Large-scale Production and Purification of an *Erwinia ananas* Ice Nucleation Protein and Evaluation of Its Ice Nucleation Activity

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The ice nucleation-active protein of *Erwinia ananas* IN-10 (inaA protein) was over-expressed as inclusion bodies in *Escherichia coli* in a yield of 15.3 mg of inaA protein from 60 mg of bacterial cells on a dry-matter basis. The inaA protein was purified from the inclusion bodies by solubilization with detergents to obtain a protein preparation free from sugar and lipid. This preparation had a distinct ice nucleation activity, indicating that the inaA protein per se is able to act as a nucleus.

Some Gram-negative bacteria act as ice nuclei to promote the freezing of water. These include *Pseudomonas fluorescens*,1 P. syringae,2 Erwinia herbicola,3 and *Xanthomonas campestris*,4 which are called ice nucleation-active bacteria. Genes responsible for the ice nucleation activity have been cloned from *P. syringae*, *P. fluorescens*, and *Erwinia herbicola*, and are named inaZ,5 inaW,6 and iceH,7 respectively. We have cloned and characterized an ice nucleation-active gene from *Erwinia ananas* IN-10 (inaA), a species that causes frost injury to tea plants and other crops in Japan.8,9

Ice nucleation bacteria have been used for some practical applications, where it would be preferable from environmental and sanitary points of view to use the ice nucleation proteins themselves rather than the bacterial cells. However, it has been reported that the product of the bacterial ice nucleation activity is isolated from the outer membrane10 and also that ina protein is associated with outer membrane phospholipids.11 On the other hand, the known ina gene structures suggest that the ina proteins do not contain signal sequences nor any transmembrane domains that might be related to their possible localization into the outer membrane.

Thus, both biochemical analysis and large-scale production of ina proteins are needed for further studies on the structure–activity relationship of ice nucleation and for practical applications. Here we reported the large-scale production and purification of inaA protein, and demonstrate that the inaA protein per se has ice nucleation activity, even in the absence of membrane components such as sugar and lipid.

Materials and Methods

**Bacterial strain.** An ice nucleation-active strain of *Erwinia ananas*1 isolated from a tea germisphere grown in Shizuoka Agricultural Experiment Station, Japan, was cultured with a *Pseudomonas* F medium12 at 22°C for 2 days. *E. coli* YA21 was used as an expression bacterium.

**Expression of inaA protein in E. coli.** The expression of inaA protein in *Escherichia coli* was performed as described by Abe et al.13 E. coli strain YA21 harboring pINAS153 was grown to the stationary phase in 2-broth with 50 µg/ml ampicillin at 37°C for 18 h. The cells were collected and suspended in medium M9 with 0.2% casamino acids, 0.2% glycerol, 50 mg/ml ampicillin, and 1 mM isopropyl-β-D-galactopyranoside, and incubated at 37°C for 1.5 h.

**Identification of inaA protein expressed in E. coli.** Cell extracts were prepared from the sonicates of transformed *E. coli* in 10 mM Tris HCl–1 mM EDTA (TE buffer, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The extracts were electrophoresed on sodium dodecyl sulfate–polyacrylamide gel by the method of Laemmli14 and the protein was stained with Coomassie Brilliant Blue R-250.

Western blotting was done as described by Deininger et al.,14 except that transverse electrophoresis was done in the blotting apparatus (Atto) at 70 V for 1 h and immunological staining of the labelled band was done with an avidin-biotin alkaline phosphatase-conjugate anti-rabbit IgG. Labeled bands were stained by a color-producing reaction of alkaline phosphatase with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as the substrate. The periodic acid schiff (PAS) reaction15 was used for detection of sugar.

**Antibody preparation.** To detect the expected expression product, an anti-inaA protein antibody was prepared. The inaA protein overexpressed in *E. coli* was used as the antigen. It was prepared from a gel slice from the corresponding band on 7.5% SDS polyacrylamide gel electrophoresis (SDS–PAGE).16 Polyclonal antisera were produced by injection of a mixture of Freund's complete adjuvant in fetal calf serum (FCS) and 100 µg of the inaA protein sample into New Zealand white rabbits followed by a booster injection two weeks after.

**Electron microscopy.** For electron microscopy, the *E. coli* cells overexpressing inaA protein were fixed with 3% glutaraldehyde, postfixed with 1% osmium tetroxide, and then block-stained with 0.5% aqueous uranyl acetate. Thin sections were stained with 3% uranyl acetate and lead citrate and then examined with a JEOL 200 CX electron microscope at 100 kV.

