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Measurement of detergent concentration using 2,6-dimethylphenol in membrane-protein crystallization

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Methods have previously been developed to measure detergent concentration in membrane-protein samples, but most have significant limitations, such as requiring specialized equipment or consuming a significant amount of precious sample. This work explores the use of 2,6-dimethylphenol in a phenol–sulfuric acid assay to accurately measure the concentration of common glycosidic-based detergents used in crystallization. This method is amenable to routine laboratory use, provides excellent sensitivity and significantly reduces the sample volume required. Using an *Escherichia coli* tyrosine kinase (Etk) construct as an example, it is shown that the crystallization potential of Etk is directly influenced by measurable changes in detergent concentration.

1. Introduction

Addition of detergent is essential for membrane-protein solubility and stability, but can be detrimental to crystallization efforts (Privé, 2007; Hitscherich *et al.*, 2000; Sonoda *et al.*, 2010). Ultrafiltration in centrifugal concentrators is the most common technique used to obtain reproducible protein concentrations in membrane-protein crystallography (Maslennikov *et al.*, 2007). Unfortunately, protein-free detergent micelles formed above the critical micelle concentration (CMC) can also be concentrated by this technique (Privé, 2007; Urbani & Warne, 2005; Shi *et al.*, 2008). These empty micelles can interfere with membrane-protein crystallization and the overall level of detergent can be crucial to crystallization success (Privé, 2007; daCosta & Baenziger, 2003; Eriks *et al.*, 2003; Strop & Brunger, 2005). In addition, the detergent concentration factor of centrifugal concentrators has been shown to vary between manufacturers and within units from the same manufacturer (Maslennikov *et al.*, 2007). Thus, the use of a detergent-detection technique to standardize detergent concentrations is a useful strategy for improving the reproducibility of membrane-protein crystallization experiments.

A wide variety of techniques have been proposed for the quantitation of detergent. These include thin-layer chromatography (Eriks *et al.*, 2003), gas chromatography coupled with flame ionization detection or mass spectrometry (Shi *et al.*, 2008, 2009), nuclear magnetic resonance (Maslennikov *et al.*, 2007), contact-angle measurement (Kaufmann *et al.*, 2006), refractive-index measurement (Strop & Brunger, 2005) and Fourier-transform infrared spectrometry (daCosta & Baenziger, 2003). Although these techniques are effective, they require specialized equipment that is not available in most laboratories for routine measurements. The detection of sugars through a phenol–sulfuric acid assay (DuBois *et al.*, 1956) has also been applied to the measurement of glycosidic-based detergents in membrane-protein samples (Urbani & Warne, 2005). However, the proposed method uses a relatively large sample volume. This work explored the use of 2,6-dimethylphenol as a colorimetric agent with increased absorbance (Mallya & Pattabiraman, 1997). This substitution has allowed the sample consumed to be significantly reduced. Moreover, this improved method maintains a fast processing time of under an hour and requires no specialized equipment. Therefore, it is ideal for the limited samples common in membrane protein crystallization.

In this work, *Escherichia coli* tyrosine kinase (Etk) was used to demonstrate the application of the 2,6-dimethyphenol reaction to membrane-protein crystallization. Etk is an inner membrane protein with poorly understood roles in capsular polysaccharide production and antibiotic resistance (Ilan *et al.*, 1999; Peleg *et al.*, 2005). The overall structure of Etk consists of an N-terminal periplasmic domain separated from a C-terminal cytoplasmic domain by two transmembrane helices. The unique structure of prokaryotic tyrosine kinases has recently been revealed through a structural study of the C-terminal domain of Etk (Lee *et al.*, 2008). However, crystallization of an Etk construct (Etk NM2) containing the N-terminal domain and the transmembrane helices has been inconsistent. The 2,6-dimethylphenol assay has been used to define an optimal crystallization 'zone' for this membrane protein when solubilized in *n*-dodecyl-β-D-maltoside.

2. Materials and methods

2.1. Chemicals

Solvent-grade *n*-dodecyl-β-D-maltoside (DDM), *n*-decyl-β-D-maltoside (DM) and *n*-octyl-β-D-glucoside (OG) were purchased from Anatrace (USA). 2,6-Dimethylphenol was obtained from Sigma (USA) and sulfuric acid from Fisher Scientific (Canada).

2.2. Expression and purification of Etk NM2

Etk NM2 is a truncation of the Etk membrane protein that contains the N-terminal periplasmic domain and the transmembrane helices, while removing the C-terminal cytoplasmic domain. The protein was expressed in *E. coli* BL21 cells using the pET-21b vector and IPTG induction at 293 K for 20 h. Cells were lysed by sonication and the remaining insoluble material was resuspended in 50 m*M* NaH_2PO_4 pH 7.8, 250 m*M* NaCl, 1% DDM for overnight solubilization of the protein at 297 K. The protein was further purified by nickel-affinity chromatography (Qiagen, USA) and size-exclusion chromatography using a Sephacryl S-300 column (GE Healthcare, USA). All buffers contained 0.01% DDM to maintain protein stability. The protein was concentrated using a 30 kDa centrifugal concentrator (Millipore, USA).

