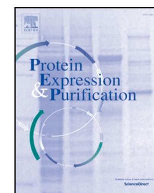
Protein Expression and Purification 170 (2020) 105591

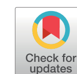
Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



Cell-free expression of natively folded hydrophobins



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ARTICLE INFO

Keywords:
Cell-free expression
Hydrophobin
NMR spectroscopy
Disulphide bond

ABSTRACT

Hydrophobins are a family of cysteine-rich proteins unique to filamentous fungi. The proteins are produced in a soluble form but self-assemble into organised amphipathic layers at hydrophilic:hydrophobic interfaces. These layers contribute to transitions between wet and dry environments, spore dispersal and attachment to surfaces for growth and infection. Hydrophobins are characterised by four disulphide bonds that are critical to their structure and function. Thus, obtaining correctly folded, soluble and functional hydrophobins directly from bacterial recombinant expression is challenging and in most cases, initial denaturation from inclusion bodies followed by oxidative refolding are required to obtain folded proteins.

Here, we report the use of cell-free expression with *E. coli* cell lysate to directly obtain natively folded hydrophobins. All six of the hydrophobins tested could be expressed after optimisation of redox conditions. For some hydrophobins, the inclusion of the disulfide isomerase DsbC further enhanced expression levels. We are able to achieve a yield of up to 1 mg of natively folded hydrophobin per mL of reaction. This has allowed the confirmation of the correct folding of hydrophobins with the use of ^{15}N-cysteine and ^{15}N–^{1}H nuclear magnetic resonance experiments within 24 h of starting from plasmid stocks.

1. Introduction

Hydrophobins are a family of surface-active proteins unique to filamentous fungi. They play a number of important roles in fungal biology including aiding aerial hyphal growth, coating of spores and mediating interactions with hosts. The proteins are produced in a soluble form, with a signal peptide directing the proteins to be exported from the cell. The mature form of the protein after cleavage of the signal peptide then self-assembles into organised amphipathic layers at hydrophilic:hydrophobic interfaces (e.g. water:air interfaces and spore surfaces) [1,2].

All hydrophobin sequences are characterised by eight cysteine residues which form four disulphide bonds [2]. The disulphide bonds are essential for the structure and function of all known hydrophobins [3,4]. Members of the hydrophobin family are ~6–20 kDa and have traditionally been classified into two categories, class I and class II, based on their sequence, the distribution of hydrophobic and hydrophilic residues, the spacing between cysteine residues and the nature of amphipathic monolayers that they form (Fig. 1) [2]. Class I hydrophobins assemble into layers with a fibrillar morphology with underlying amyloid characteristics. The fibrils are known as rodlets and can only be dissociated with certain concentrated acids [5–7]. In contrast, class II hydrophobins form assemblies that are less robust [8,9]. More recently, hydrophobins with intermediate properties were reported; they have been termed class III hydrophobins [10].

The unique physical properties of hydrophobins have suggested a range of biotechnological applications ranging from implant coating to surfactants and drug delivery systems [11–13]. In particular, engineered surfaces that allow good integration with human tissue are in high demand [12]. An easy-to-use and robust production system that can yield correctly folded wild-type and engineered hydrophobin variants with novel properties will streamline the characterisation and engineering of hydrophobins.

There have been a number of reports demonstrating homologous and heterologous methods for hydrophobin production. For example, genetic engineering techniques were trialled by Schuurs et al. [14] to enhance the native expression of SC3, a class I hydrophobin from *Schizophyllum commune*. While *S. commune* has been the host of choice

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https://doi.org/10.1016/j.pep.2020.105591
Received 19 October 2019; Received in revised form 28 January 2020; Accepted 30 January 2020
Available online 04 February 2020
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A

Class I

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EAS      PNTCSIDDYKPYCCQSMSGPAGSPGLLNLIPVDLSASL--------------GC---------------VVGVIGSQCGASVKCCKDDVTNTGNSFLIINAANCV--
EASΔ15   PNTCSIDDYKPYCCQSMSGSASL-----------------------------GC---------------VVGVIGSQCGASVKCCKDDVTNTGNSFLIINAANCV--
DewA/Y   GTTCNV--GSIACCNSPAETNNDSLLSGLLGAGLLNGLSGNTGS--------ACAKASLIDQLGLLALVDHTEEGPVCKNIVACCPEGTTN------------CVAV
MPG1     QQKCGAE-KVVSCCNSKELKNSKSGA-E--IP-----IDVLSGE---------CKN-IP---INILTINQLIPINNFCSDTVSCCSGEQIGLV-------NIQCTPI
RodA     TEKCGDQ-AQLSCCNKATYAGDVTDIDEGILAGTLKNLIGGGSGTEGLGLFNQCSKLDL--QIPVIGIPIQALVNQKCKQNIACCQNSPSDASGSLIGL-GLPCIAL
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Class II

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HFBI     -NVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGA-
HFBII    -AVCPTGLFSNPLCCATNVLDLIGVDCKTPTIAVDTGAIFQAHCASKGSKPLCCVAPVADQALLCQKAIGTF
NC1      -TPC-SGLYGTAQCCATDVLGVADLDCANPPATLANATHFESTCAAIGQRARCCVLPILGQDILCQTPAGL-
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B

