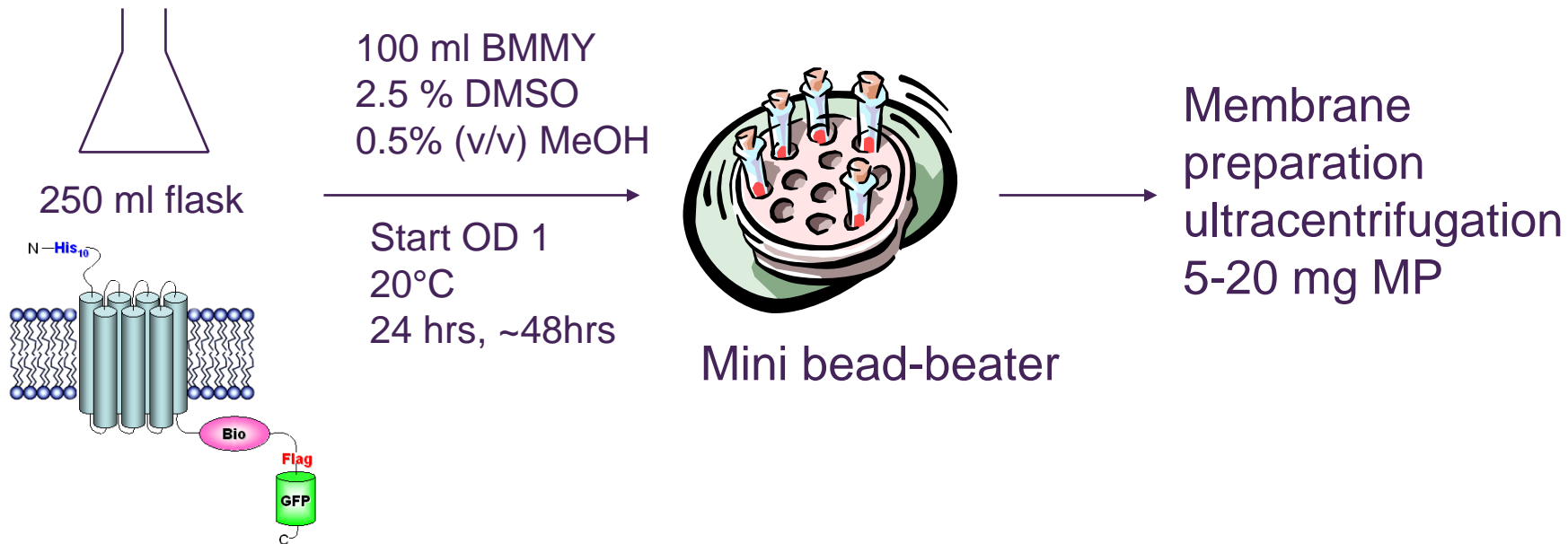


Figure 1 Expression screening process. Constructs have an N-terminal His tag, on the C-terminal side proteins are decorated with a Bio-tag and FLAG tag, a GFP is optional. Three to five colonies (SMD1163) are selected and grown in 100 ml BMMY supplemented with DMSO. Samples are retrieved after 24 and ~48 hrs. Cells are broken in a glass bead-beater and membranes are prepared using ultracentrifugation. 50 ml of culture results in approximately 1-1.5 gr wet weight cells and 5 to 20 mg of membrane proteins.

