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A non-foaming proteosurfactant engineered from Ranaspumin-2

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ABSTRACT

Advances in biological surfactant proteins have already yielded a diverse range of benefits from dramatically improved survival rates for premature births to artificial photosynthesis. Presented here is the design, development, and analysis of a novel biosurfactant protein we call Surfactant Resisting Foam formatioN (SRFN). Starting with the Tungara frog's foam forming protein Ranaspumin-2, we have engineered a new surfactant protein with a destabilized hinge region to alter the kinetics and equilibrium of the protein structural transition from aqueous globular form to an extended surfactant structure at the air/water interface. SRFN is capable of approximately the same total surface tension reduction, but with the unique property of forming quickly collapsible foams. The difference in foam formation is attributed to the destabilizing glycine substitutions engineered into the hinge region. Surfactants used specifically to increase wettability, such as those used in agricultural applications would benefit from this new proteosurfactant since foamed liquid has greater wind resistance and decreased dispersal. Indeed, given growing concern of organsilicone surfactant effects on declining bee populations, biological surfactant proteins have several unique advantages over more common amphiphiles in that they can be renewably sourced, are environmentally friendly, degrade readily into non-toxic byproducts, and reduce surface tension without deleterious effects on cell membranes.

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1. Introduction

Surfactants are a diverse range of molecular and biological structures which permit the mediation of fluid/fluid interfaces due to relative hydrophobicity or hydrophilicity of spatially separated moieties within their structure [1]. As a result of this architecture, surfactant molecules align preferentially along particular interfaces and act to reduce the surface tension and thus permit the activity of otherwise unavailable processes [1]. Biological surfactants are ubiquitous in nature and perform a host of critically important roles in growth, development, immunity, and motility [2,3]. Based on their unique chemical characteristics and biocompatibility, biosurfactants are widely used in industries ranging from agriculture to food production to soil and water remediation [4–6].

While biosurfactants generally have a similar physical role in the mediation of aqueous and hydrocarbon interfaces, their structure and function are rather diverse [2] and instead, are generally characterized by chemical constituents and organism of origin. For example, biological proteosurfactacts share amino-acids as a building block of varied molecular structures that include lipopeptides, lipoproteins, polymer biosurfactants, and particulate biosurfactants [2]. Another common feature amongst the diverse range of biosurfactants is the formation of lipoprotein or lipid-mimetic structures capitalizing on the separated hydrophobic and hydrophilic structures in order to reside at the air/water interface [7,8]. This includes the formation of extensive stable lipoprotein complexes of the pulmonary surfactant proteins SP-B and SP-C, and the lipid-mimetic head–tail structure of surfactin and Ranaspumin-2 (Rsn-2), with corresponding hydrophobicity and hydrophilicity of the opposing regions [9–11].

A widely occurring and critically important biosurfactant is the cocktail of four constitutive pulmonary surfactant proteins, referred to as surfactant proteins (SP) A-D of which SP-B and SP-C, in conjunction with essential lipids, form the lipoprotein mixture that prevents pulmonary alveoli collapse via significant lowering of the lung fluid surface tension during respiration [10,12]. Analysis of surfactant protein activity, such as pulmonary SP-B which is known to be necessary for proper respiration [13] and has conserved sequence homology [10], demonstrates increased surface tension

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reduction in mixtures with additional amphiphilic molecules such as lipids [14]. This increased surfactant activity is attributed to complexing of pulmonary surfactant proteins with lipid molecules [15]. Structurally, SP-B, a hydrophobic protein with significant cationic residues, contains considerable alpha-helical character as determined by circular dichroism; sequence homology to the saposin family suggest its conformational flexibility may relate to interfacial activity [16].

Another important biological surfactant class is the ranaspumins, which are a selection of proteins found in foam nests of various frog species, which perform the functional role of providing a protective, homeostatic environment for egg development prior to eclosion, or hatching [9,17]. Ranaspumins with significant surfactant activity that exceeds globular proteins such as bovine serum albumin (BSA) have been isolated from Central and South American frog *Physalaemus pustulosus* [9] and the Northeast Brazilian frog *Leptodacylus vastus* [18]. Ranaspumins from both of these species exhibit similar surface tension reducing and foam forming capabilities [19]. In the case of Ranaspumin-2 (Rsn-2), isolated from *P. pustulosus*, structural analyses have demonstrated a lipid-mimetic protein configuration capable of existing in two conformations: one a closed, water soluble globular form and the other an "open" state at the air/water interface [9]. The latter conformation has surfactant activity and foams at the interface when agitated which parallels the behavior of the complex mixture of proteins and complex carbohydrates found in the frog foam nests [9,17]. Most importantly, the structure and physiological behavior of the ranaspumins renders them biologically labile in the aqueous phase, a necessary trait in the natural application, but also useful in engineering applications [20] wherein the usage of more biologically destructive cell membrane penetrating surfactins remains unfavorable.