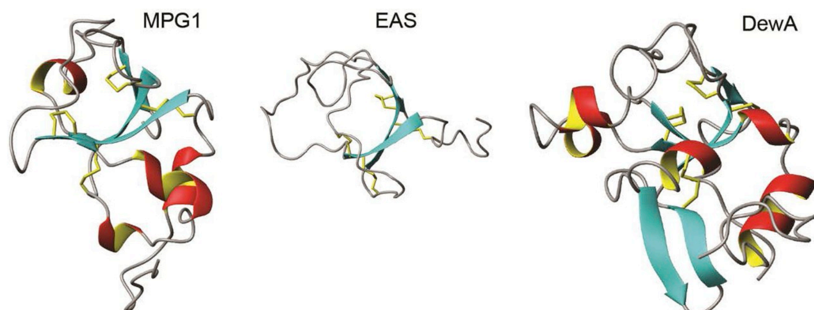

Fig. 1. (A) Sequence alignment of selected class I and class II hydrophobins. Class I hydrophobins include EAS (*Neurospora crassa*) and $EAS_{\Delta15}$, DewA (*Aspergillus nidulans*) and DewY, MPG1 (*Magnoportha oryzae*) and RodA (*Aspergillius fumigatus*). Class II hydrophobins include HFBI (*Trichoderma reesei*), HFBII (*Trichoderma reesei*), NC2 (*Neurospora crassa*). Cysteine residues are coloured in orange. Only two residues are shown before the first cysteine residue as the N-terminus varies substantially in length. Black brackets represent disulphide bonds between cysteine residues. (B) Solution structure of MPG1, EAS and DewA in ribbon display showing the disulphide bonds in the core hydrophobin fold.

for SC3 expression, both the endogenous and introduced copies of the SC3 gene were found to be silenced [14]. In contrast, the overexpression of the class II hydrophobins HFBI and HFBII in the native host, *Trichoderma reesei*, has been much more successful with a yield of up to 600 mg/L [15]. Generally, far higher yields have been observed for class II than class I hydrophobins and this has been attributed to the more effective folding of class II hydrophobins and their more conserved protein sequences, although the precise reason is not known [12].

Heterologous methods using plants or plant cell cultures for hydrophobin production have also been reported [13,16–20]. In some cases, hydrophobins have been used as fusion tags to increase expression and aid purification of other proteins [21]. Lahtinen et al. [22] have reported the successful expression of HFBI using an insect cell expression system. However, *Escherichia coli* is the most common heterologous host for hydrophobin expression due to its highly suitable and well-established growth characteristics, including fast growth, high cell densities and the availability of defined media and reagents that allow isotopic protein labelling and introduction of non-native amino acids. In addition, many molecular tools and protocols exist for modifying and transforming exogenous DNA into the bacterial cell. We have previously reported *E. coli* expression and site-directed mutagenesis to identify the regions of the class I hydrophobin EAS from *Neurospora crassa* that are responsible for assembly into rodlets [23]. The *E. coli* recombinant protein expression system has been used by us and others for the production of a diverse range of hydrophobins for structural and functional analysis [24–28].

Nonetheless, achieving a high yield of fully folded hydrophobins in the soluble fraction from recombinant expression proves to be a challenge for most hydrophobins. With notable exceptions, for example the hydrophobin DewA from *Aspergillus nidulans* [27], hydrophobins have been found to be expressed predominantly in the insoluble fraction of *E. coli*. Even for DewA, the expression switched to the insoluble fraction with use of a defined media, as required for isotopic labelling for Nuclear Magnetic Resonance (NMR) studies. The reducing environment inside the cytoplasm of a bacterial cell prevents the formation of disulphide bonds integral to the structure of hydrophobins and is thought to drive the aggregation of misfolded hydrophobins into inclusion bodies [4,29].

Thus, production of fully folded and functional hydrophobins, involves a reagent-intensive and time-consuming refolding process whose requirements include solubilisation of the inclusion bodies using high concentrations of denaturants and then gradual removal of the denaturants in a series of redox buffers that allow refolding of the hydrophobin into its native structure [25–27,30,31]. We previously attempted to sidestep this *in vitro* refolding step by utilising *E. coli* SHuffle® strain capable of periplasmic protein expression [32] but could not detect any hydrophobin expression and hence we have returned to the *E. coli* cell-free (CF) system.

