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An ice nucleation protein from *Fusarium acuminatum*: cloning, expression, biochemical characterization and computational modeling

Milad Lagzian · Ali Mohammad Latifi ·
Mohammad Reza Bassami · Morteza Mirzaei

Received: 6 March 2014 / Accepted: 23 May 2014
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Abstract Ice nucleation proteins (INP) are a major cause of frost damage in plants and crops. Here, an INP gene from *Fusarium acuminatum* was optimized, synthesized, expressed in *E.coli* and subsequently purified and characterized. The protein belongs to the second class of ice nucleation proteins with an optimum pH 5.5, relative activity and stability between pH 5 and 9.5 and up to 45 °C. The protein was fully active and stable in the presence of dimethyl sulfoxide (DMSO), dioxane, acetone and ethyl acetate. Moreover, it retained over 50 % of its original activity in the presence of polyvinyl alcohol. The 3D structure model of the INP-F indicated the protein had three distinct domains as exist in other ice nucleation proteins with some variations. Considering

these promising results, INP-F could be a novel candidate for industrial applications.

Keywords Cloning and expression · *Fusarium acuminatum* · Ice nucleation protein · Protein 3D structure

Introduction

Unlike the popular belief, pure water does not freeze at 0 °C. It can remain in the liquid state down to -41 °C (Wolber 1993). However, different impurities can increase the freezing point up to zero, by a process called ice nucleation. These impurities are categorized in several groups (Margaritis and Bassi 1991), including minerals, dust, pollen, crystalline particles and biological ice nucleators. The last group is composed of, but not limited to, lichen, fungal and bacterial ice nucleators. Members within this group are the most powerful ice nucleators can nucleate water close to 0 °C. Ice nucleation activity of these organisms is due to a proteinaceous component, the Ice Nucleation Protein (INP) (Kieft and Ruscetti 1990).

INPs are the primary cause of frost injury to many economically important plants in cold seasons. Although, it is a disaster to agriculture, there are attractive applications of INPs that might compensate their damage (Margaritis and Bassi 1991; Li and Lee 1995). Some of these are as follows

M. Lagzian · M. R. Bassami
Biotechnology Division, Faculty of Veterinary Medicine,
Ferdowsi University of Mashhad, Mashhad, Iran
e-mail: milad.lagzian@gmail.com

M. R. Bassami
e-mail: bassami@um.ac.ir

M. Lagzian · M. R. Bassami
Institute of Biotechnology, Ferdowsi University of
Mashhad, Mashhad, Iran

A. M. Latifi (✉) · M. Mirzaei
Applied Biotechnology Research Center, Baqiyatallah
University of Medical Sciences, Tehran, Iran
e-mail: amlatify@yahoo.com

M. Mirzaei
e-mail: mraga85@gmail.com

- Artificial snow making is the major commercial application of ice nucleation proteins. They are added to the supercooled water that is used for producing snow. This results in significant saving of electrical energy because it elevates the freezing point temperature of the water droplets.
- Cloud seeding is one of the most interesting applications of INP. When they are dispersed in the clouds, they can act as powerful ice nuclei, which can raise the chance of precipitation in warmer temperatures.
- Ice nucleation activity is suitable as the basis for a new reporter gene system whereby a promoter-less ice nucleation gene is fused with a gene of interest to monitor its transcriptional activity.
- Freezing is frequently used in the manufacturing and processing of food materials. Therefore, INPs can be employed as an efficient source of cold generation with the advantages of saving time and cost. Regarding to these applications, there is a growing demand for production of these proteins from new sources such as fungi and bacteria.