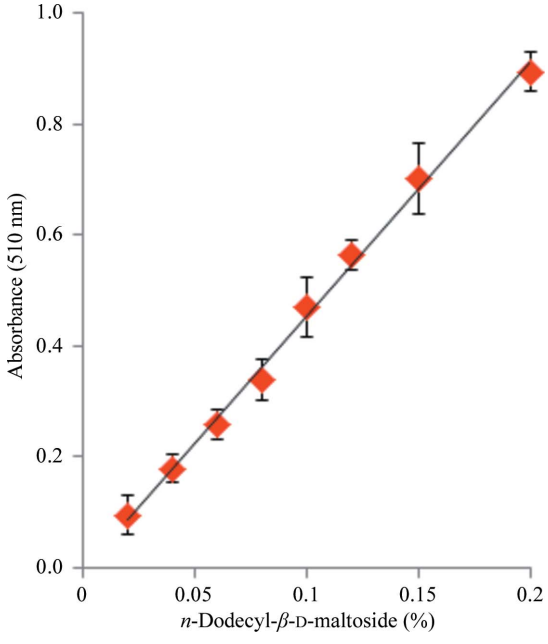

Figure 1
Standard curves for the quantitation of *n*-dodecyl-β-D-maltoside (DDM). The assay was performed in triplicate; data represent the average ± standard deviation for each concentration.

2.3. 2,6-Dimethylphenol detergent assay

Based on the work of Mallya & Pattabiraman (1997) and of Urbani & Warne (2005), a 2,6-dimethylphenol–sulfuric acid reaction was explored. Initially, 5 µl protein sample was diluted with 220 µl deionized water in a SafeSeal 1.5 ml tube (Starstedt, Germany). Then, under a fume hood, 25 µl 20% 2,6-dimethylphenol dissolved in absolute ethanol was added, followed by 750 µl concentrated sulfuric acid. The reaction tube was closed and mixed by inversion before incubation at room temperature for 40 min. During this time a strong exothermic reaction occurs and the sample returns to room temperature. Subsequently, the optical density (510 nm) was measured and corrected using an appropriate blank. Each sample was tested in triplicate and samples were diluted to achieve a reading within the range 0–1. Control reactions were performed with a sample volume of 5 µl.

2.4. Crystallization of Etk NM2

Crystallization conditions for Etk NM2 in DDM were originally determined by high-throughput screening in 96-well sitting-drop plates. In this work, Etk NM2 was crystallized using the hanging-drop method at room temperature, a well solution consisting of 0.1 *M* MES pH 6.0, 1–5% PEG 3000, 20–30% PEG 200 and a drop ratio of 2 µl protein solution plus 2 µl well solution.

3. Results

The 2,6-dimethylphenol assay was tested with three glycosidic detergents commonly used in membrane-protein crystallization. In all cases, the standard curves obtained showed a linear trend which could be fitted with a correlation coefficient of above 0.97 (Fig. 1, Supplementary Fig. S1[1]). When tested with water alone, the resultant absorbance was 0.16 ± 0.02. Therefore, subtraction of the absorbance resulting from water is required. To ensure that the 2,6-dimethylphenol reaction can be used on complex protein samples, cross-reaction with common buffer components and several protein standards was also tested (Supplementary Fig. S2[1]). Glycerol was the only additive which showed additional reactivity, and this contribution can be removed by subtraction. In addition, this technique is not recommended for the measurement of detergent in the presence of glycosylated proteins, which do cross-react. Finally, a reaction time course was performed to monitor the development and decay of absorbance at 510 nm. The standard deviation of readings taken between 40 and 120 min (0.055) was less than the average standard deviation in the triplicate measurements (0.078). Therefore, it was concluded that measurements taken within this time period are stable and should provide accurate measurements of detergent concentration.

The Etk NM2 construct produces hexagonal prism crystals as showers of small crystals (Fig. 2*a*) and medium-large single crystals (Fig. 2*b*). Without prior measurement of detergent concentration, the success rate for its crystallization was less than 10%. By comparing detergent concentration with crystallization success, an optimal range

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: NJ5133). Services for accessing this material are described at the back of the journal.