Here we report that the *E. coli* CF system can provide an alternative method that can overcome refolding issues encountered during recombinant hydrophobin production. The CF system uses a biological machinery outside of living cells to produce proteins. The *E. coli* extract is the most commonly used lysate containing the transcription and translation machinery required for protein production [33–35]. Coupled transcription and translation of protein is achieved by incubating the lysate with a gene encoding the desired construct and necessary cofactors in a dialysis setting [36,37]. Since folded hydrophobins are highly surface active, the proteins can be detrimental to the host cells if overexpressed in a folded and active form. However, this is not a problem for CF expression. The CF system is also more flexible than recombinant production because the reaction environment can be controlled directly. Thus, more variables may be optimised to allow for correct folding and to increase protein yield. The CF protein synthesis system also provides other advantages over the traditional recombinant system. For example, multiple hydrophobins and/or hydrophobin mutants can be produced and screened in parallel during engineering of hydrophobins. The addition of stable-isotope labelled proteins for NMR can be more readily achieved [38]. In addition, non-natural amino acids can also be introduced for functional characterisation studies without potentially harming the host during production as occurs with the recombinant system [39]. Other laboratories have successfully used

the CF system to overexpress a number of cysteine-rich proteins [34,40,41]. Here, we have used the CF system based on an *E. coli* extract to produce six well-studied hydrophobins covering both class I members: $EAS_{\Delta15}$, EAS, DewY – a DewA mutant, RodA, MPG1 [24,25,27,30,31,42] and a class II hydrophobin NC2 [26]. In all cases, after minor optimisation of the CF protocol, the proteins can be directly produced in the soluble fraction and are correctly folded. In addition, with the use of ^{15}N-cysteine in NMR experiments, an assessment of correct hydrophobin folding can be made within 24 h of starting from purified plasmids.

2. Materials and methods

2.1. Cloning

All hydrophobin constructs used in this study encode for a His_6-ubiquitin tag at the N-terminus as we have found this tag to aid recombinant expression and purification via Ni-NTA affinity chromatography. Unless otherwise stated, all expressed proteins referred to from this point have an N-terminal His_6-ubiquitin tag, followed by a TEV protease cleavage site. The six hydrophobins of interest: $EAS_{\Delta15}$, EAS, DewY, NC2, MPG1 and RodA, along with an N-terminal ubiquitin tag, were subcloned into pET-MCSIII vectors. The pET-MCSIII vector, which lacks the lac operator and expresses protein constitutively, was chosen as it had successfully been used for CF synthesis of other proteins [36,43].

2.2. DsbC expression and purification

The DsbC plasmid was kindly provided by the Kigawa laboratory and expression and purification was carried out as described previously [33]. After elution, DsbC was dialysed into PBS and stored at 4 °C until use or after addition of 30% glycerol, at −80 °C for long-term storage. We have found purified DsbC to be stable and active when stored at 4 °C for up to 2 years.

2.3. Bacterial strains and extract preparation

E.coli strain BL21 (DE3) star cells were used to make the S30 lysate for the CF expression system based on the protocol previously described [36].

2.4. Cell-free (CF) (in vitro) protein synthesis

CF protein synthesis consists of coupled transcription and translation of protein by incubating the S30 lysate with a gene encoding the desired construct and necessary cofactors in a dialysis setting with inner and outer mixtures. CF hydrophobin expression was performed using a modified procedure of the dialysis method [36,37]. To minimise the use of reagents during screening, each reaction was scaled down from 300 µL to 50 µL (inner volume) and 3 mL–0.5 mL for outer volume. Previously a knot was tied at the ends of a small dialysis tube holding the inner reaction mix. However, this was prone to leakage. Therefore, micro-buttons that mimic commercial 1-mL dialysis buttons were prepared by using the lid of a cut 0.45-mL PCR tube to hold 50 µL when placed inside a flat-bottom plastic tube.

Briefly, 6 ng/µL of plasmid DNA, the 20 amino acids, 0.8 mM rNTP, 55 mM Hepes (pH 7.58), 68 µM folinic acid, 0.64 mM cAMP, 27.5 mM NH_4OAc, 1.2 mM ATP, 80 mM creatine phosphate, 208 mM potassium glutamate, 250 µg/mL creatine kinase (Roche), 15 mM $Mg(OAc)_2$ (Ajax), 0.175 mg/mL tRNA (Roche), 30% (v/v) S30 lysate, were prepared as the inner and outer mixture, with the absence of DNA and S30 in the latter. Varying concentrations of dithiothreitol (DTT, from 0.1 to 1.7 mM), GSH (0–1 mM), GSSG (0–0.1 mM) and DsbC (0–0.3 mg/mL) as well as cOmplete®, EDTA-free protease inhibitor cocktail (Roche, used according to manufacturer's instructions) were also included as indicated in the different reactions. A small stretch of 10,000 MWCO SnakeSkin® dialysis membrane was wedged between the two reaction chambers. The mixture was incubated for 4–20 h at 30 °C and 180 rpm. To verify protein expression, SDS-PAGE was performed using NuPAGE™ 4–12% Bis-Tris Protein Gels (Invitrogen) at 200 V for 19 min in MES buffer (Invitrogen). For ^{15}N-cysteine protein production, unlabelled cysteine was replaced by ^{15}N-cysteine as part of the amino acid mixture. To produce sub milligram quantities of hydrophobin for NMR and mass spectrometry experiments, the reactions were scaled up 20 × using 1-mL dialysis buttons for the inner mixture.

