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Biophysical characterization of soluble *Pseudomonas syringae* ice nucleation protein InaZ fragments

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ABSTRACT

Ice nucleation protein (INP) with its functional domain consisting of multiple 48-residue repeat units effectively induces super-cooled water into ice. Circular dichroism and infrared deconvolution analyses on a soluble 240-residue fragment of *Pseudomonas syringae* InaZ (InaZ240) containing five 48-residue repeat units indicated that it is mostly composed of β-sheet and random coil. Analytical ultracentrifugation suggested that InaZ240 behaves as a monomer of an elongated ellipsoid. However, InaZ240 showed only minimum ice binding compared to anti-freeze proteins. Other *P. syringae* InaZ proteins with more 48-residue repeat units were made, in which the largest soluble fragment obtainable was an InaZ with twelve 48-residue repeat units. Size-exclusion chromatography analyses further suggested that the overall shape of the expressed InaZ fragments is pH-dependent, which becomes compact as the numbers of 48-residue repeat unit increase.

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1. Introduction

Water in a metastable state resists ice formation to a super-cooled state well below the 0 °C freezing point. The research field of bacterial ice nucleation was established when *Pseudomonas syringae* bacteria on the surface of agricultural plants were found to function as ice crystal nucleating centers [1]. This function in *P. syringae* was attributed to ice nucleation protein (INP) which is a membrane protein at the outer membrane [2–4]. INP induces ice formation by acting as ice crystal nuclei (seeds) for super-cooled water molecules to assemble, which triggers water freezing into ice. Because most plants lack tolerance in intracellular ice formation, bacteria with ice nucleation capability bring severe frost damage to the plants [5–7].

Among the many INPs, the protein product of *P. syringae inaZ* gene was the first to be characterized in detail [3,8,9]. The structure of InaZ is divided into an N-terminal domain (175 residues), a central domain (~1000 residues), and a C-terminal domain (49 residues). While the N- and C-terminal domains function to anchor the protein to the outer membrane of the bacteria, the central domain which contains the highly repetitive sequences is responsible for the functional ice nucleation activity of InaZ (Fig. 1). The central repetitive domain consists of twenty-five 48-residue repeat units, in which the repeat units themselves contain three blocks of 16-residue repeat (AGYGSTxTAxxxSxLx, x is a non-conserved residue) (Fig. 1). Theoretical models of this central repetitive domain suggested that it folds into planar arrays of anti-parallel β-sheets [10] or to a β-helix [11,12]. However, either X-ray crystal or NMR spectroscopy structures which can elaborate on the ice nucleation mechanism have not yet been provided. Since, both structural determination techniques require milligram amounts of protein, the lack of structure can be partly due to the limited amounts of InaZ proteins obtainable.

Other than the studies performed on chemically synthesized peptides of InaZ [13,14], an attempt to make highly soluble InaZ

Abbreviations: AUC, analytical ultracentrifugation; AFP, anti-freeze protein; CD, circular dichroism; FPLC, fast protein liquid chromatography; INP, ice nucleation protein; IR, infrared; LeIBP, Leucosporidium ice-binding protein; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; NMR, nuclear magnetic resonance; RMS, root-mean-square; RPM, rotation per minute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography.

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Fig. 1. The amino acid sequence of *Pseudomonas syringae* InaZ in the central repetitive domain. The sequence of InaZ in the repetitive central domain (InaZ residues 176–1151) is shown with indication of repeating 16-residue units in shades. The region of initially expressed InaZ240 is underlined, and the sole tryptophan residue (W220) located at the third 16-residue repeat unit is indicated in bold type. Other soluble fragments of InaZ used in this study all start from the residue 176, and their C-terminal ends are indicated with the construct names.

fragments using the *Escherichia coli* expression system has been previously reported on a 96-residue InaZ fragment of the central repetitive domain termed "INP96" [9]. INP96 was reported to be mostly β-strands (45%) and random coil (41%) with positive ice-binding ability [9]. Herein, much larger fragments of *P. syringae* InaZ in the central repetitive domain such as the 240-residue fragment (InaZ240) as well as others containing 432, 480, 528 and 576 residues were successfully expressed in large quantities using *E. coli*, in which the amount and the concentration of the proteins acquired are sufficient for future structural determination attempts. While InaZ240 was characterized using circular dichroism (CD), infrared (IR) spectroscopy, and analytical ultracentrifugation (AUC), the pH-dependent folding properties of the larger fragments were studied using size-exclusion chromatography (SEC).