Pichia pastoris expression screening

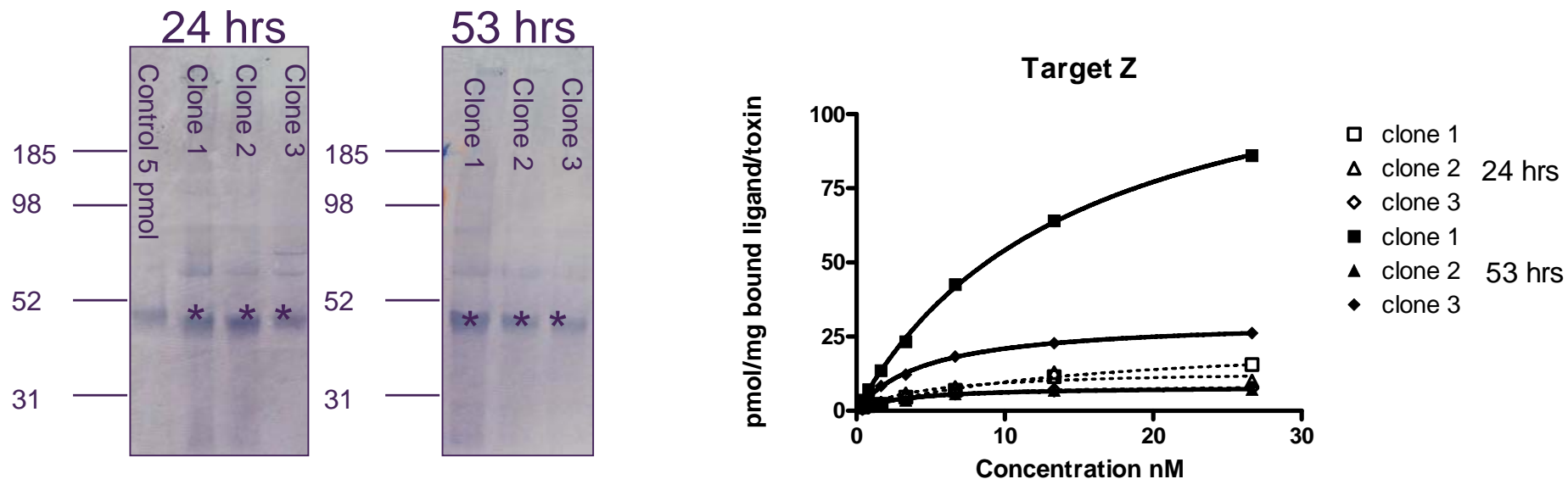


Figure 2 Analysis of expression screening for a GPCR. Expression screening is analysed by western blot using antibodies aimed at both N- and C- terminal tags, asterixes indicate bands positive to both His and Bio-tags. Expression is followed by binding assays when assays are available, correlation between western blot signals and binding activity are poor, see also (ref MEPNET). Substantial clone-to-clone variation is observed and levels of binding affinity, western blot signals and fluorescent signals (GFP) at longer incubation times are in general higher.

GFP-tag tool for membrane proteins

GFP (green fluorescent protein) has a wide application range and can serve as an excellent tool for membrane protein expression, solubilization and purification. Expression has been analysed for 7 GPCRs and 2 ICs both with and without GFP. Although GFP constructs show a tendency for proteolytic degradation, the tag is suitable for establishing a purification strategy.

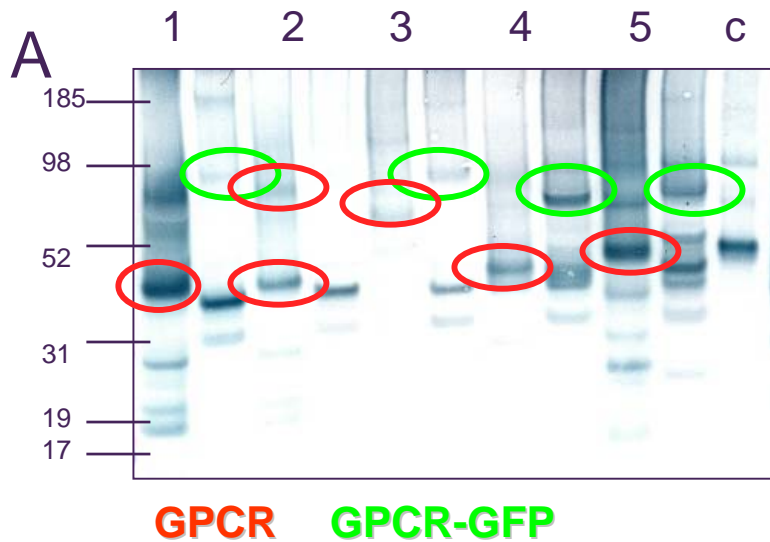
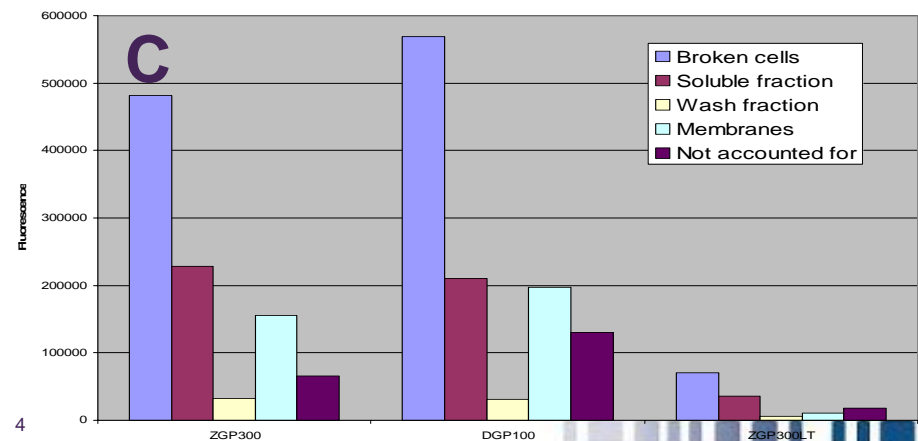
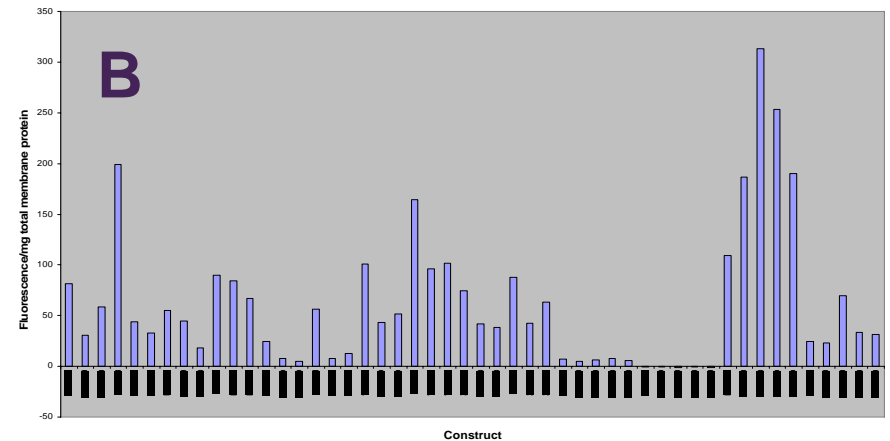