There are a number of publications on identification, production, purification and characterization of INPs (Maki et al. 1974; Lindemann et al. 1982; Lindow et al. 1982; Gross et al. 1983; Orser et al. 1985; Corotto et al. 1986; Wolber et al. 1986; Abe et al. 1989; Cirvilleri et al. 1990; Kieft and Ruscetti 1990; Li and Lee 1995; Hwang et al. 2001; Kumble et al. 2008). Most researches have focused on bacterial INPs (bINP), especially those from *Pseudomonas* and *Xanthomonas*. In spite of a strong ice nucleation activity, bINPs have some deficiencies that must be addressed. For example, they are adversely affected above 20 °C or in acidic and basic environments, or by harsh conditions during the purification process, organic solvents and ice nucleation inhibitors. In addition, the sizes of encoded genes for bINPs are relatively large (Orser et al. 1985) which may cause some problems for their efficient cloning, engineering and expression.

An alternative to bINP are fungal INPs, especially those purified from *Fusarium* genus. They have a broader range of pH and thermal stability, better chemical solvent tolerance and more appropriate gene size. However, publications and technical information about these INPs and their cloning and expression are limited (Pouleur et al. 1992; Hasegawa et al. 1994).

In the present work, we report gene optimization, cloning and expression of an ice nucleation protein from the epiphytic fungus, *Fusarium acuminatum*, for the first time in a bacterial expression system. We also describe purification, characterization and 3D structure modeling of this recombinant protein.

Materials and methods

Materials

All molecular biology enzymes, used in our experiments, were purchased from Thermo Fisher Scientific. PerfectPro Ni-NTA magnetic agarose beads for protein purification were obtained from 5PRIME company (5PRIME, Germany). All other chemicals and reagents were analytical grade and acquired from various international vendors.

Candidate protein selection and optimization

The UniProtKB database was searched to find all relevant full-length ice nucleation proteins, and the retrieved records were refined based on their source organism. Subsequently, all fungal sequences (10 sequences) were selected that belonged to the Leotiomyceta super-class (filamentous ascomycete fungi). Thereafter, all records, which their function was derived by computational predictions (nine sequences), were removed from the collection. The last remaining item, accession number Q71SX2, was selected as the candidate for fungal INP cloning. This record was identified during a wide screening of *Fusarium* for the presence of ice nucleating activity by Dr E Anastassopoulos (Technological Research Centre of Thessaly, Greece). This protein belongs to a ubiquitous phytopathogen, *Fusarium acuminatum* (recently re-named as *Gibberella acuminata*). It has 445 amino acids with a molecular weight ~ 50 kDa. The protein is named INP-F. The corresponding gene of INP-F was then optimized (Bai et al. 2011). Briefly, the protein was reverse-translated to its cognate DNA and all its codons were changed according to the *E. coli* codon usage table. Following transcriptional and translational optimization, the INP-F gene was cloned into the expression vector, pET28a + (Novagen, USA). The gene was also synthesized by Shyngen Co. Ltd (Shyngen Co., South Korea).

Sub-cloning in pET28

pUC57-INP construct was digested by *Bam*HI/*Hind*III restriction enzymes. The digested products were electrophoresed on 0.8 % (w/v) agarose gel, and the corresponding band around 1450 bp was excised and extracted from the gel by AccuPrep Gel Purification Kit (Bioneer, South Korea). The purified fragment was directly ligated to pET28a. The ligated product was transformed into *E.coli* TOP10 by heat shock. Success of the sub-cloning procedure was validated by restriction enzyme mapping and PCR assay. Finally, pET28a-INP vector was transformed into *E.coli* BL21 (DE3) pLys by the previous method.

Protein expression

Initially, 3 ml LB broth medium with appropriate antibiotic was inoculated with a single colony of the transformant *E.coli* BL21. This culture was grown overnight at 37 °C with shaking at 150 rpm and 0.5 ml was used for inoculating 50 ml expression medium. The inoculated expression medium was grown at 37 °C with shaking at 180 rpm until the OD₆₀₀ reached 0.4. The protein expression was then induced by adding 1 mM IPTG while the temperature and shaking were reduced to 20 °C and 100 rpm, respectively. Then the expression allowed to continue for 14 h. Finally, the medium was immediately cooled and kept on ice for further use.