2.5. Purification proteins from cell-free synthesis for mass spectrometry and NMR spectroscopy

Completed cell-free expression reaction mixture for each hydrophobin was centrifuged (14,000 rpm, 10 min, 25 °C) and the supernatant was loaded onto a gravity flow Ni-NTA agarose column (Qiagen) and incubated at 25 °C for 1 h followed by addition of four column volumes of buffer A containing 50 mM NaH_2PO_4 (Ajax), 500 mM NaCl (Chem-Supply), 10 mM imidazole (Sigma), pH 8. The column was washed with five column volumes of buffer A containing 20 mM imidazole and eluted by four column volumes of buffer A containing 250 mM imidazole.

For NMR data collection, the eluted hydrophobins were dialysed into NMR buffer (20 mM NaH_2PO_4, pH 5.5, 5% D_2O, 20 µM sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS)) overnight. The samples were concentrated to a final volume of 300 µL using Vivaspin® 6 Polyethersulfone 10-kDa concentrators and placed into 5-mm Shigemi tubes (SHIGEMI Co. Japan). Where indicated, 20 µg/mL of TEV protease was added to the sample to cleave the His_6-ubiquitin tag.

For mass spectrometry, the cleaved elution fractions or NMR samples were subjected to reverse-phase High Pressure Liquid Chromatography (rpHPLC) as previously described for each of the hydrophobins. Freeze-dried purified protein was then analysed using a Bruker AmaZon or Thermo LCQ instrument [24–27,30,31,42].

2.6. NMR spectra

All NMR spectra were acquired on Bruker Avance III 600 MHz or 800 MHz spectrometers equipped with 5-mm triple resonance TCI cryogenic probe heads (Bruker, Karlsruhe, Germany), and processed using Topspin 2.1/3.5 (Bruker Biospin Ltd).

3. Results and discussion

We first tested the CF expression protocol on the hydrophobin $EAS_{\Delta15}$, a 15-residue truncation mutant of the class I hydrophobin EAS from *Neurospora crassa*. $EAS_{\Delta15}$ was chosen as the removal of 15 residues from the large disordered Cys3-Cys4 loop led to improved expression and refolding compared to full-length EAS but did not affect its core structure or assembly properties [30]. Expression of $EAS_{\Delta15}$ in the soluble fraction was observed in the standard CF condition even though expression level was initially very low (Fig. 2). Because the relatively high concentration of DTT (1.7 mM) in the standard CF condition might interfere with the correct folding of hydrophobins, lower DTT concentrations were tested. Reduction of the DTT concentration to ~0.21–0.42 mM, substantially increased the amount of soluble $EAS_{\Delta15}$ produced (Fig. 2).

We next tested whether this optimised CF condition with 0.2 mM DTT could be used to produce MPG1, a hydrophobin from the rice blast fungus, *Magnaporthe oryzae* and associated with infection of rice [19]. Typically, *in vitro* refolding of MPG1 gives a much lower yield of fully folded protein than that of $EAS_{\Delta15}$ with only $< \sim 20\%$ of the MPG1 protein remaining soluble after oxidative refolding. The level of MPG1 in the soluble fraction was initially low under CF expression conditions optimised for $EAS_{\Delta15}$ with the protein expressing mostly in the

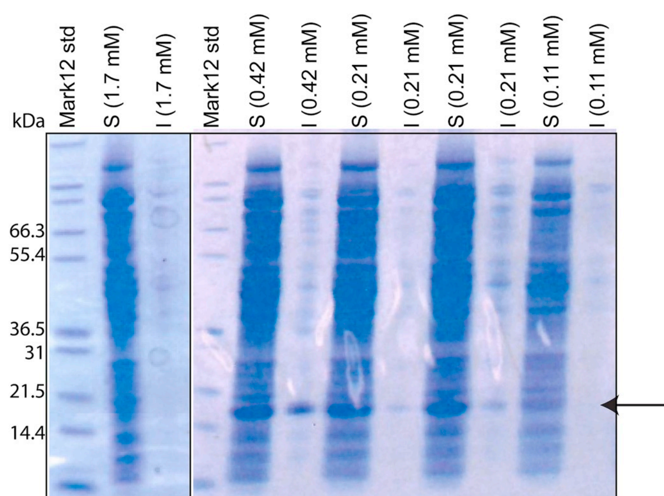

Fig. 2. SDS-PAGE showing the overexpression of $EAS_{\Delta 15}$ in the soluble and insoluble fractions as the concentration of DTT in the CF reaction is varied. Replicates were carried out for all concentrations tested but only the 0.21 mM concentration replicates are shown. S and I represent the soluble and insoluble fractions, respectively. Lane with Mark12™ protein standard is indicated. Arrow indicates expected position of His_6-Ub-$EAS_{\Delta 15}$. The overexpression of $EAS_{\Delta 15}$ can be enhanced substantially by reduction of the DTT concentration from 1.7 mM to ~0.2–0.4 mM.

insoluble fraction (Fig. 3A). Since the insoluble fraction probably contained misfolded MPG1 with incorrect disulphide bonds, we next tested whether the addition of GSH:GSSG could increase the amount of soluble protein produced. The addition of GSH:GSSG has previously been shown to increase the correct folding of proteins with disulphide bonds [44]. The level of soluble MPG1 expression could be enhanced substantially with GSH:GSSG added at the molar ratio of 2:3 (Fig. 3B). However, it was noted that on occasions at longer incubation times (> ~4 h), the amount of soluble protein decreased, so an inhibitor cocktail was added to the CF reactions, which allowed for an unchanged level of overexpression of MPG1 in the soluble fraction between 4 h and 16 h (Fig. 3C).