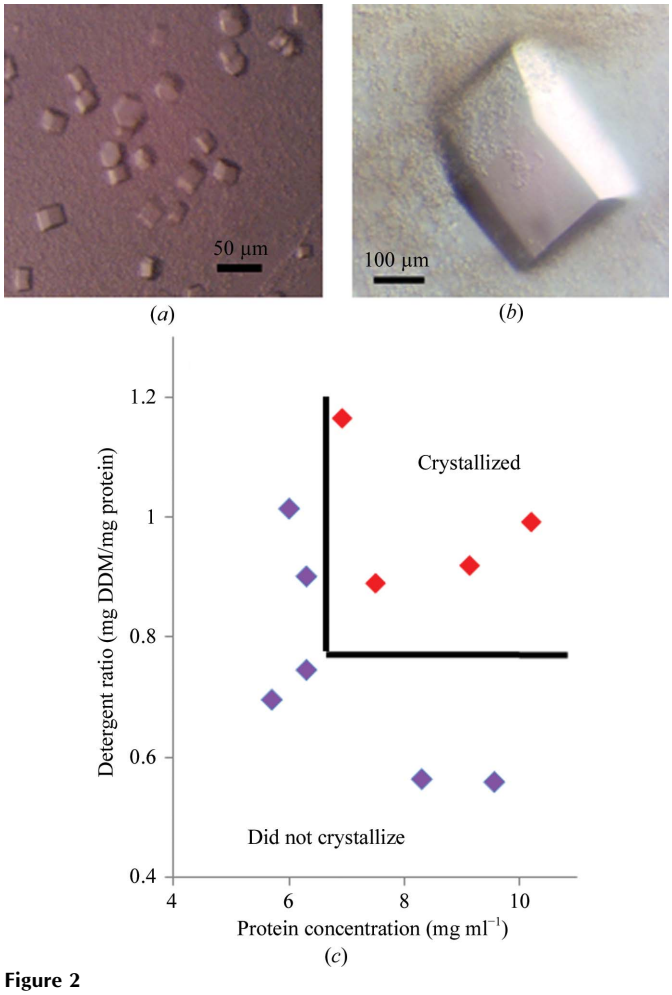

Figure 2
(*a*) Typical shower of small Etk NM2 crystals. (*b*) Large hexagonal prism crystal of Etk NM2. (*c*) Correlation of DDM and protein concentrations with Etk NM2 crystallization success.

of protein and detergent concentrations for crystallization was defined (Fig. 2*c*). Optimal crystallization conditions required a minimum protein concentration of 6.9 mg ml^{-1} with a minimum detergent:protein ratio of ∼0.8 mg DDM per milligram of protein.

4. Discussion

The 2,6-dimethylphenol assay presented here demonstrates linearity within the range 0.01–0.2% detergent content, or 0.5–10 µg DDM assayed. Therefore, this method can quantitate smaller amounts of detergent than the previously published phenol method, which has a detection limit of 2.5 µg DDM (Urbani & Warne, 2005). This reduces the amount of sample required, which is ideal for membrane-protein crystallization, in which the final sample volume is typically small (*i.e.* <500 µl). Despite the wide diversity of detergents that are available, DDM, DM and OG are collectively used in ∼45% of successful crystallizations [Membrane Protein Data Bank (MPDB); http://www.mpdb.tcd.ie]. This assay can also be extended to an additional 12% of cases in which less common glycosidic-based detergents have been used (MPDB). Unfortunately, there are still some popular detergents which are not detected by 2,6-dimethylphenol, including octyl tetraethylene glycol ether and lauryldimethylamine-*N*-oxide.

Previous reports have suggested that the minimization of excess detergent micelles favours crystallization success (daCosta & Baenziger, 2003; Wiener, 2004) and techniques have been developed to determine the minimum detergent required for protein stabilization under specific conditions (Jumpertz *et al.*, 2011). In contrast, this work found that higher detergent concentrations appeared to be essential for the crystallization of Etk NM2. Previously, it has also been suggested that concentrations near the cloud point of a detergent (*i.e.* the phase boundary where intermicellar attractive forces drive micelles into a separate phase) can show correlation with membrane-protein crystallization (Hitscherich *et al.*, 2001; Wiener, 2004). The detergent requirements observed for Etk NM2 may be another example of this phenomenon. Most detergents do not 'phase-separate' between 273 and 373 K, but this property can be influenced by common reagents in the crystallization cocktail such as PEG and/or salts (Hitscherich *et al.*, 2001). Although conditions near the cloud point can be favourable for crystallization, phase separation was not observed in any of the trials that led to successful crystallization of Etk NM2 in DDM.

The results presented here exemplify the critical importance of tracking the detergent concentration during crystallization efforts. The reduction in sample volume achieved upon substitution of 2,6-dimethylphenol for phenol in this assay represents a significant improvement that is particularly relevant to the field of crystallography. Reproduction of the protein:detergent ratios in crystallization samples was shown to be essential to the crystallization success of Etk NM2. Thus, this technique could be readily used by a majority of researchers working in membrane-protein crystalloraphy.

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