**Purification of inaA protein.** The cells expressing inaA protein were broken by 5 x 30 s bursts of ultrasonication. The suspension was centrifuged at 13000 g for 20 min. The precipitate was resuspended in TE buffer containing 2% Triton X-100 and 1 M NaCl and placed on ice for 2 h. The suspension was then centrifuged at 13000 g for 20 min and the precipitate containing inaA protein was collected by centrifugation. This procedure was repeated four times. The protein sample thus obtained was detected by SDS–PAGE and Western blotting.

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Enzyme assays. Succinate dehydrogenase activity was assayed as described by Koyasu et al.\(^{10}\) The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.5), 990 μg of p-iodophenyl-tetrazolium violet, 25 μg of phenazine methosulfate, 770 μg of gelatin, and 50 μl of the supernatant or the precipitate by 1300×g centrifugation after the sonication of inaA protein expressing E. coli in 0.9 ml. After incubation at 30°C for 10 min. The mixture was rapidly cooled at 0°C and the absorbance at 520 nm was measured. One enzyme unit is defined as an increase in \(A_{548}\) of 0.01 min\(^{-1}\).

2-Keto-3-deoxyoctanoate (KDO) was measured by the thiorbituric acid assay of Osborn.\(^{17}\) One hundred and fifty μl of each sample was suspended in 0.08 N \(H_2SO_4\) and hydrolyzed at 100°C for 20 min. The samples were incubated with 0.25 ml of 0.125 N \(H_2SO_4\) containing 0.4 N \(HIO_4\) at room temperature for 20 min and then with 0.5 ml of 0.5 N HCl containing 0.2 M NaAsO\(_2\) at room temperature for 3 min.

After they were hydrolyzed with 2 ml of 0.3% thiobarbituric acid (pH 2) at 100°C for 20 min, cooled on ice and extracted into 1.5 ml of cyclohexanone. The KDO was measured by the absorbance at 548 nm.

Amino acid composition and sequence analysis. The amino acid composition was analyzed with an Applied Biosystem model 120A amino acid analyzer. The N-terminal sequence was analyzed by Edman degradation using an Applied Biosystem model 470A protein sequencer.

Ice nucleation assays. Bacterial cultures were prepared from single colonies in 2-ml culture tubes of \(\chi\)-broth and Pseudomonas F medium, growing for 48 h with aeration at 22°C. The bacterial suspensions (1.0×10\(^8\) cells/ml) and inaA protein (50 μg/ml) were diluted to 10\(^{-2}\) in increments of 10\(^{-2}\) in sterile distilled water. Thirty replicate 10-μl drops of each dilution were placed on a refrigerated cold plate (thermoelectric plate Mituwa model K-1). Droplet freezing was tailored through stepwise reductions of temperature, and ice nucleation frequencies at each step were calculated as described by Vai.\(^{18}\) Each graph point presented below is a mean of results from two or three replicates of such as assay.

Results and Discussion
Localization of inaA protein expressed in E. coli
The result of electron microscopy showed that the inaA protein was in inclusion bodies in the cytoplasm (Fig. 1) and was not secreted into the medium nor into the periplasm, though a recent observation by immunofluorescent microscopy has shown that both inaZ and inaW proteins are present in clusters on the surface of E. coli cells expressing them.\(^{19}\) To find whether our inaA protein was membrane-associated, the E. coli cells expressing inaA protein were sonicated and the supernatant and precipitate were separated by centrifugation at 1300×g. SDS-PAGE showed that the inaA protein was absent in the supernatant, but present in the precipitate (Fig. 2, lanes 1 and 2). Western blot analysis of both the precipitate from the E. coli overexpressing inaA protein and the total protein from Erwinia ananas IN-10 cells gave a single immunoreactive band of 130 kDa (Fig. 2, lanes 3 and 4). On the other hand, succinate dehydrogenase and 2-keto-3-deoxyoctanoate which are intrinsic to the inner and outer membranes, respectively, were both detected mostly in the supernatant fractions (Table 1). These results indicate that most membrane components are not associated with inaA protein.

Purification of inaA protein
Since the 130-kDa protein was mainly in the insoluble fraction after disruption of the cells, it was purified from Table 1. Localization of Succinate Dehydrogenase and 2-Keto-3-deoxyoctanoate in the Supernatant and Precipitate Fractions Obtained by Centrifugation of Sonicated E. coli Cells Expressing the inaA Protein

<table>
<thead>
<tr>
<th>Maker enzymes</th>
<th>Precipitate</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.008* (6.7%)</td>
<td>0.111* (93.3%)</td>
</tr>
<tr>
<td>2-Keto-3-deoxyoctanoate</td>
<td>0.1* (8%)</td>
<td>1.0* (92%)</td>
</tr>
</tbody>
</table>

* Values represent the amounts of succinate dehydrogenase in units of activity.

* Values represent the amounts (μg) of 2-keto-3-deoxyoctanoate.