The optimised conditions used for MPG1 expression were then applied to the expression of DewY, EAS, NC2 and RodA hydrophobins. These proteins have been chosen as they are of scientific interest and have been previously studied in our laboratory. For example, the spores of *Aspergillus fumigatus*, an opportunistic human pathogen, are coated by the hydrophobin RodA, enabling them to evade the host immune response [45]. DewY is an engineered mutant of DewA, a hydrophobin from *A. nidulans,* which has been used in a number of biotechnological applications [46]. NC2 is a Class II hydrophobin from *A. nidulans* for which the structure has been determined [26]. The optimised CF conditions could be used to successfully overexpress DewY, EAS, NC2 and RodA hydrophobins to a reasonable yield in the soluble fraction ranging from 0.1 to 1 mg/mL of CF reaction based on the intensity of the overexpressed bands on the SDS-PAGE after minor variations in the plasmid concentration used (Fig. 4). Interestingly, in all cases a substantial reduction in DNA concentration from the original recommended DNA concentration of ~16–20 μg/ml [38] to ~1–4 mg/mL gave the highest expression for the hydrophobins tested in the soluble fraction (Fig. 4 and Supp. Figure 1). This is probably due to lower DNA concentrations allowing for a slower rate of protein production which gives time for the four disulphide bonds to form correctly and for the hydrophobins to fold, thereby avoiding protein aggregation and precipitation.

Next we tested if the expression levels of hydrophobin proteins in the soluble fraction are further enhanced by the addition of a disulphide bond isomerase, namely DsbC. DsbC acts to proofread disulphide bond formation [47] and has been used as a chaperone in protein expression

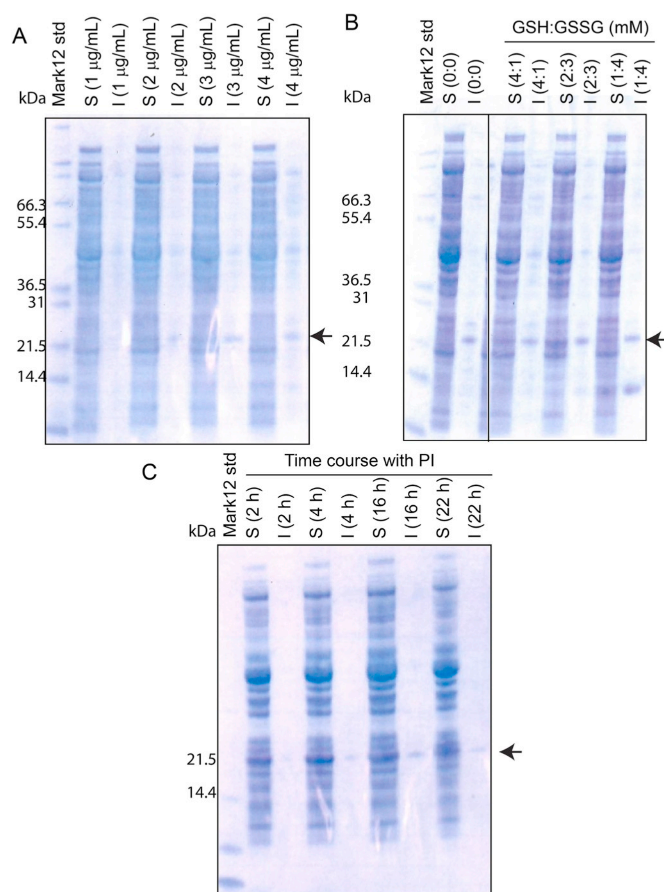

Fig. 3. (A) SDS-PAGE showing the overexpression of MPG1 in the soluble and insoluble fractions as the concentration of plasmid DNA in the CF reaction is varied. The DTT concentration was constant at 0.21 mM. (B) SDS-PAGE showing the overexpression of MPG1 in the soluble and insoluble fractions as the concentration of GSH:GSSG in the CF reactions is varied with the most MPG1 being expressed in the soluble fraction at GSH:GSSG of 2:3 mM. (C) SDS-PAGE showing the overexpression of MPG1 in the soluble and insoluble fractions as the CF reaction was incubated from 2 to 22 h. The optimised concentration of GSH:GSSG was used and PI indicates the presence of protease inhibitor in the reaction mix. S and I represent the soluble and insoluble fractions, respectively. Lane loaded with Mark12™ protein standard is indicated. Arrows indicate expected position of His_6-Ub-MPG1.

systems. DsbC is able to rearrange non-native disulphide bonds to their native forms. It has been reported that the addition of DsbC under oxidative conditions increases the yield and efficiency of refolding for proteins that contain disulphides, whilst decreasing protein aggregation [44,48,49].