Protein purification

Induced cells were centrifuged at 4,000×*g* for 5 min. The pellet was re-suspended in 10 ml lysis buffer containing 1 mg lysozyme/ml, 50 mM NaH₂PO₄, 0.3 M NaCl, 10 mM imidazole and 1 mM PMSF. The suspended cells were disrupted by two rounds of sonication. The lysate was then centrifuged at 10,000×*g* for 15 min to obtain a clear crude protein extract. The extract was subjected to affinity purification by PerfectPro Ni-NTA magnetic agarose beads (5PRIME, Germany) according to the manufacturer's guidelines. Concentration of the protein was determined during the purification by Bradford's method. Finally, homogeneity of the purified protein was analyzed on the 12 % SDS-polyacrylamide gel.

INP activity assay

Ice nucleation activity of expressed INP-F was assessed by droplet freezing method (DFM) (Vali 1971) with some modifications. Briefly, a flat surface was prepared by spraying aluminum foil with 1 % (w/v) paraffin in xylene and it was floated on methanol/water maintained in a refrigerated bath. A solution was then prepared by mixing 50 μl purified protein (1 μg/μl) with same volume of ultrapure water. The solution was divided into 10 μl droplets over the foil and the temperature was gradually reduced to −9 °C. The temperature at which the droplets froze was selected as nucleation point and further assays were done at this temperature. One unit of INP activity was defined as amount of the protein that can nucleate 10 μl of −5 °C supercooled water in 1 min.

Protein characterization

Effect of pH, temperature, organic solvents and some inhibitors was investigated on the activity and stability of INP-F. Finally, 3D structure of the protein was predicted. The detailed information is given in the legend of the corresponding figures.

Results and discussion

Overexpression and purification of the recombinant INP

The INP-F was purified with fourfold enrichment in specific activity and 50 % recovery in one-step affinity purification. Summary of the purification steps are demonstrated in Table 1. The purified protein was also appeared as a single band around 49 kDa on SDS-PAGE, indicating the homogeneity of the protein (Fig. 1).

pH Profile and stability

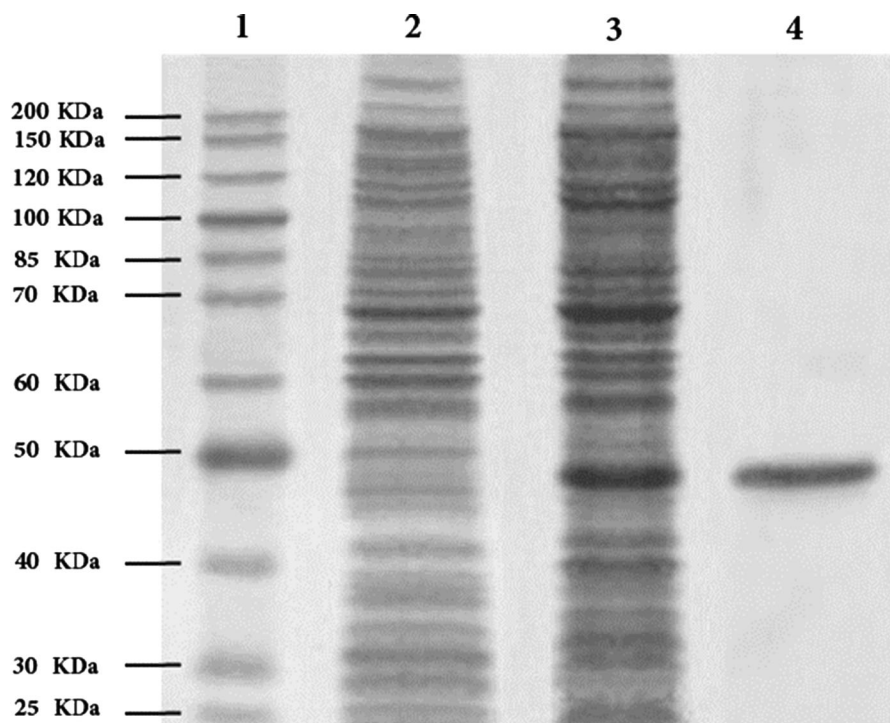
INP-F activity was little affected by the most pH examined. The protein was able to nucleate supercooled water at pH between 4.5 and 9 with an optimum at 5.5. This range, however, was narrower than that reported for native INP from *Fusarium avenaceum* which was completely stable from pH 2 to 12 (Hasegawa et al. 1994) although in shorter time

