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Development of a bacteriophage-based system for the selection of structured peptides

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ABSTRACT

Short structured peptides can provide scaffolds for protease-resistant peptide therapeutics, serve as useful building blocks in biomedical and biotechnological applications, and shed light on the role of secondary structure elements in protein folding. It is well known that directed evolution is a powerful method for creating proteins and peptides with novel properties, and a system for the selection of short peptides based on structure from a randomized library would be an important advancement. In this study, phage particles monovalently displaying a short peptide and an N-terminal 6×His tag on their P3 coat protein were bound to nickel agarose resin and were subsequently challenged with a protease that specifically cleaves at a site within the peptide. The extent to which phage is proteolytically released from the resin was found to be dependent on the structural properties of the inserted peptide sequences. As proofs-of-concept, a structured peptide has been isolated from a pool of flexible peptides using a trypsin selection, and a flexible peptide has been isolated from a pool of structured peptides using a chymotrypsin selection. This selection system will be a strong technological platform for the creation of short peptides with interesting structural properties using directed evolution.

Novel structured polypeptide sequences have the potential to impact several critical areas in biotechnology and bioengineering [1–5]. Short peptides with stable structures can serve as model systems to enhance our understanding of protein folding and protein misfolding. Recent examples include designed "zinc finger" motifs [6], β-hairpin motifs [7], trp-cages [8], and tryptophan zippers [9]. The study of intramolecular interactions within these polypeptides can provide insights into key fold-determining interactions in larger, more complex proteins, thus aiding efforts in protein design and engineering. Small structured peptides can also serve as scaffolds for engineering biomolecular recognition [10–12], and there has been increasing interest in microbodies and miniproteins such as cystine knots [13,14] and the avian pancreatic polypeptide (aPP) [15,16] for this purpose. These sequences have the potential to act as proteolytically stable scaffolds for the creation of novel peptide therapeutics which often exhibit poor in vivo stability due to degradation by blood proteases [17–19]. Finally, peptides with interesting structures and properties are proving to be valuable building blocks for applications in biotechnology, nanotechnology, and synthetic biology. Structured peptides are currently being explored in the development of biomaterials, nanodevices, tissue engineering scaffolds, and as targeted drug delivery vehicles [3,20,21].

There are several approaches for obtaining short structured peptide sequences. Interesting peptides can be identified within natural protein structures, and they can be further engineered once they are isolated. Examples of this approach include the cystine knot proteins [14], elastin-like peptides [22], and the hinge peptide from the hemagglutinin protein [23]. Another approach is to use rational protein engineering to introduce structure into peptides. For example, cysteine side chains are routinely incorporated into phage displayed libraries to ensure that selected peptides are disulfide constrained [24]. And chemical tethers [25] or staples [26] can be added to synthetic peptides to introduce secondary structure. Computational design can also be used to create peptides that adopt defined structures in solution. This approach has led to the engineering of several peptides exhibiting mainly β-hairpin conformations [27–30].

There are fewer examples in the literature where combinatorial and directed evolution methods are used for discovering new structured peptides. A combinatorial approach for the discovery of short β-hairpin structured peptides has been proposed [31], but this is a computational method that relies on preexisting knowledge of β-hairpin-forming interactions in order to generate a computationally screenable library. Recently, a screening system for the identification of structured peptides based on a change in intracellular FRET signal in a peptide linking two fluorescent proteins was reported [32]. This system was successfully used to stabilize a tryptophan zipper β-hairpin scaffold and may be effective for probing structure in

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short peptides. Finally, a method for monitoring short peptide conformational changes using a single chain antibody system has been reported, but this system has not yet been used for directed evolution experiments [33,34].

Here, we describe and demonstrate a bacteriophage-based system for the selection of short peptides based on structure-induced resistance to proteolytic cleavage (Fig. 1). The selection system involves the monovalent display of a peptide of interest between a 6×His tag and the P3 coat protein of M13 bacteriophage, followed by immobilization of phage on nickel-functionalized agarose resin, and selective release from the resin of phage carrying unstructured peptides by a specific protease. Protease-based systems have previously been employed to select for structural traits in larger proteins [35,36]. For example, filamentous bacteriophages have previously been used to select structured proteins based on protease-mediated modulation of either phage infectivity [37–39] or selective phage adhesion to a solid support [40–42]. However, to the best of our knowledge, no study has attempted to evaluate phage-based proteolytic selection for the identification of structure in short polypeptides.

In this study, we developed a structural selection that involves probing protease-resistance properties within 12- or 13-mer peptides (Table 1) containing a limited number of cleavage sites for the highly specific proteases, trypsin and chymotrypsin. The ability of the selection system to enrich a structured 12-mer peptide diluted in a mixture of unstructured peptides is probed. The ability of the selection system to enrich for a readily cleavable peptide when diluted in a mixture of poorly cleaved peptides is also demonstrated. These results suggest that this method can be used to separate peptides based on structure, and this will be a valuable technique for the directed evolution of new structured peptides.