Under the DsbC concentrations tested, the expression level of soluble $EAS_{\Delta 15}$ did not appear to increase probably due to the already high level of $EAS_{\Delta 15}$ expression even without DsbC (Fig. 5A). In contrast, the expression of soluble MPG1 increased when DsbC was added at a range of concentrations to the CF reactions (Fig. 5B). In particular, the inclusion of DsbC at concentrations ranging from 0.15 to 0.3 mg/mL was found to significantly improve the expression level of soluble MPG1 and further enhanced the expression of RodA and EAS (Fig. 5C), the two hydrophobins which had a low yield (Fig. 4). A summary of the optimised conditions used for the six hydrophobins is provided in Table 1.

During hydrophobin engineering, it is highly desirable to determine if a hydrophobin variant is correctly folded, without the use of large-scale expression and multiple purification steps. In hydrophobins, the correct pattern of disulphide bond formation is critical for the correct folding of the protein, and the position of cysteines labelled with ^{15}N in ^{15}N-HSQC NMR spectra can inform if the protein has folded correctly.

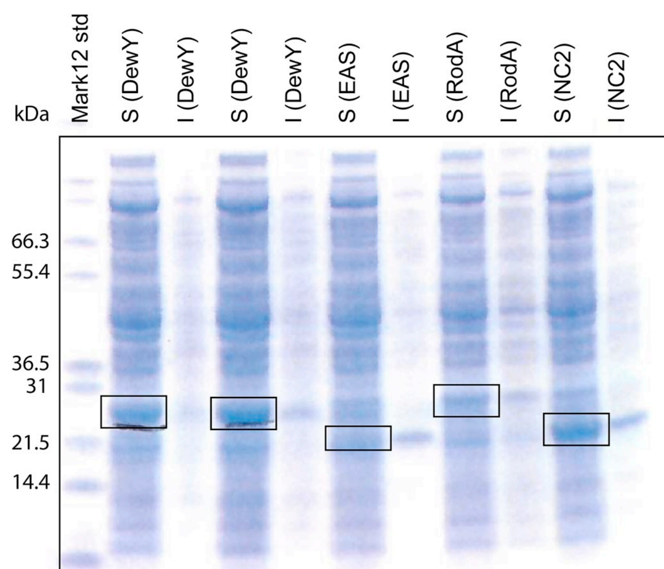

Fig. 4. SDS-PAGE showing the overexpression of DewY, EAS, RodA and NC2 in the soluble and insoluble fractions under the CF condition optimised previously for MPG1 expression. Mark12™ protein standard was loaded onto the leftmost lane. Boxed regions indicate expected position of the His_6-Ub-hydrophobin fusions.

Unlabelled cysteine in the acid-soluble amino acid stock solution was replaced with ^{15}N-cysteine in the CF expression to allow cysteines to be visualised in ^{15}N-HSQC spectra. Because other components in the CF expression are not enriched with ^{15}N, they are conveniently invisible in ^{15}N-HSQC spectra, which means they do not need to be removed from the sample to determine if the expressed hydrophobin is folded. In addition, as the His_6-ubiquitin tag does not contain any cysteines, it is also NMR-invisible, which makes its cleavage and removal optional for assessing folding of the attached hydrophobin.

There was no difference in protein expression levels between the ^{15}N-cysteine labelled and unlabelled CF reactions for all hydrophobins tested. Following CF expression, reaction mixtures containing overexpressed hydrophobins with ^{15}N-cysteines were subjected to Ni-affinity chromatography, dialysed into NMR buffer and ^{15}N-HSQC spectra were directly recorded or after a concentration step using a 3-kDa centrifugal concentrator. The ^{15}N-HSQC spectra showed the ^{15}N-cysteine labelled proteins are correctly folded, with the positions of ^{15}N-cysteines agreeing well with those from uniformly ^{15}N-labelled proteins produced previously using conventional *E. coli* expression (Fig. 6A and B for $EAS_{\Delta15}$ and MPG1, respectively, and Supp. Fig. 2A, C and E for DewY, RodA and NC2, respectively). However, in some cases only 5–7 cysteines, instead of all 8 contained within the amino acid sequence, could be observed in the ^{15}N-HSQC spectra for each of the hydrophobins. This has been attributed to cysteine residues in disulphide bonds having unusual bond geometry and complex dynamics. As a result, broad or missing resonances are sometimes observed for cysteines in disulphide bonds [50]. In all cases, missing cysteine peaks in the ^{15}N-labelled samples produced using CF are also not visible or are weak and broad in the uniformly labelled samples obtained from recombinant expression indicating the disulphide bonding pattern and the fold of the hydrophobins are the same. TEV protease was then added to the NMR samples, cleaving the His_6-ubiquitin from the hydrophobin (confirmed by SDS-PAGE), and for some hydrophobins (e.g. DewY), this led to an improvement in the signal-to-noise ratio and narrowing of the peaks in ^{15}N-HSQC spectra but no change in peak positions (Fig. 6C for MPG1, and Supp. Fig. 2B, D and F for DewY, RodA and NC2, respectively), indicative of cleavage of the His_6-ubiquitin tag and maintenance of the hydrophobin fold. In some cases, extra signals are also observed in the ^{15}N-HSQC spectra, including one resonance at ~8.5 ppm/123 ppm.