Table 1 Summary of the INP purification

Purification step	Protein concentration (mg)	INP activity (unit)	Specific protein activity (unit/mg)	% Recovery	Enrichment fold
Crude extract	85	135	1.58	100	1
Ni-NTA beads	16.5	67	4	50	2.53

All purification steps were done at 4 °C. The cells were participated and lysed by centrifugation and lysozyme–sonication treatment, respectively. Subsequently INP-F was purified by metal affinity precipitation with Ni-NTA magnetic agarose beads. The activity was measured at –5 °C as nucleation point. One unit of INP activity was expressed as amount of protein that can nucleate 10 µl of supercooled water in one min. The specific protein activity is result of dividing total unit of the protein per its concentration in mg

Fig. 1 SDS-PAGE analysis of INP-F during production and purification steps. *Lane 1* Mass ruler protein ladder, *Lane 2* the uninduced bacterial cell lysate, *Lane 3* the induced bacterial cell lysate, *Lane 4* the purified protein. Amount of the loaded proteins, were 200 µg for lane 2 and 3, and 50 µg for lane 4



(10 min) in comparison with this study (30 min). Beyond the aforementioned range, the ice nucleation activity was declined dramatically as compared to the optimum pH (Fig. 2a). In addition, INP-F was quite stable between pH 5 and 7.5. However, it lost its stability in pH lower and higher than 4.5 and 9.5 respectively (Fig. 2b).

Thermal stability of the protein

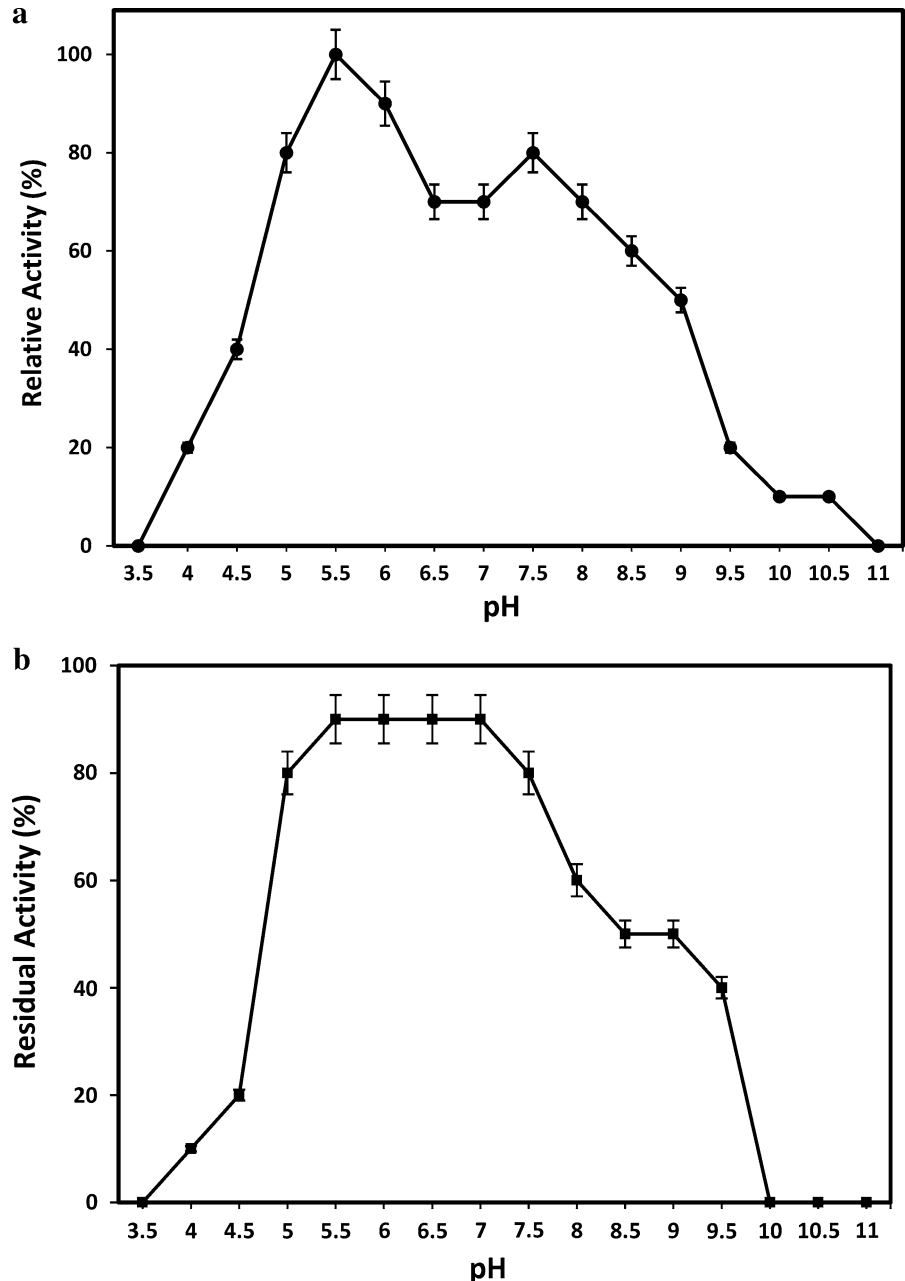
Temperatures over than 15 °C are devastating for most biological ice nucleators. Interestingly, INP-F was highly stable at these temperatures. The protein retained about 80 % of its initial activity after 60 min at 0 to 40 °C. However, above 40 °C, the protein