Figure 3 GFP as tool in protein production, expression analysis and solubilization. A) anti-bio western blot of 5 different GPCRs with (green) and without (red) tag. C-terminal degradation of GFP constructs is apparent. B) Expression analysis of different constructs judged by fluorescence prepared membranes. C) Approximately 30% of the total fluorescence in the cells is recovered in the membrane fraction.



Fluorescence Size Exclusion Chromatography

Fluorescence size exclusion chromatography (FSEC) combines detection of a fluorophore (GFP) with size-exclusion (Figure 4a, Kawata & Gouaux 2006). Here, FSEC is applied to discriminate between GPCR preparation in *Pichia* or mammalian cells; the GPCR is entirely aggregated when solubilized from *Pichia* membranes but shows little aggregation when solubilized from mammalian cell membranes. FSEC gives an early biophysical quality control that allows improved design of expression and purification strategy.

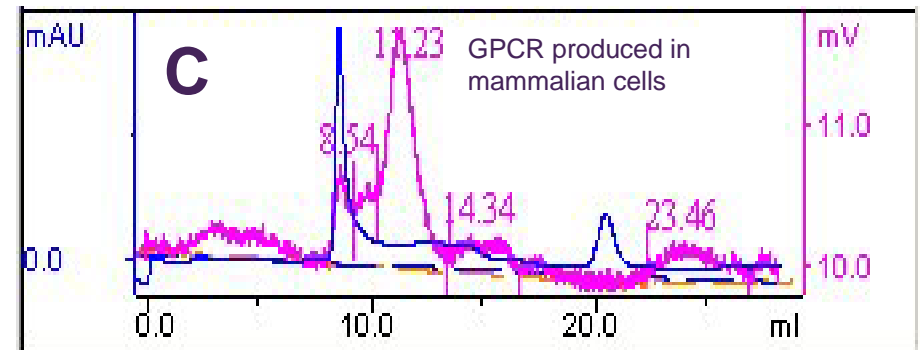
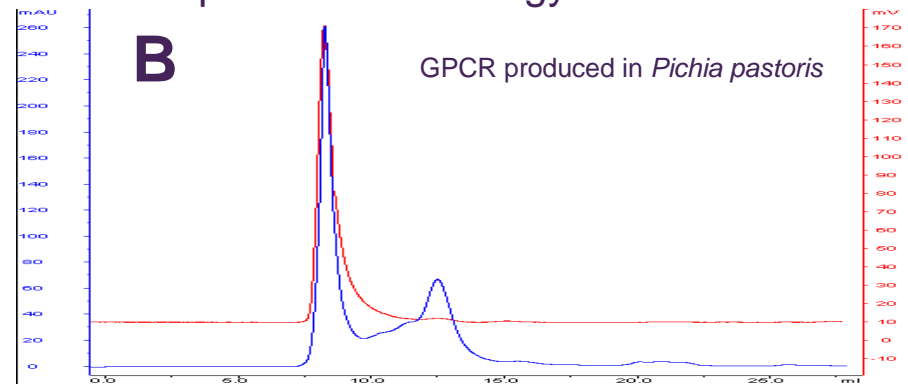
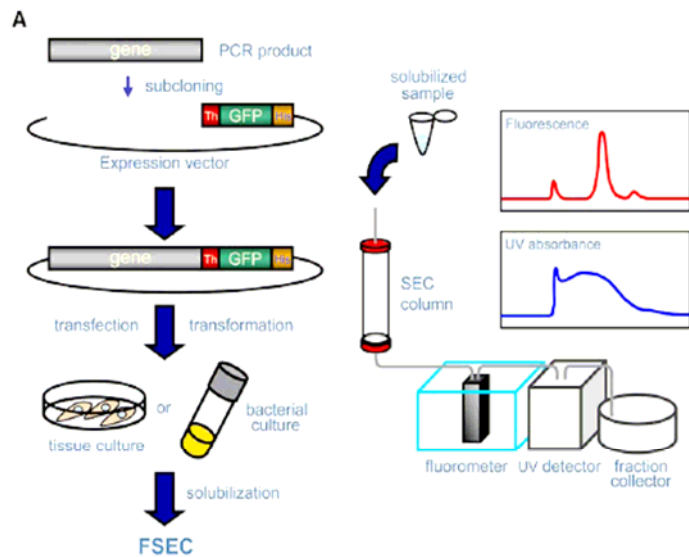
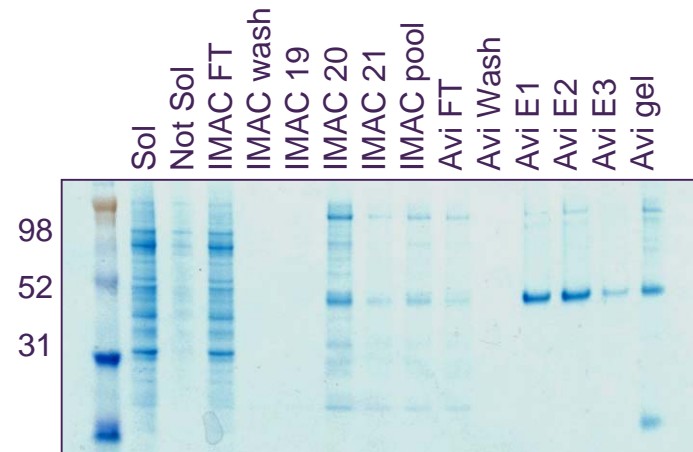
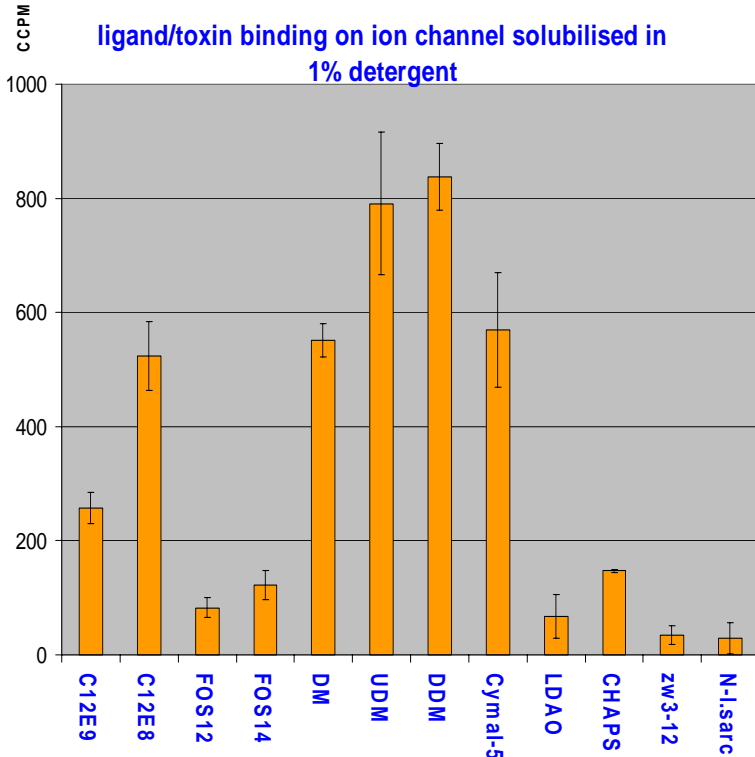


Figure 4 FSEC. A) Principle of FSEC method (Kawata & Gouaux, 2006) B) Solubilized GPCR produced in *Pichia pastoris* is aggregated, 100 μ g total protein C) The same GPCR produced in mammalian cells is non-aggregated, 20 μ g total protein