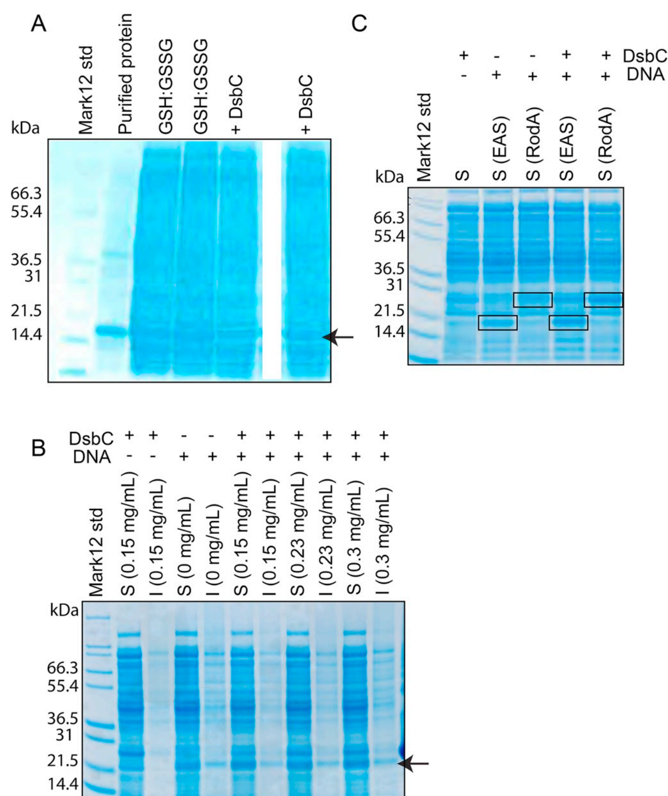

Fig. 5. (A) SDS-PAGE showing the overexpression of $EAS_{\Delta15}$ in the soluble fraction produced by CF expression with either the addition of GSH:GSSG (2:3 mM) or DsbC (0.15 mg/mL). Replicates are shown. Purified $EAS_{\Delta15}$ from a previous recombinant expression was loaded onto Lane 2 for comparison. (B) SDS-PAGE showing the overexpression of MPG1 in the soluble and insoluble fractions as the concentration of DsbC in the CF reactions is varied, with a substantial increase in MPG1 expression in the soluble fraction at a DsbC concentration of 0.15–0.3 mM. (C) SDS-PAGE showing the overexpression of EAS and RodA in the soluble fraction can also be enhanced by including 0.15 mg/mL DsbC in the reaction. A negative control reaction without any plasmid DNA but containing DsbC is loaded onto Lane 2. S and I represent the soluble and insoluble fractions, respectively. Arrows indicate expected position of His_6-Ub-MPG1. Lane loaded with Mark12™ protein standard is indicated. Boxed regions indicate expected position of the His6-Ub-hydrophobin fusions.

Table 1
Summary of optimised conditions for hydrophobin production using CF.

Hydrophobin	$EAS_{\Delta15}$	EAS	DewY	MPG1	NC2	RodA
[DNA] µg/mL	1–4	0.3–1	0.3–1	0.3–1	0.3–1	0.3–1
DTT (mM)	0.2	0.14	0.14	1.7	0.14	1.7
GSH:GSSG (mM)	[a]	2:3	2:3	1:10	2:3	1:10
DsbC (mg/mL)	[a]	0.2	–	0.2	–	0.4

[a] Note that for $EAS_{\Delta15}$, a range of conditions gave similarly good expression and the inclusion of GSH:GSSH or DsbC did not affect expression levels.

These extra signals are located in the central part of the ^{15}N-HSQC spectra corresponding to flexible regions and are likely to arise from hydrophobin degradation products or partially translated fragments, consistent with the presence of lower molecular weight bands on SDS-PAGE gels (for example, see Supp. Figure 3A). Varying amounts of lower molecular species are also commonly observed in recombinant hydrophobin preparations which can be removed using rpHPLC if required.

Lastly, hydrophobin fusion proteins produced under CF were also subjected to Ni-affinity chromatography, TEV cleavage and rpHPLC (Supp. Figure 3) with purification properties and elution profiles comparable with hydrophobins oxidatively refolded after purification