quickly lost its stability as there was only 10 % of its original activity at 50 °C, and no activity was measurable at 60 °C (Fig. 3). This result was in agreement with the previous studies, which had shown a higher thermal stability for fungal, and lichen INPs comparing to bacterial counterparts (Hasegawa et al. 1994).

Effect of the organic solvents and the ice nucleation inhibitors on INP-F activity and stability

INP-F was fully active in presence of solvents at 1 % (v/v). However, at 5 % (v/v), DMSO and dioxane decreased the INP activity for about 30 and 50 %, respectively.

Fig. 2 Effect of pH on the protein activity (a) and stability (b). To determine optimum pH for the INP activity, the protein was dissolved at final concentration of 0.5 $\mu\text{g}/\mu\text{l}$ in different buffers, which covered a broad range of pH from 3.5 to 11. The following buffers were used: sodium acetate buffer pH 3.5–5.5, sodium phosphate buffer pH 6.0–7.5, Tris/HCl buffer pH 8.0–9.5, $\text{Na}_2\text{HPO}_4/\text{NaOH}$ buffer pH 10–11. The relative INP activity was determined as described above and the maximum activity point was considered as optimum pH. To determine pH stability, the protein was incubated at each pH for one hour at 4 °C, and then the residual activity was determined. Activity at pH 5.5 and at the beginning of the experiment was considered as 100 % for protein activity and stability respectively. The result suggested that the protein must be used in the mild acidic and basic pHs, although the main activity and stability is around the neutral pHs. Each data point represents a mean of three independent experiments. The error bar was less than 5 % of the mean



respectively. On the other hand, acetone and ethyl acetate had a negligible effect on the nucleation activity even at 5 % (v/v) (Fig. 4). In addition, the protein was also fully stable after 1 h at 1 % (v/v) solvents. At the same time, at 5 % (v/v), DMSO and dioxane completely abolished the nucleation activity. Conversely, acetone had no measurable effect on INP-F stability and ethyl acetate decreased it by only 30 %.

These variations may be attributed to density of the solvents, because the activity and stability of INP-F increased alongside of reduction of the solvent density, although it has yet to be proven. In addition, although the protein can retain more than 50 % of its initial activity in the presence of 1 % PVA, the activity was strongly inhibited in the presence of 1 % PGL (Fig. 4).