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Fig. 1. Electron Microscopic Appearance of the Inclusion bodies Containing inaA Proteins Expressed in E. coli.
Expression and Purification of an Ice Nucleation Protein

Fig. 2. Detection of inaA Protein in Erwinia ananas IN-10 and Escherichia coli Carrying the inaA Gene.

The precipitate (lanes 1 and 3) and supernatant (lane 2) fractions obtained by centrifugation at 13000 x g of sonicated inaA-protein expressing E. coli cells and the total protein of Erwinia ananas IN-10 (lane 4) were electrophoresis to SDS-PAGE and the gels were stained with Coomassie brilliant blue (lanes 1 and 2) or analyzed by Western blotting analysis (lanes 3 and 4).

Fig. 3. Purification of inaA Protein Expressed in E. coli.

SDS-PAGE patterns at each purification step are shown in lanes 1 to 4, and immunological identification of the purified inaA protein is shown in lane 5. Lane 1, the precipitate obtained from sonicated E. coli cells expressing inaA protein treated with 2% Triton X-100 and 2% Nonidet P-40. The precipitate obtained after the detergent treatment was repeated twice is shown in lane 2; repeated three times in lane 3; repeated four times in lane 4. Purified inaA protein (lane 4) was analyzed by Western blot with anti-inaA protein antibody (lane 5).

The precipitate of the bacterial cell lysate as described in Materials and Methods. Protein samples at each purification step were analyzed by SDS-PAGE (Fig. 3, lanes 1 to 4).

Table II. Amino Acid Composition of inaA Protein

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>Nucleotide sequence</th>
<th>Experimental value</th>
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<tbody>
<tr>
<td>Asx</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>Thr</td>
<td>194</td>
<td>179</td>
</tr>
<tr>
<td>Ser</td>
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<td>Glx</td>
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<td>Gly</td>
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<td>236</td>
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<td>Ala</td>
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<td>173</td>
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<tr>
<td>1/2Cys</td>
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<td>N.D.</td>
</tr>
<tr>
<td>Val</td>
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<td>14</td>
</tr>
<tr>
<td>Ile</td>
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<tr>
<td>Trp</td>
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<td>N.D.</td>
</tr>
<tr>
<td>Total</td>
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<td>(1310)</td>
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</table>

N.D., not determined.

Fig. 4. Ice Nucleation Spectra of Erwinia ananas IN-10 (△), E. coli Expressing inaA Protein (●) and Purified inaA Protein (○).

Erwinia ananas and E. coli carrying the inaA gene were grown in Pseudomonas F medium and shake-broth, respectively, at 20℃ for 48 h inaA protein was purified as described in the text.

The resultant precipitate showed a single stained band at 130 kDa, and also immunologically (Fig. 3, lanes 4 and 5). An inaA protein preparation (15.3 mg) was obtained from E. coli (60 mg) on a dry-matter basis. The purified inaA protein fraction should contain no lipid since it has been treated many times with detergents. The absence of sugar was confirmed by the observation that the PAS reaction was negative.

Protein characterization

The N-terminal amino acid sequence of the inaA protein produced by E. coli YA21 carrying pINA6S13 was analyzed by a protein sequencer. The first 9 residues were found to be Met-Lys-Glu-Asp-Lys-Val-Leu-Ile-Leu. This sequence agreed exactly with that predicted from the inaA gene. An amino acid composition analysis of the purified inaA protein was also done, and the results corresponded approximately with that predicted from the sequence of the inaA gene.
(Table II); the small deviation from the predicted value may result from the fact that the inaA protein has a very large molecular mass, 130 kDa. As expected from the repetition sequence of the inaA protein, the purified protein was rich in serine, threonine, alanine, and glycine.

Ice nucleation activity of purified inaA protein

Freezing spectra of Erwinia ananas IN-10, E. coli expressing inaA protein, and inaA protein obtained by the droplet freezing method of Vali\textsuperscript{18} are shown in Fig. 4. The nucleation frequency is defined as the fraction of cell or protein activity (i.e., the number of ice nuclei per cell or protein) at a given temperature. The ice nucleation activity of the purified protein was clearly observed. The purified inaA protein lacks ice nucleation activity at warmer temperatures, compared with Erwinia ananas IN-10 and E. coli expressing inaA protein, but the cumulative number of detectable ice nuclei is not affected so drastically. Though it has recently been reported that the elicitation of some bacterial ice nucleation protein activity involves the binding of lipids, probably in a non-covalent fashion,\textsuperscript{20} our inaA protein per se has a potent ice nucleation activity, although it is a weaker ice nucleus than any Erwinia ananas IN-10 or E. coli expressing inaA protein.

In conclusion, this study establishes a simplified and enlarged system for the production of a pure inaA protein preparation. The protein could be used for the mild freeze-concentration of foodstuffs and other materials sensitive to deep-freezing.

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References