Materials and methods

Reagents and materials

The pS1602a phagemid [43] was generously provided by Genentech (South San Franscisco, CA). XL1-Blue *Escherichia coli* cells were purchased from Stratagene (La Jolla, CA). TPCK-treated trypsin (13,500 U/mg) and α-chymotrypsin (83.9 U/mg), both from bovine pancreas, were purchased from Sigma–Aldrich (St. Louis, MO), and M13K07 helper phage was obtained from New England Biolabs (Ipswich, MA). NTA agarose resin was purchased from Qiagen (Valencia, CA) and charged with 100 mM $NiSO_4$ solution according to the manufacturer's guidelines. Unless otherwise stated, all enzymes were purchased from New England Biolabs, and all other reagents were from Sigma–Aldrich. Disposable 5-ml polypropylene columns for phage binding to resin were obtained from Pierce Biotechnology (Rockford, IL).

Cloning and bacteriophage production

The P3-encoding gene in the pS1602a phagemid contains a human growth hormone (hGH) gene in place of the native N1 and N2 domains. The hGH gene in pS1602a was replaced with a peptide/6×His-tag insert created by overlap extension PCR with a DNA sequence encoding the following polypeptide: STSHHHHHGTGGSGG TGAGGGS[peptide of interest]GGGSGGSGGAGTSS. The amino acid sequences of the peptides of interest were GTGASGGKGTGS (Tryp-flex), GTGASGGSGTGS (Tryp-NK), SWTWENGKWTWQ (Tryp-rig), KPVQYWTQMFYT (Chym-flex), and GTGACWYGSYMFC (Chym-rig) (Table 1). The *Nsi*I and *Nhe*I sites in pS1602a were used for insertion of the overlap extension PCR-generated insert. All bacteriophage manipulations were carried out as described [44], with XL1-Blue *E. coli* as the infection host. In a typical phage amplification, a single colony of XL1-Blue *E. coli* was picked from a freshly streaked 2XYT agar plate containing 5 μg/ml tetracycline and grown in 2.5 ml 2XYT at 37 °C for 4.5–5 h (final OD_{600} = 0.6–0.8). One to 2 ml of the phage from either the cleaved or the eluted fraction (see section titled *Selection system* below) of the protease selections was added to the freshly grown XL1-Blue *E. coli* cells and incubated with shaking at 37 °C for an additional 15 min. The infected cells were then added to a flask containing 100 ml 2XYT containing 5 μg/ml tetracycline and 50 μg/ml carbenicillin, and grown overnight (15–17 h) with gentle shaking at 37 °C. Phages from these amplifications were subsequently precipitated with PEG as described [44], and redissolved with 3 ml NTA binding

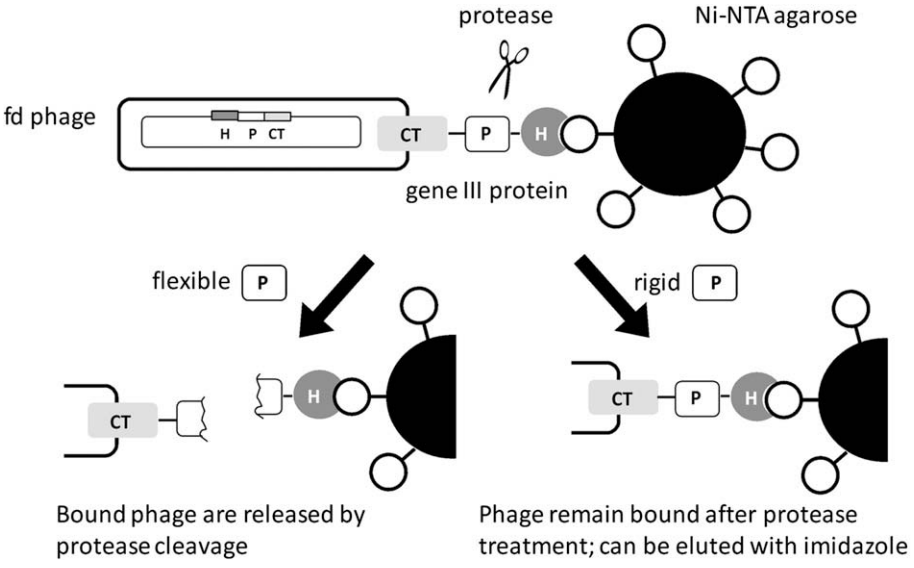

Fig. 1. Overview of the bacteriophage-based method for the selection peptides based on structure-mediated protease resistance. A peptide sequence containing a protease cleavage site and a 6×His tag is added to the N-terminus of the P3 gene. Phages are then bound to a nickel column, and the bound phages are exposed to a specific protease. Structure in the peptide region inhibits proteolytic cleavage, and this effect is used to separate rigid and flexible peptides.