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Intensified expression and purification of a recombinant biosurfactant protein

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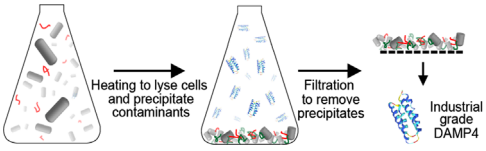
HIGHLIGHTS

- Developed a parallel molecular and process design strategy to produce biosurfactant protein.
- No tag protein, enzymatic cleavage, mechanical cell disruption, or chromatography was required.
- Cell disruption and precipitation of contaminants achieved by thermal treatment.
- Biosurfactant expression yield of 15 mg L^{-1} $OD_{600}{}^{-1}$ in fully synthetic minimal media.
- Biosurfactant recovered by filtration and precipitation units only.

GRAPHICAL ABSTRACT



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ABSTRACT

Proteins and peptides are emerging as components for novel materials that are switchable in response to their environment, and have enhanced sustainability over traditional materials. Proteins and peptides are known to be surface active and are widely used to stabilise foams and emulsions. However, designed surfactant proteins and peptides are presently produced using non-industrial processes based, for example, on costly chromatographic approaches developed for biopharmaceuticals. Here we report an intensified chromatography-free process for protein and peptide surfactant manufacture, for a recently-reported helix-bundle class of biosurfactants. The helix bundle structure is shown to remain stable and soluble under temperature and salt conditions that disrupt cells and precipitate cellular proteins. This finding opens a process route which simply involves heating cells, in fermentation media, to high temperature (e.g. 95 °C), leading to release of soluble biosurfactant with simultaneous precipitation of contaminants. This "bake-to-break and precipitate" (BPP) process allows recovery of purified biosurfactant through simple thermal treatment followed by solid–liquid separation. Experiments were conducted with the four-helix bundle protein DAMP4, which was expressed intracellularly at a level of 15 mg L^{-1} $OD_{600}{}^{-1}$ in fully synthetic minimal media. Thermal treatment of 100 mL of *E. coli* suspension at OD_{600}=4 produced 4.8 mg of functional protein surfactant at a yield of 84% following simple microfiltration. Further polishing by precipitation and filtration gave 2.4 mg of highly-pure biosurfactant. This work demonstrates that co-considered molecular and intensified process design can be used to progress the development of new biological products into low-cost industrial sectors such as those based on surfactants.

1. Introduction

New bio-based chemical products will play a significant role in improving the sustainability of current materials and chemicals

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manufacture, particularly in industrial applications where greener, more sustainable alternatives to existing, non-renewable or biodegradable components are needed, or where new functionality is desired. In the transition to this sustainable future, bioprocess-derived peptides and proteins are emerging as key building blocks for such novel bio-derived products (Banat et al., 2010; Black et al., 2012; Dexter et al., 2006; DiMarco and Heilshorn, 2012; Hauser and Zhang, 2010; Jin and Kaplan, 2003; Kim et al., 2010; Koopmans and Middelberg, 2009; Lakshmanan et al., 2012; Liu et al., 2012; Luo and Zhang, 2012; Omenetto and Kaplan, 2010, 2012; Qiu et al., 2003; Zhang, 2003, 2004; Zhang et al., 2002, 2010; Zhao and Zhang, 2007). There is considerable engineering potential to design both products and processes (e.g. Abdollahi and Dubljevic, 2012; Telen et al., 2012; Tuchscherer et al., 1998) that facilitate translation of these emerging products to industrial reality, through new approaches in bioprocess engineering and synthetic biology (Rollie et al., 2012). This approach of co-considered design of product and process relies on 'process intensification', defined as '…a set of often radically innovative principles ("paradigm shift") in process and equipment design, which can bring significant benefits in terms of process and chain efficiency, capital and operating expenses, quality, wastes, process safety, and more. '(European Federation of Chemical Engineering Working Party of Process Intensification, 1999).

Within the range of bio-based products being developed, products for the surfactant industry, which currently relies on petrochemically-based products, are receiving increasing attention (Banat et al., 2000; Desai and Banat, 1997; Fiechter, 1992; Marchant and Banat, 2012b; Winterburn and Martin, 2012). Extensive research directed at developing and studying a range of biosurfactants, including microbially-produced lipid-based biosurfactants (Cortes-Sanchez et al., 2013; Henkel et al., 2012; Langer et al., 2006; Luna et al., 2013; Marchant and Banat, 2012b; Morita et al., 2009; Nott et al., 2013; Van Bogaert et al., 2011) naturally-occurring surface active proteins such as ranaspumins (Mackenzie et al., 2009), rationally designed protein/peptide-based biosurfactants (Dexter and Middelberg, 2008; Middelberg and Dimitrijev-Dwyer, 2011), and analysis of these (Middelberg et al., 2008; Chen et al., 2013; Penfold et al., 2012), is ongoing. For lipid-based biosurfactants, process economy issues are being addressed by attempts to decrease substrate costs, optimise fermentation, improve downstream processing, and identify high-producing strains (Desai and Banat, 1997; Marchant and Banat, 2012a; Mukherjee et al., 2006). While these efforts are yielding tangible results, there remains need for improvements in product yield (Mukherjee et al., 2006; Marchant and Banat, 2012a), due to limitations in the indirect synthesis pathways for lipid-based biosurfactants (Kaar et al., 2009).