2. Results and discussion

2.1. Expression and purification of P. syringae InaZ240

The previous studies on short InaZ fragments have shown that at least two-fold repetition of the 48-residue repeat unit (eg. INP96) is essential in order to establish the inherent structural feature of *P. syringae* InaZ [9,13,14]. In this study, a 240-residue fragment called InaZ240, which starts from Ala^{176} to Ile^{415} of the central repetitive domain was successfully expressed in *E. coli* and purified (Fig. 2A). Because the N-terminal domain of InaZ which accounts for ~15% of the full-length InaZ is composed of mainly hydrophobic residues, it was excluded in the fragment to minimized hardship during protein expression. Instead, the first 240 residues of the central repetitive domain, which contains five-fold repetition of the 48-residue repeat unit, were chosen for expression (Fig. 1). The amino acid sequence of the central repetitive domain in InaZ suggests that the domain is negatively charged at pH 7 due to the low occurrence of the basic residues (~3% Arg/Lys) in comparison to the acidic residues (~5% Glu/Asp). Based on the amino acid sequence, InaZ240 containing ~6% Glu/Asp and ~1% Arg/Lys leads to the estimated pI of 3.9 and the estimated charge of −14 at pH 7.

For initial purification of the expressed InaZ240, the N-terminal His_6-tag was utilized for Ni-NTA affinity chromatography. Subsequent purification was performed using SEC (Fig. 2A). The elution fractions under the peak were concentrated to ~20 mg/mL (~1 mM) with no precipitation, and the protein was observed as a single band on SDS-PAGE (Fig. 2A *inset 1*). Further mass spectrometry analysis of the chymotrypsin-treated protein suggested that the expressed protein is indeed *P. syringae* InaZ showing 98.8% coverage within the InaZ residues 176–415 (Table 1). The acidic nature of InaZ240 likely caused the apparent molecular weight shifted to ~35 kDa from the calculated molecular weight of ~25 kDa under the SDS-denaturing condition (Fig. 2A *inset 1*). It is interesting to note that having five 48-residue repeat units in InaZ240 had ten-fold enhanced solubility compared to INP96 which dissolved in water with at most ~100 μM solubility [9].

The intrinsic fluorescence emissions of tryptophan (maximum at 330–350 nm) and tyrosine (maximum at 300–310 nm) have been widely used to infer the protein unfolding dynamics in solu-

Table 1
List of peptide fragments found from mass spectrometry analysis of the expressed InaZ240.

Peptide Sequence[a]	MH^+ (Da)
IAGYGSNETAGNHSDLIAGY	2009.92
GSTQTAREGSNLTAGY	1612.75
GSNETAGNHSDLIAGY	1605.71
GSTQTAQEGSNLTAGY	1584.71
TAGYGSTGTAGSDSSLIAGY	1835.83
IAGYGSNETAGNHSDL	1605.71
TAGYGSTGTAGSDSSLI	1544.71
TAGYGSTGTAGVDSSLIAGY	1847.86
GSTLSGDNHSRLIAGY	1647.81
GSTQTAQEGSNL	1192.54
TAGYGSTGTAGSDSSL	1431.62
GSTQTSGSDSALTAGY	1502.66
GSTQTAREGSNL	1220.58
GSTQTAGGDSALTAGY	1456.65
TAGYGSTQTAREGSNL	1612.75
GSTQTSGGDSSLTAGY	1488.64
GSTGTAGSDSSLIAGY	1443.66
GSTGTAGVDSSLIAGY	1455.70
GSTGTAGSDSWLVAGY	1528.69
GSTQTSGSDSAL	1110.49
TAGYGSTQTAQEGSNL	1584.71
GSTQTAQEGSNLTAGYGSTGTAGVDSSL	2617.19
GSTQTAGGDSAL	1064.48
GSTQTSGGDSSL	1096.47
TAGYGSTQTAQEGSNLTAGY	1976.87

[a] Peptide hits are listed based on the sorted cross correlation score (XCorr) starting from 6.0 to 2.2 [XCorr defined by Proteome Discoverer 1.3 (Thermo Scientific, USA)].

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