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A straight-forward method of optimising protein solubility for NMR

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Abstract

Maximising solubility is a key step in applying solution-state NMR to proteins. The 'microbatch' crystallisation screening method can be adapted to screen for protein solubility. In this approach, drops of test solutions are placed under paraffin oil in 96-well screening plates. This requires very small amounts of protein, is easy to set up and is readily automatable.

Introduction

Protein solubility is the main obstacle for liquid-state protein NMR. Structure determination by NMR typically requires a protein concentration of 0.5 mM or greater for several days at temperatures over 20 °C. NMR studies of ligand binding require concentrations of at least 0.05 mM. Therefore, the first step in any NMR study of a protein is to identify conditions where the protein is soluble and stable. This brief paper proposes a simple method of screening for solution conditions which minimise protein precipitation.

Protein solubility is affected by many different factors. The dominant ones are typically temperature, pH and salt concentration. However, a wide range of other factors have been reported as improving the solubility of one protein or another (for example, non-ionic detergents and osmolytes). As well as these factors which affect all proteins, the solubility of any individual protein is likely to be affected by any ligands which bind to it. The net result of this is that the NMR spectroscopist is presented with an enormous set of potential solution conditions. Evaluating all of these by NMR would require unrealistic amounts of protein and spectrometer time.

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One approach to the problem is to screen for protein solubility using methods developed for protein crystallisation (reviewed in detail by Bagby et al., 2001). In these methods, small volumes of protein solution are mixed into different solution conditions. These are then monitored by light microscopy; protein precipitation is obvious as clouding of the solutions. Although this approach only identifies protein precipitation (and not denaturation), it is a useful first step because of the small amounts of protein that are required.

Microdialysis (Bagby et al., 1997) and hanging-drop vapour diffusion (Lepre and Moore, 1998) are the two methods that have been proposed for screening protein solubility. They are both difficult to set up and difficult to automate. The hanging-drop method has the additional disadvantage that diffusion alters the solution conditions within the drop.

An alternative approach is 'microbatch' crystallisation (Chayen et al., 1992). It is simple to set up, compact and screens solution conditions that are precisely known. In this method, small drops of solution are pipetted into wells under a layer of paraffin oil (see Figure 1). The paraffin oil prevents the loss of water vapour, so tiny volumes of solution can be used without evaporation being a problem (Note that in crystallisation screening, oil mixtures are used to enable solvent

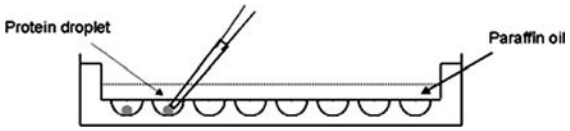

Figure 1. Schematic diagram of microbatch screening. The test solutions are pipetted into wells under paraffin oil.

Table 1. Buffer and pH combinations for the initial screen of ACP-ER solubility

Buffer	pH			Plate row
None		7		1
Imidazole	6.5	7	7.5	2
Phosphate	6.5	7	7.5	3
MOPS	6.8	7.2	7.7	4
Tris	7.1	7.6	8.1	5
Bicine	7.3	7.8		6
Plate column	A	B	C	

Samples contained 0.28 mM ACPER protein monomer, 0.05 mM Sodium fluoride, 1.4 mM NAD^+ and 50 mM of the specified buffer. MOPS: 3-(*N*-morpholino)propanesulfonic acid.

evaporation and allow a gradual increase in concentration (D'Arcy et al., 1996). This can be avoided when screening NMR conditions by using pure paraffin oil). A possible disadvantage is that the approach may not be applicable to solutions containing hydrophobic molecules because such molecules may partition out of the test droplets into the paraffin. However, previous work on crystallisation using detergents suggests this is not a severe problem (Loll et al., 2003).

Materials and method

The microbatch method was used to optimise the solubility of two proteins, ACP-Enoyl Reductase (ACP-ER) from *Brassica napus* and a 50 kDa protein of interest to Syngenta which will be referred to as Protein N. ACP-ER was purified as described (Rafferty et al., 1995). An initial set of 14 buffer/pH conditions (Table 1) was selected based on previous work showing that the protein was active between pH 6 and 8 (Slabas et al., 1986). Once the optimum pH and buffer had been identified, four different salt conditions were screened (Table 2). The solubility of Protein N was screened across a broad pH range (Table 3) in the presence and absence of a tightly binding substrate.

Table 2. Salt concentrations used in follow-up screen of ACP-ER

Salt	Concentration (mM)
None	–
Potassium Bromide	50
Magnesium Sulphate	50
Sodium Chloride	100

Samples also contained 0.25 mM ACPER protein, 0.05 mM Sodium fluoride, 3 mM NADH and 10 mM, pH 7.4 Tris–HCl.