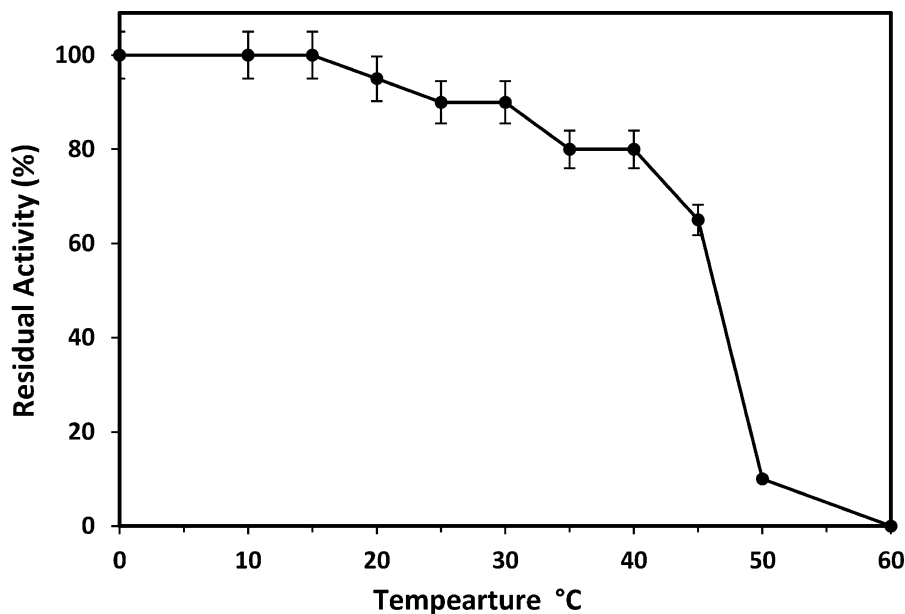


Fig. 3 The effect of temperature on the INP stability. Influence of temperature on the stability of the purified INP was investigated by incubating the protein at various temperatures ranged from 0 °C to 50 °C at optimum pH, for 1 hour and cooled immediately to 4 °C on melting ice. Thereafter, the

residual ice nucleation activity was measured as previously described. The protein was relatively stable at temperatures up to 40 °C. The activity at 0 °C was considered as 100 %. Each data point represents a mean of three independent experiments. The *error bar* was less than 5 % of the mean

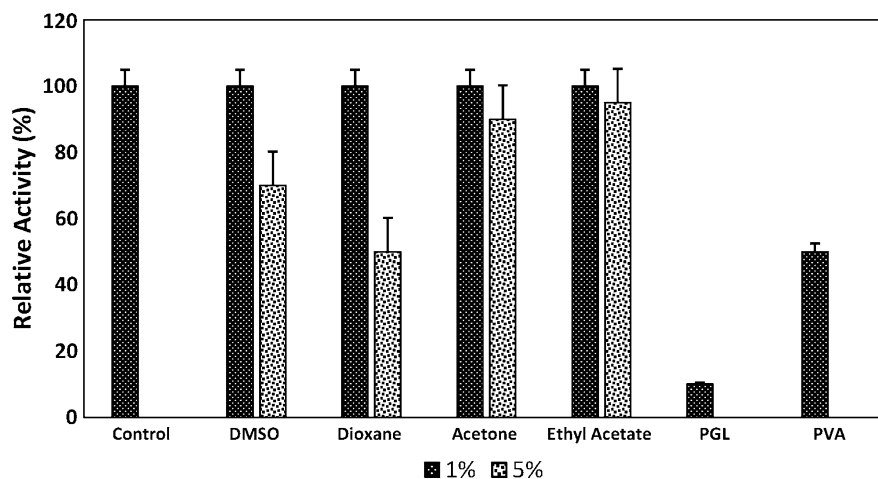


Fig. 4 The effect of organic solvents and ice nucleation inhibitors on the ice formation ability of the purified INP. Impact of four solvents, DMSO (dimethyl sulfoxide), acetone, dioxane and ethyl acetate on the ice nucleation activity of the protein were investigated at 1 and 5 % (v/v) concentration. The protein was initially diluted tenfold at each of these solvents. Subsequently, the relative activity was determined according to DFM. The protein stability in presence of these solvents was determined by extending the incubation time to one hour at optimum pH before performing the drop-freezing assay. The inhibitory effect of well-known ice-nucleation

