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High yield recombinant production of a self-assembling polycationic peptide for silica biomineralization

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ABSTRACT

We report the recombinant bacterial expression and purification at high yields of a polycationic oligopeptide, P5S3. The sequence of P5S3 was inspired by a diatom silaffin, a silica precipitating peptide. Like its native model, P5S3 exhibits silica biomineralizing activity, but furthermore has unusual self-assembling properties. P5S3 is efficiently expressed in *Escherichia coli* as fusion with ketosteroid isomerase (KSI), which causes deposition in inclusion bodies. After breaking the fusion by cyanogen bromide reaction, P5S3 was purified by cation exchange chromatography, taking advantage of the exceptionally high content of basic amino acids. The numerous cationic charges do not prevent, but may even promote counterion-independent self-assembly which in turn leads to silica precipitation. Enzymatic phosphorylation, a common modification in native silica biomineralizing peptides, can be used to modify the precipitation activity.

Introduction

Silaffins are highly post-translationally modified polypeptides used by diatoms to deposit silica when building their mineralized cell wall [1]. Silaffins are also capable of silica precipitation *in vitro*, either in their highly modified native state [2,3] or in non-modified recombinant and synthetic versions [4–6]. Since their discovery, much attention has been drawn to biomimetic, peptide-controlled mineral formation [7,8]. Hydrophilic and especially cationic amino acids are characteristic key factors for silicification by silaffins [2,9–12] and also serve as targets for post-translational modification. Phosphorylations, sulfations, as well as glycosylations with acidic sugar-compounds [3,9,12] introduce anionic charges which mediate electrostatic self-assembly of silaffins, a prerequisite for their precipitation activity. However, presence of polyanions such as phosphate and sulfate free in solution can compensate the lack of the covalent modification [3]. Lysines often are post-translationally methylated or connected with polyamines [2,13], increasing the silaffin's cationic charge and hence its interaction with weakly acidic silica. Polyamines themselves are able to precipitate silicic acid and are not necessarily bound to a peptide compound in diatoms [14]. Vice versa, the polycationic silaffin peptide part without polyamines attached can as well be active in biomineralization, but with a different pH optimum [2].

Short silaffin-polypeptides with and without modifications have been produced by solid phase peptide synthesis [5,6,15,16]. However, due to incomplete coupling reactions, yields of chemical syntheses will decrease with increasing peptide size [17]. Recombinant protein expression in *Escherichia coli* is hence an attractive alternative to achieve larger polypeptides and proteins, of uniform length and sequence [18], but post-translational modifications generally have to be performed after protein expression and purification. Among the silaffin-modifications, phosphorylations can easily be introduced by utilizing kinases. This modification changes the peptide net charge and affects its assembly-state by mediating electrostatic interactions [3]. Silaffins have been partially phosphorylated *in vitro* by a silaffin-specific diatom kinase [19]. Other peptides with biomineralizing activities have been phosphorylated by a variety of commercially available kinases [20–22] or extracts from pig golgi apparatus which contains a set of mammalian kinases [23]. To study the general impact of phosphorylations on biomineralizing peptides, recombinant silaffins can be engineered to contain kinase recognition sites. Especially kinases that phosphorylate sequences which contain basic amino acid residues, such

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Table 1
Primer sequences used in this study. All primers were received from Biomers GmbH (Ulm (Germany)). Abbreviations: s – sense, as – antisense, f – forward, r – reverse.

Primer name	Sequence (all: 5′–3′)	Function
PIN 1_s	GATCCTGTCGACGTGCTTCCTTA	Target gene, 5′ region
PIN 1_as	CCCTAAGGAAGCACGTCGACAG	
PIN 2_s	GGGAAATCTAAAAAGCTGCGTCGCGCTAGCCTA	Target gene, repetitive unit
PIN 2_as	CCCTAGGCTAGCGCGACGCAGCTTTTTAGATTT	
PIN 3_s	GGGAAGCTGCGTCGCGCTAGCC	Target gene, 3′ region
PIN 3_as	TCGAGGCTAGCGCGACGCAGCTT	
T7PrompET22b(+)fw	TAATACGACTCACTATAGGG	Sequencing pET22 b(+)
pET22b(+)rev	GCTCAGCGGTGGCAGC	Sequencing pET31 b(+)

as protein kinase A (PKA)[1] [24,25], appear suitable for the highly cationic silaffins.

Recombinant production of polypeptides with numerous cationic charges is usually challenging in bacterial expression systems. Presumably, this is due to interactions with anionic cellular compounds such as nucleic acids or anionic membrane lipids, which render such polypeptides toxic to bacteria [26,27]. Additionally, these polypeptides may become degraded by cellular proteases [28]. The polycationic domain of the silaffin precursor-protein Sil1p from *Cylindrotheca fusiformis* has been expressed in *E. coli*, albeit with yields in the low milligram range per liter of culture [4]. In the case of cationic, amphiphilic antimicrobial peptides [29,30], fusion strategies have been established for detoxification and overexpression in bacteria [31,32]. One efficient fusion is ketosteroid isomerase (KSI) [33], a hydrophobic protein that induces the formation of inclusion bodies. This fusion was established with a small peptide mating factor from yeast [34], and efficiently adapted for a hydrogel-forming peptide [35,36], antimicrobial partial sequences from bovine milk lactophoricin [37], antimicrobial human dermcidin [38], an aggregating amyloid protein [39], a highly-charged antimicrobial peptide with sequence PFWRIRIRR [40] as well as an intrinsically disordered domain of human cardiac troponin [41].