As an alternative to existing biosurfactants, surface-active peptides and proteins can be rationally designed and produced directly from recombinant DNA. This approach allows for molecular design and hence the introduction of new function, for example functional switching in response to physicochemical triggers, which is of growing interest (Carl and von Klitzing, 2011). The use of recombinant microbial systems also unleashes the full power of recombinant industrial biotechnology into the area of biosurfactants, allowing engineering-based solutions to problems of yield, cost and process scale-up. Nevertheless, the earliest generation of switchable peptide-based biosurfactants, for example AM1 (Dexter et al., 2006; Dexter and Middelberg, 2007; Malcolm et al., 2006 Middelberg et al., 2008), proved difficult to produce recombinantly in an efficient manner. For example, the bio-production of a recombinant variant of AM1 involved co-expression of a carrier protein (Kaar et al., 2009), which is a common strategy for the expression of peptides in general, as due to their small size and lack of tertiary structure, peptides are difficult to express alone (Kyle et al. 2009). However, this strategy increases cost as valuable substrate is diverted into a non-product carrier protein which must then be cleaved, purified and disposed of. While the interfacial behaviour of other peptides has been investigated (Lu et al., 2004; Pan et al., 2010; Vauthey et al., 2002), to the best of our knowledge, research into the bioproduction of these peptides has not been undertaken.

The dominant use of carrier-protein strategies and conventional purification methods such as chromatography currently limits the application of peptides into high-value sectors (Hartmann et al., 2009; Huang et al., 2009; Kaar et al., 2009; Kyle et al., 2009, 2012; Moers et al., 2010; Palczewski et al., 2000; Riley et al., 2009). Although considerable research has been directed at improving the efficiency of peptide manufacture, methods to date, without exception, represent small improvements targeted to high-value biopharmaceutical processes. There are presently no methods for the large-scale efficient manufacture of surfactant proteins and peptides at a price that would enable new materials to significantly impact large-scale industrial sectors. To address this translational barrier, we have adopted a new approach based on a family of designed biosurfactant proteins. Surface-active proteins are common in nature, such as those isolated from milk (β-lactoglobulin, whey proteins, caseins), and egg (lysozyme), which have important roles in the food industry (Bos and van Vliet, 2001; Dickinson, 1999; Wilde et al., 2004; Wilde, 2000), as well as non-food proteins (Cooper and Kennedy, 2010). Proteins act as surfactants by imparting a range of stabilising mechanisms to fluid interfaces, including steric repulsion, electrostatic repulsion and viscoelasticity. These properties are responsive to solution conditions such as pH and salt, and therefore have inherent controllable stability. The first non-naturally occurring designed protein surfactant is DAMP4, having amino acid sequence MD(PSMKQLADS-LHQLARQ-VSRLEHAD)$_4$ (Middelberg and Dimitrijev-Dwyer, 2011). DAMP4, which comprises a repeating sequence of four AM1 peptide modules, can be either be used as a biosurfactant itself (Middelberg and Dimitrijev-Dwyer, 2011) or can be acid-cleaved into individual peptides (Dimitrijev Dwyer et al., 2013). Circular dichroism measurements showed DAMP4 has a highly helical secondary structure (Middelberg and Dimitrijev-Dwyer, 2011) consistent with a four-helix bundle structure in bulk, as designed (Fig. 1). Four-helix bundle structures are known to have good post-expression stability (Akiva et al., 2008; Hecht et al., 1990), and due to this design, DAMP4 was

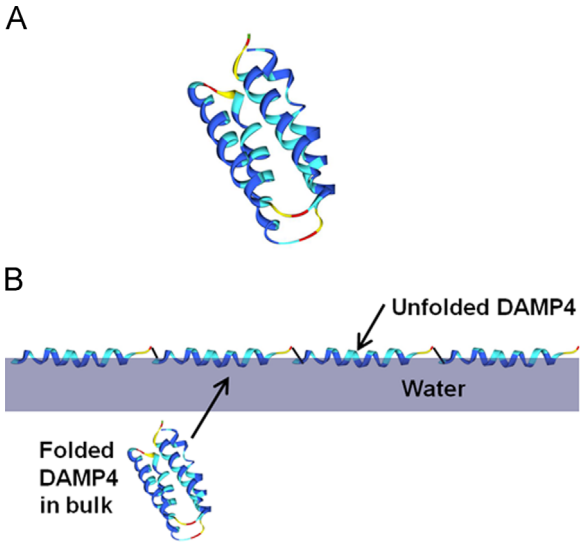

Fig. 1. (A) Cartoon of DAMP4 4-helix bundle – generated using VMD software, Available at: http://www.ks.uiuc.edu/Research/vmd Humphrey et al. (1996) and (B) cartoon of DAMP4 unfolding at an interface.