Solubilisation & purification



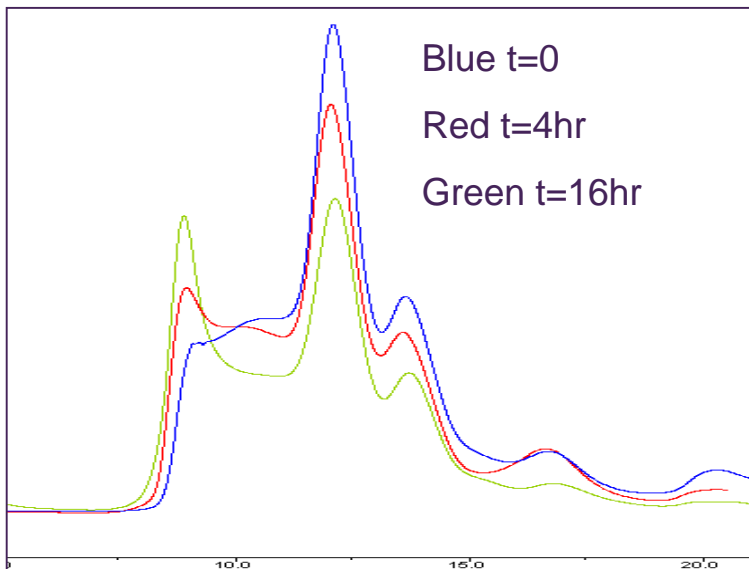
Purified GPCR: 2.5 mg/liter

Membrane protein purification generally requires the liberation of the proteins from the membrane using detergents or detergent-lipid mixtures. Activity is used as golden standard for solubilization. In the above example optimal ligand binding is obtained in undecylmaltoside and dodecylmaltoside.

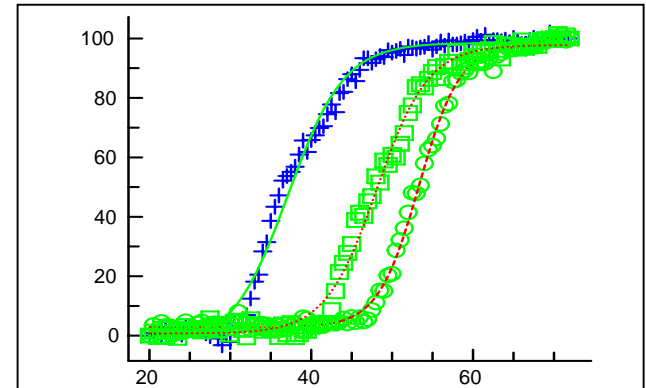
Protein expression typically is low and dual affinity purification schemes are required. For a GPCR the combination of IMAC and monomeric avidin agarose results in purity over 95% as judged by SDS-PAGE. Ligand affinity serves as excellent step both in purification as well as quality control.

Characterization/Quality Control

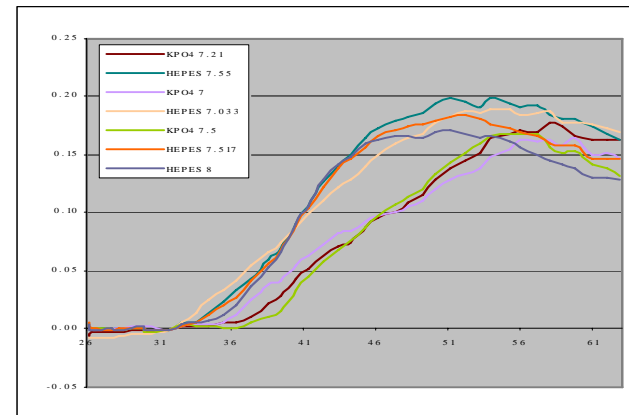
Purity, homogeneity and stability are crucial factors for crystallization success. Buffer formulations are optimized using methods as analytical size exclusion chromatography, circular dichroism and other thermal unfolding methods.



A pure GPCR preparation proved to have short stability. Changes in buffer composition increased stability from hours to days.



Thermal stability of an IC measured by CD was increased by addition of ligand or toxin (above). A further enhancement was obtained by buffer variation (below).



Conclusion/summary

- *Pichia pastoris* produces pure protein (2.5-5 mg/liter)
- Binding data crucial for expression&solubilization screening
- FSEC early quality control to establish expression purification strategy
- Dual affinity purification required
- Buffer composition and ligand addition tune protein stability

References

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