inhibitors, PGL (poly glycerol polymer) and PVA (polyvinyl alcohol), on the ice formation ability of the protein, were also studied (Wowk and Fahy 2002). Both inhibitors were included in the ice formation mixture at final concentration of 1 % v/v. Subsequently ice nucleation activity in the presence and absence of these compounds was determined. Activity in the absence of any solvent, at the beginning of experiment and in the absence of any additive was considered as 100 % for these studies, respectively. Each *bar* represents a mean of three independent experiments. The *error bar* was less than 5 % of the mean

Three-dimensional structure of the recombinant INP-F

The modeled structure of INP-F is mainly composed of β -strands, which is consistent with other studies. Three common domains of other INPs were found in this model. The model has an *N* and *C*-terminal domains that are unique for each ice nucleation protein (Fig. 5). The *N*-terminus domain consists of an alpha-helix with 14 residues, which may have a role in secretion pathway and membrane anchoring (Khodi et al. 2012). In addition, as demonstrated in other ice nucleation proteins, the carboxyl terminus contains many hydrophilic and charged residues that may play a role in the protein folding (Wolber 1993; Khodi et al. 2012). Indeed, the most important segment of ice nucleation proteins is the core domain or repetitive domain, which is composed of β -strands. This domain

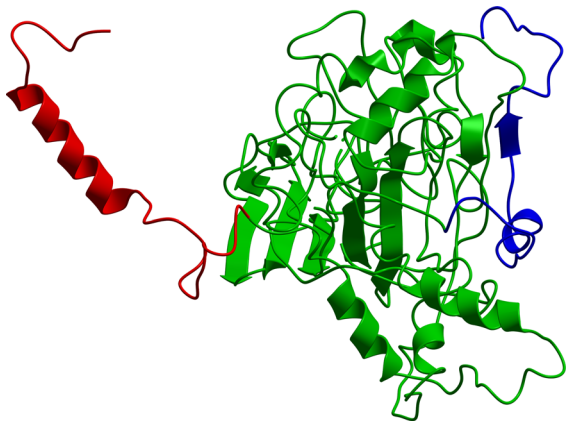


Fig. 5 Computational model of INP-F produced by Accelrys Discovery Studio 2.5.5. Initially, the signal sequence of the protein was trimmed. The remaining sequence, which had 459 amino acids in length, was searched against PDB databases by PSI-BLAST. Top two hits, 2UWA and 1UMZ were selected as partial 3D templates for building homology model. Other segments of the protein, which were not derived from any templates, was de novo modeled by “Ab Initio” algorithm of the software. All parameters were set to highest level for protein optimization and loop refinement. Five models were produced and ranked based on their DOPE scores (Discrete Optimized Protein Energy) and the reliability score that was calculated by Biologic module of Schrodinger suite 2013-1. Finally, the best-fitted model was used as the structural model of INP-F. The result shows there are three distinct domains, which are color-coded in the protein. These domains exist in other ice nucleation proteins with some variation. The red segment demonstrates the *N*-terminal domain. Green is the core domain and blue indicate the *C*-terminal end

has a key role in the ice formation. In almost all bINPs, this domain consists of four subdomains of multiple copies of an 8-amino-acid repeat. This was clearly demonstrated that the number of 8-amino-acid repeats is directly linked to the ability of ice formation in temperatures close to 0 °C. However, architecture of this domain is unknown for other INPs such as fungal type. A deep inspection of the INP-F core domain reveals there is no repetitive scheme. Nevertheless, it is mainly composed of β -stands that is in accordance with the other investigations (Wolber 1993).