In this contribution, we report on the expression of a designed polycationic, biomineralizing polypeptide, P5S3, by fusing it to ketosteroid isomerase (KSI). Introduction of a kinase target side allows specific *in vitro* phosphorylation with protein kinase A (PKA). P5S3 is modeled after *C. fusiformis* silaffin, bearing numerous basic amino acid side chains, which are either lysine-clusters as in silaffin, or arginine-doublets being part of the PKA phosphorylation site.

Materials and methods

Gene design and plasmid construction

We designed a polypeptide based on silaffin motifs from *C. fusiformis* [2] (Genbank AF191634.1). Numerous construction details are of no special importance for the present work but served other purposes. To keep this section compact, only the major steps will be described here. Readers interested in the detailed cloning procedure are referred to ESI. The cloning procedures were done following standard protocols for molecular biology procedures [42]. All restriction enzymes were from New England Biolabs GmbH (Frankfurt (Germany)) and used with appropriate buffers according to the supplier.

Gene construction was achieved by ligating three primer hybrids (PIN 1, 2, and 3, all primer sequences are given in Table 1). Corresponding primer pairs (sense and antisense, each at 40 pmol/l) were hybridized by denaturation (10 min at 100 °C) and subsequent cooling to room temperature, in annealing buffer composed of 40 mmol/l Tris/HCl pH 7.5, 20 mmol/l magnesium chloride and 50 mmol/l sodium chloride. To 5′ phosphorylate the DNA double strands, hybridization samples were diluted 10-fold with buffer to achieve a final buffer composition of 9 mmol/l Tris/HCl pH 7.5, 3 mmol/l magnesium chloride, 5 mmol/l sodium chloride, 1 mmol/l dithiothreitol and 1.1 mmol/l adenosine triphosphate, and reaction with T4 polynucleotide kinase (2 units, New England Biolabs GmbH, Frankfurt (Germany)) was run for 1 h at 37 °C. Afterwards, the enzyme was heat-inactivated (20 min at 65 °C). Each of the primer hybrids contained single strand ends complementary to its neighbor to allow for oriented sticky end ligation (ESI, Fig. S1A). Hybrid PIN 2 was intended to be inserted multiple times to create repetitive sequences with variable lengths. Therefore, it was self-ligated for 30 min at 16 °C (T4 DNA Ligase, New England Biolabs GmbH, Frankfurt (Germany)), before hybrids PIN 1, 3 and (pre-ligated) PIN 2, together with the target plasmid were combined. The primary target plasmid was pET22b(+) (Merck Chemicals GmbH, Schwalbach (Germany)). Because of an earlier insertion into the multiple cloning site, the plasmid was double-digestable with *Bam*HI and *Xho*I for sticky end ligation with the corresponding 5′ and 3′ end of the assembled PIN construct. For ligation, the components were combined at molar ratios plasmid: PIN 1: PIN 2: PIN 3 of 1:2:6:2 with T4 DNA ligase. The new construct had *Ava*I restriction sites immediately 3′ and some bases 5′ of the P5S3-coding region for excision from the plasmid and insertion into the *Ava*I-digested expression plasmid pET 31b(+) (Merck Chemicals GmbH, Schwalbach (Germany)) which contains a ketosteroid isomerase (KSI) coding region upstream of the multiple cloning site, later serving as N-terminal fusion for recombinant fusion protein expression. The plasmid map and sequence of the expression vector containing KSI-P5S3 are shown in Fig. 1 and ESI Fig. S1, respectively. Please note that some single site mutageneses (codon exchange, codon insertion) were made after gene construction and are described in detail in Supplementary Information. Plasmid constructs were used to transform *E. coli* strain DH5-α (Life Technologies GmbH, Darmstadt (Germany)). All constructs were verified by sequencing (StarSeq GmbH, Mainz (Germany)) with primers T7PrompET22b(+) or pET22b(+)rev (Table 1).

Protein expression

Protein expression was performed in *E. coli* strain Rosetta (Merck4Biosciences GmbH, Schwalbach (Germany)). All LB media [43] for protein expression were supplemented with 0.1% (w/v) glucose, 100 μg/ml ampicillin and 34 μg/ml chloramphenicol.

Bacteria of an LB overnight culture (37 °C and 185 rpm shaking) were harvested (5 min at 5000g) and suspended in LB-medium at a 25-fold volume of the original culture. Cultures were grown (37 °C,

[1] *Abbreviations used:* KSI, ketosteroid isomerase; PKA, protein kinase A; Tris, Tris(hydroxymethyl)aminomethane; Bis-Tris, Bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane.