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A radish seed antifungal peptide with a high amyloid fibril-forming propensity

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ABSTRACT

The amyloid fibril-forming ability of two closely related antifungal and antimicrobial peptides derived from plant defensin proteins has been investigated. As assessed by sequence analysis, thioflavin T binding, transmission electron microscopy, atomic force microscopy and X-ray fiber diffraction, a 19 amino acid fragment from the C-terminal region of *Raphanus sativus* antifungal protein, known as RsAFP-19, is highly amyloidogenic. Further, its fibrillar morphology can be altered by externally controlled conditions. Freezing and thawing led to amyloid fibril formation which was accompanied by loss of RsAFP-19 antifungal activity. A second, closely related antifungal peptide displayed no fibril-forming capacity. It is concluded that while fibril formation is not associated with the antifungal properties of these peptides, the peptide RsAFP-19 is of potential use as a controllable, highly amyloidogenic small peptide for investigating the structure of amyloid fibrils and their mechanism of formation.

1. Introduction

Amyloid fibrils are highly ordered fibrillar species in which the polypeptide chain is arranged in a cross β-sheet conformation [1]. They have attracted considerable interest of late due to their association with many diseases, their role as functional biological structures and their potential uses in nanotechnology [2,3]. Certain peptides and proteins are inherently amyloidogenic, preferentially forming misfolded intermediate structures that lead to ordered aggregation after minimal destabilization (such as a slight increase in temperature). Increasingly, peptides are being designed with amyloid fibril-forming propensities, to facilitate the *in vitro* study of amyloid fibril formation under physiological temperature and pH conditions [4]. In this study, two peptides originally derived from plant defensin proteins, which exhibit antifungal properties [5], were investigated for their amyloid fibril-forming ability.

Plant defensins are proteins produced in seeds, flowers and pathogen-stressed leaves. Their role is to provide protection for the plant against a variety of potentially damaging agents, including fungal and bacterial pathogens. Plant defensins are small proteins, 45–54 amino acids in length, which are rich in cysteine residues and include four or five intramolecular disulfide bonds [6,7]. The disulfide bonds are arranged in a specific conformational motif, known as the 'cysteine-stabilized alpha beta' (CSαβ) motif, whereby three disulfide bridges stabilize a triple-stranded antiparallel β-sheet, one strand of which is associated with an α-helix (Fig. 1). The CSαβ region is common to many insect and plant defensins and may act as a scaffold incorporating a range of important functional regions from these proteins [8–10]. To date, there is no known association between this motif and amyloidogenicity, although amyloid fibril formation by other small proteins (such as insulin) occurs following the cleavage of disulfide bonds [11].

Defensins have a range of potential commercial applications, including uses as antifungal and antimicrobial agents and as enzyme inhibitors [8]. These applications arise for both the native proteins and for their small peptide fragments. RsAFP-19, a 19 amino acid C-terminal peptide from the radish seed (*Raphanus sativus*) antifungal protein 1 or 2 (RsAFP1 or RsAFP2), is one such bioactive plant defensin sequence (Fig. 1). Previously, RsAFP-19 was used to examine the effect of disulfide-mediated stability on the function of the intact protein, where it was established that the cysteine residues were essential for

Abbreviations: AFM, atomic force microscopy; CSαβ, cysteine-stabilized alpha beta; NaD1, *Nicotiana alata*; RsAFP, *Raphanus sativus* (radish seed) antifungal protein; ThT, thioflavin T; TEM, transmission electron microscopy; XRD, X-ray diffraction

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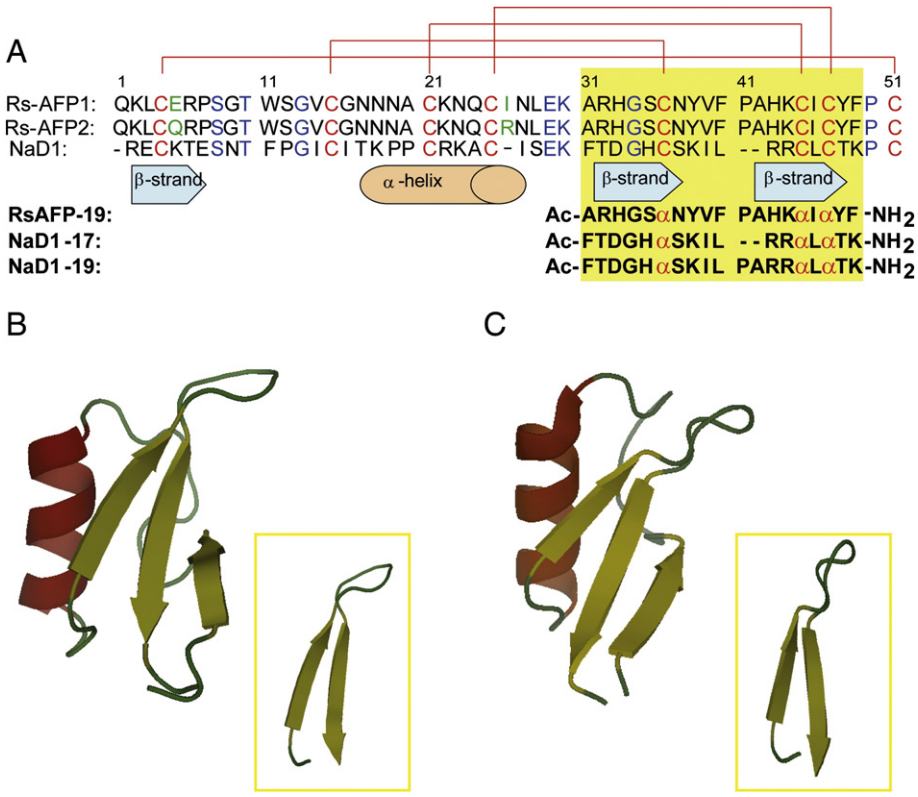