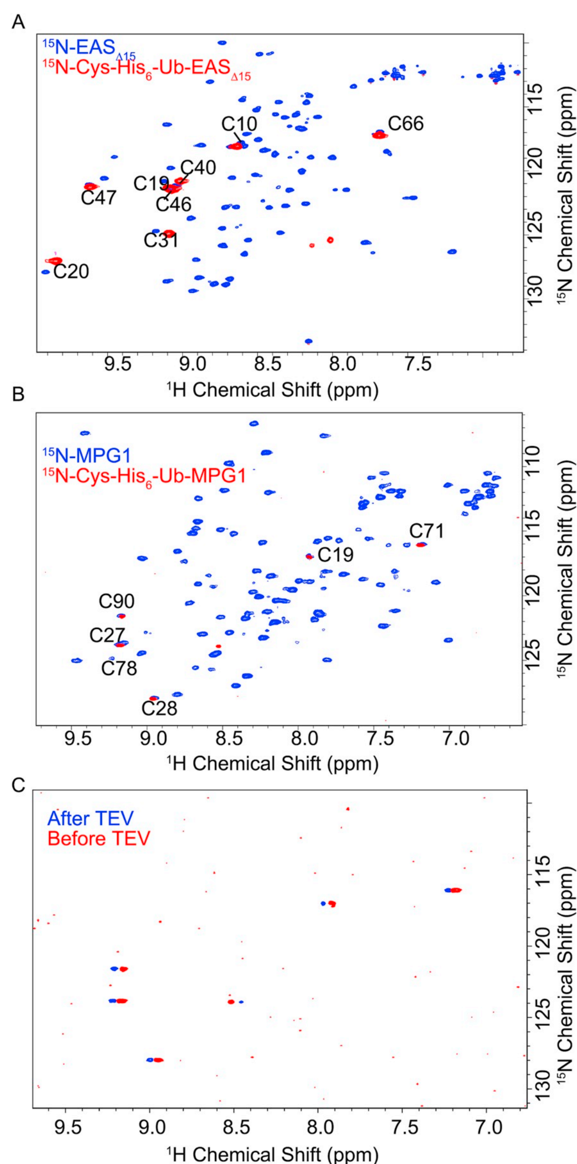

Fig. 6. (A and B) Overlay of ^{15}N-HSQC spectra recorded on purified recombinant ^{15}N-labelled hydrophobin produced from *E. coli* with those recorded from ^{15}N-Cys-His$_6$-ubiquitin-hydrophobin fusions produced using CF synthesis. (A) EAS$_{\Delta15}$ and (B) MPG1. (C) Overlay of ^{15}N-HSQC spectra recorded before (red) and after (blue) the addition of 20 μg/mL of TEV to ^{15}N-Cys-His$_6$-ubiquitin-MPG1. The red spectrum has been shifted to the right by ~0.04 ppm to aid visual comparison of the linewidths as otherwise the peaks corresponding to cysteine residues in the two spectra overlap completely.

from inclusion bodies. Mass spectrometry confirmed that the mass of the unlabelled hydrophobins produced using the CF protocol is consistent with formation of four cystines.

In conclusion, we have established an easy-to-use and rapid CF expression system for hydrophobin expression that directly yields natively folded hydrophobins with four disulphide bonds without the need of a series of oxidative *in vitro* refolding steps following protein expression. While the yield of folded hydrophobin is relatively low (0.1–1 mg purified protein/mL extract based on SDS-PAGE analysis and Abs280 nm after rpHPLC), CF coupled with selective isotope labelling and NMR spectroscopy provides a time efficient and powerful tool where proteins can be produced directly from DNA and structurally characterised in as little as 24 h, compared with ~2 weeks using conventional recombinant overexpression. By carefully adjusting DTT, GSH:GSSG and DsbC concentrations where required, the yield of individual hydrophobins can be improved substantially. This system provides direct control of the reaction environment and streamlines the process of screening the refolding characteristics of new hydrophobin mutant constructs. In addition, it offers the opportunity to perform selective isotopic labelling on hydrophobins which can be applied to studying the rodlet forms of hydrophobins. Due to the heterogeneous nature of these large, insoluble and non-crystalline rodlet structures resulting in broad overlapping signals in the solid-state NMR spectra, the incorporation of stable isotopes in selected amino acids using the CF system can be used to reduce the complexity of solid-state NMR spectra. The CF system has also been used by others to incorporate non-natural amino acids to proteins [39]. The addition of non-natural amino acids with unique functional groups or fluorescent tags [51,52] can potentially be used to further functionalise hydrophobins and yield additional insights about their assembled structures and provide new biotechnological applications.

Funding

This work was supported by the Australian Research Council (DP120100756).

CRediT authorship contribution statement

Rezwan Siddiquee: Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Investigation. **Samuel Sung-chan Choi:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Investigation. **Shirley Siuley Lam:** Methodology, Investigation. **Patrick Wang:** Formal analysis, Visualization, Writing - original draft. **Ruhu Qi:** Methodology, Investigation, Formal analysis, Writing - review & editing. **Gottfried Otting:** Methodology, Formal analysis, Writing - review & editing. **Margaret Sunde:** Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Ann Hau-yu Kwan:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Investigation, Supervision, Project administration, Funding acquisition.

Acknowledgement

We acknowledge Sarah Webb for providing assistance with making the S30 extract, Hadrien Chéreau for expression and purification of DsbC, Rengen Parlane for preliminary screening of DsbC conditions, Takanori Kigawa for the kind gift of the DsbC clone and Bill Bubb for proofreading the manuscript. We acknowledge access to the NMR facility at School of Life and Environmental Sciences, University of Sydney.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2020.105591.

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