Conclusion

INPs are proteinaceous substances that act as an efficient nucleus for ice formation in temperatures close to 0 °C. They exist in many organisms including bacteria, lichen and fungi with different characteristics. Here, we cloned, expressed and characterized an ice nucleation protein from *Fusarium acuminatum*. The results showed the ice nucleation activity of this protein was relatively lower than those reported for *Pseudomonas* species such as *P. syringae* elsewhere (Warren and Corotto 1989; Obata et al. 1993; Cochet and Widehem 2000). However, the protein remains active and also stable in a broader range of pHs compared to the aforementioned bacterial counterparts. The activity in presence of the ice nucleation inhibitors is comparable to that reported for *P. syringae*. In the other site, the nucleation was occurred at -5 °C compared to -2 °C for *P. syringae*. Therefore, this protein could be categorized as second class of INPs, with the ability to nucleate water at temperatures between -4.6 and -5.8 °C (Turner et al. 1990).

In addition, the lower ice nucleation activity of this protein can be attributed to its primary structure. Usually, bacterial ice nucleation proteins such as those in *Pseudomonas* species have a repetitive pattern in their sequences comprising 48-meric amino acid motif (Fig. 6a). These repetitious motifs have a propensity for β -sheet formation, which can form an initial seed for ice-crystallization and previous studies have clearly shown deletion of even one of the motifs had a serious impact on the ice nucleation temperature (Wolber 1993). The described pattern did not exist in the INP-F sequence (Fig. 6b). The other reason for the lowest nucleation activity of this protein compared to the bacterial counterparts is the glycosylation state. The native form of INP-F might be glycosylated,

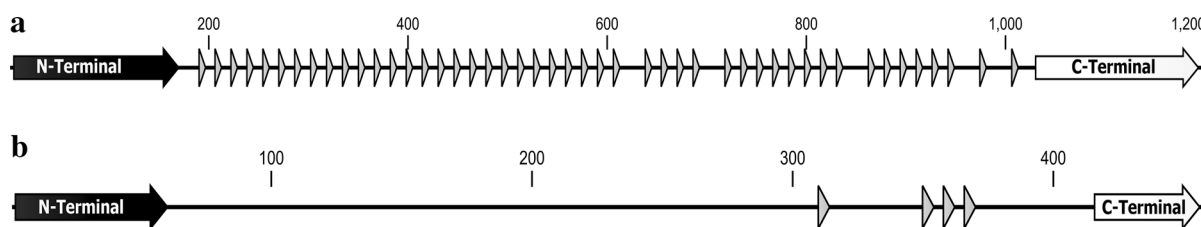


Fig. 6 A schematic representation of ice nucleation protein from *P. syringae* (a) and INP-F from *F. acuminatum* (b). The repetitious motif is shown by grey triangles. This motif is rare in *F. acuminatum* ice nucleation protein compared to *P. syringae*

because it is an extracellular protein. However, this modification does not occur when the protein expressed in a prokaryotic host, especially *E. coli*. Even, if it does, it would be in a very different pattern. Obviously, the posttranslational modifications had severe impacts on the final folding and activity of the target protein. Unfortunately, there is no study on this subject and proof of this hypothesis is postponed for the future work. Likewise, the *N*-terminal segment of INP-F that has a role in the secretion pathway may not have existed in the native form of the protein. This may be an additional significant factor on INP-F activity. In the future, it is valuable to elucidate the native crystal structure of this protein, which can help us to resolve issues discussed above.

Acknowledgments We greatly appreciate the Biotechnology Division members of Ferdowsi University of Mashhad for their help. We especially thank Dr. Hakimeh Ebrahimi Nik for critical review of the manuscript. This work was supported by grants from Baqiyatallah University of Medical Sciences, which is gratefully acknowledged by the authors.

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INP, which may point to the weaker ice nucleation activity of INP-F. The carboxyl and amino domains of the proteins were indicated in each figure

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