Fig. 1. Amino acid sequence and structure of three defensin proteins, RsAFP1, RsAFP2 and NAD1, and three peptides derived from them. A) Sequence similarity of RsAFP1, RsAFP2 and NaD1 (adapted from Lay et al. [9]); disulfide linkages are indicated in red, sequence differences in green and conserved residues in blue [12,60]. The regions from which the peptides RsAFP-19 and NaD1-17 are derived are highlighted in yellow. The peptide sequences for RsAFP-19, NaD1-17 and NaD1-19 are given in bold, where Ac is an acetyl group, NH_2 is an amino group and α is α-aminobutyric acid which replaces the three cysteine residues in these peptides [10]. B) Tertiary structure of RsAFP1 as determined by NMR spectroscopy, with inset (yellow edged box) of the RsAFP-19 region. C) Solution structure of NaD1, determined by NMR spectroscopy with inset (yellow edged box) of the NaD1-19 region. Images were drawn using PyMOL [61] from co-ordinates available from PubMed for the molecules RsAFP (PDB ID: 1AYJ_A) [13] and NaD1 (PDB ID: Q8GTM0) [8,9].

maintaining the secondary structure of RsAFP proteins [12]. RsAFP-19 was also investigated with regard to its potential as a commercial antifungal peptide [12]. The RsAFP-19 peptide forms the C-terminal double β-strand region of RsAFP proteins (Fig. 1), as determined by solution NMR spectroscopy [13]. It includes one of the primary regions associated with the antifungal activity of RsAFP1 and RsAFP2 [6], *i.e.* the nine-amino acid region between residues 38 and 46. NaD1-19 is another plant defensin peptide, derived from the ornamental tobacco plant *Nicotiana alata* [9] and is related in structure and biological activity to RsAFP-19 (Fig. 1). Notably, although they feature quite different modes of action, both peptides are derived from a conserved fold in their respective parent proteins that is known to form β-sheet secondary structure [8].

Amyloid fibril-forming peptides have the useful traits of a short amino acid sequence and readily understood folding and unfolding behavior, in contrast to large proteins that often display complex structures and folding behavior [14]. Thus, such peptides have been used to study aspects of amyloid fibril formation in detail, from the protofilament level to higher order assemblies [14–18]. Small amyloid fibril-forming peptides have contributed greatly to defining the structural details of amyloid fibrils, as they are more amenable to examination *via* solid-state NMR and X-ray diffraction (XRD) techniques at a higher resolution than larger peptides or proteins [2]. Amyloid fibril-forming peptides, including designed peptides, have been used in the production of nanomaterials with specific morphological or structural characteristics [3,15,18,19]. They have also been used to explore specific interactions which occur during the amyloid fibril formation process, including intermolecular [20,21], and solid-phase surface-molecule interactions [22]. In addition, many amyloid fibril-forming peptides display relatively rapid aggregation kinetics and can therefore be used for *in vitro* assays to identify potential inhibitors of amyloid fibril formation [14,23]. Finally, peptides have been used to explore the addition of biological function to amyloid fibrils, either through the incorporation of functional protein domains [24,25] or *via* the attachment of biologically active molecules to previously formed peptide-based amyloid fibrils [26].

Investigations into the structure and stability of these short peptides derived from defensin sequences revealed a tendency for gel formation by RsAFP-19 (Schirra H.J., unpublished results), which initiated a study of the amyloidogenicity of these peptides and speculation as to whether the fibril-forming propensity may be related to their biological role. A range of destabilization and fibril-forming conditions were examined for the two defensin peptides, RsAFP-19 and NaD1-19, and the resulting fibrillar structures were characterized. The findings provide insight into whether fibril formation is linked to their biological activity and the potential applications of these peptides as useful nanostructures.

2. Results

2.1. RsAFP-19 peptide

Over the past decade predictive algorithms have been developed to analyze the amyloid fibril-forming propensity of peptides and proteins. TANGO [27] is one such statistical mechanics algorithm which incorporates not only the structure of the peptide or protein but also such physico-chemical parameters as concentration, pH, ionic strength and the effect of adding a destabilizing agent (such as trifluoroethanol). The higher the score, the more likely a peptide or protein is to have a tendency to aggregate into β-sheet structures. TANGO also enables a view of which regions in a protein or peptide are most likely to be involved in such aggregation (Fig. 2). To examine their β-sheet aggregation propensity, RsAFP1 and RsAFP-19 were analyzed using TANGO. The RsAFP-19 peptide has three cysteine residues (Fig. 1). The synthetic