Table 3. Buffer and pH combinations used to screen Protein N

Buffer	pH			Plate row
Acetate	5.2	4.8	4.4	1
MES	6.6	6.2	5.8	2
Bis-Tris	6.9	6.5	6.1	3
Imidazole	7.4	7.0	6.6	4
Tris	7.9	7.6	–	5
Phosphate	7.5	7	6.5	6
None added	7	–	–	7
Plate column	A	B	C	(Apoprotein)
	D	E	F	(With substrate)

Samples contained 50 mM of buffer, 0.3 mM Protein N, 0.05 mM Sodium fluoride and (where used) 0.3 mM substrate. MES: 2-(*N*-morpholino)ethanesulfonic acid.

'Microbatch' 96-well screening plates and paraffin oil were obtained from Douglas Instruments (product number VBATCH 1/1 PHO-10). Proteins were concentrated to the point of precipitation at 4 °C. 5 µl aliquots of protein solution were pipetted under the paraffin oil into the plate wells using the method described by Douglas Instruments (http://www.douglas.co.uk/vb_inst.htm). Each set of solution conditions being tested was prepared by adding 6 × concentrate to a well. The plate was then incubated at 20 °C and checked daily for precipitation for 3 days. The temperature of the incubator was then increased by 4–5 °C and the plate was checked for a further 3 days. The process of increasing temperature and incubating was repeated until optimum conditions were identified.

Results

Figure 2 shows photographs of the plate at the end of screening. Clear wells (no precipitation)

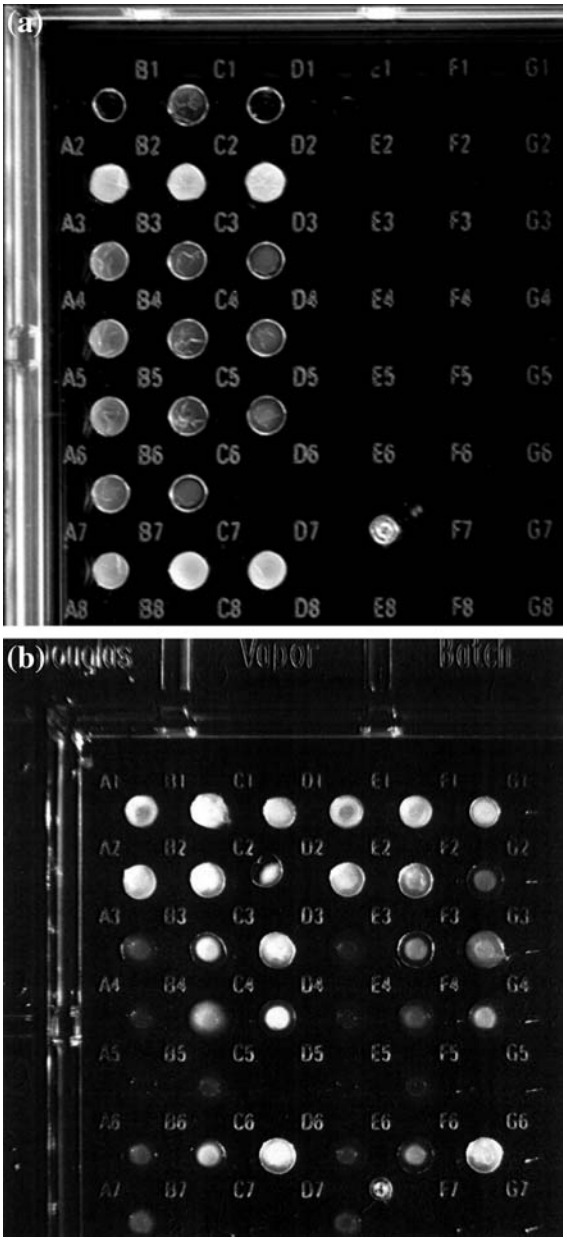

Figure 2. Photograph of microbatch screening plates containing (a) ACP-ER protein and (b) Protein N. Each well contains approximately 6 µl.

and cloudy wells (precipitation) are immediately obvious. For ACP-ER, the initial pH/buffer screen showed that maximum protein solubility was in 50 mM pH 7.4 Tris buffer. A second round optimisation of the salt concentration showed that 50 mM KBr gave maximum solubility. In the final sample conditions, the protein was indefinitely stable at up to 0.2 mM at 28 °C. This optimisation required only 80 µl of protein, and could have been achieved with as little as 40 µl using smaller pipette volumes.

For Protein N, least precipitation occurs at higher pHs and adding substrate reduces precipitation further (compare cells B5 and E5). In pH 7.9 Tris with substrate, the protein is indefinitely stable at 33 °C at a concentration of 0.3mM. Other additives will be investigated to see if solubility at lower pHs can be improved.

Discussion and conclusions

This example demonstrates that the 'under-oil' method is an effective way of optimising protein solubility. It is simple to set up and requires little material, so should be considered during the initial stages of studying any protein. It would be particularly appropriate for NMR-based structural genomics projects because robots capable of setting up 'under-oil' screens are commercially available.

There is one clear disadvantage of the method; it only monitors protein precipitation. Denaturation and aggregation are not monitored, so need to be investigated by other approaches until methods are developed for monitoring them under paraffin oil. However, this disadvantage also applies to other proposed methods of screening protein solubility.

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