In addition to the aforementioned applications, biosurfactants have the capacity to increase both hydrophobic surface wettability and the spreadability of aqueous solutions along surfaces of diverse hydrophobic and hydrophilic characteristics [21,22]. An example of this type of surfactant function is latherin, a non-glycosylated PLUNC (palate, lung, and nasal epithelium clone) family protein [23]. Latherin is produced in both equine sweat and saliva [24] and is responsible for the critical wettability of the external surface of the horse pelt, which is otherwise waterproof and therefore extremely hydrophobic, in order to promote evaporation and essential dissipation of heat for thermoregulation [23]. Neutron reflectivity and infrared reflectance experiments at the air/water interface suggest that latherin undergoes a significant conformational change from a folded globular protein to a component of the interfacial layer [24].

Biosurfactant molecules, which by definition reduce the surface tension in aqueous solutions, often have a variety of conformational states that confer functionality and can contribute to stable foam and bubble formation such as the ranaspumins [9,19], provide increased wettability and interaction along the interface as in latherin and surfactin [11,21–23], or both functions, which occurs in the pulmonary surfactant cocktail [15]. Despite significant structural and functional differences, surfactant proteins from distinct groups such as latherin and Ranaspumin-2, have shown similar activity in terms of the reduction of surface tension as a function of protein concentration [9,24].

Because of these unique chemical properties, biosurfactants are being adapted and used for a wide range of applications [4–6]. Biological impact and the potential for future contamination are essential criterion to consider when selecting surfactants for environmental applications [25]. Recently, there has been an increasing body of evidence demonstrating that the "inert" but persistent organosilicones used as agriculture adjuvants are contributing to honeybee declines [26–29]. Biodegradable and cell friendly surfactants like SRFN may be a helpful alternative to present silicone technology by meeting the need for increased wettability while minimizing environmental impact. Support for biologically sourced surfactants as opposed to synthetically generated molecules may also reside in performance. For example, biosurfactants are often employed for bioremediative purposes such as cleansing soil of hydrophobic contaminants and dispersion of excess hydrocarbons from oil spills [25].

Previous work has demonstrated that synthetic and engineered biological synthesis of known biosurfactants has the potential to generate active surfactant molecules with properties similar to naturally produced and collected biosurfactants and thus may provide a feasible source of surfactant molecules for diverse and widespread real-world applications [30]. To this end, we have designed a new surfactant protein inspired by the Ranaspumin-2 structure, which in its most basic form, consists of a short alpha helix capped by a beta sheet. SRFN retains the biologic compatibility of Rsn-2, ultimately the same surface tension reduction, but contains a more flexible hinge point which reduces foaming. The latter is particularly useful when wettability and spreading is desired, such as in agricultural applications, since foams can have greater wind resistance and reduced coverage. This unique combination of surface tension reduction with destabilized foam formation was engineered by altering the free energy of motion available for the region linking the helix and beta sheet.

NMR studies have demonstrated a structural rearrangement similar to a clamshell-like extension when Rsn-2 approaches the air/water interface which places the helix in the air [9]. For this conformational change to occur at the interface, we postulated that the amino acid residues in the moment arm between these two domains must play a critical role, since this location contains a relatively stable hydrogen bonded turn in globular form [9]. The positions of the first 18 amino acid loop form a link with the beta sheet in globular form (Fig. 1, left) but remain undocumented (dotted line Fig. 1) in surfactant form (Fig. 1, right). We estimated the hinge region (Fig. 1, amino acids highlighted in yellow) to be resistant to opening [9] based on simulated free energy changes with the Site Directed Mutator [32]. To destabilize this hinge point, allowing the helix greater freedom of movement, we replaced amino acids 39–41 with glycines. This triple amino acid mutation alters the protein's ability to shift from aqueous globular form to the extended surfactant structure at the air/water interface by increasing the freedom of motion at the hinge point. The cost of this destabilization was slower equilibria at the air/water interface (Fig. 4), but wettability and surface activity remained similar to that of Rsn-2.

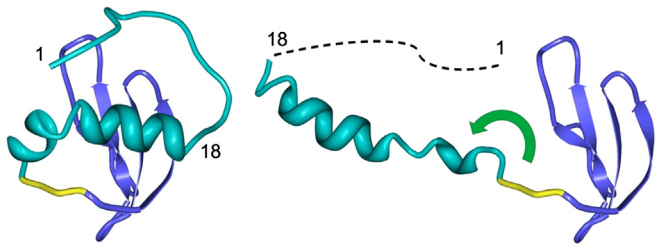

Fig. 1. Illustration of Rsn-2 conformational change (green arrow) of the helix (aqua) separating from the beta sheet (purple) at the hinge point (yellow) adapted from Kennedy [23] using Rsn-2 structure 2WGO [9] and rendered with Protein Workshop [31]. The dotted line represents the first 18 amino acids which form an interacting loop between the helix and beta sheet in globular form (left), but remain ambiguously located [9] after shifting to surfactant form (right).

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