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Functional evaluation of candidate ice structuring proteins using cell-free expression systems

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ABSTRACT

Ice structuring proteins (ISPs) protect organisms from damage or death by freezing. They depress the non-equilibrium freezing point of water and prevent recrystallization, probably by binding to the surface of ice crystals. Many ISPs have been described and it is likely that many more exist in nature that have not yet been identified. ISPs come in many forms and thus cannot be reliably identified by their structure or consensus ice-binding motifs. Recombinant protein expression is the gold standard for proving the activity of a candidate ISP. Among existing expression systems, cell-free protein expression is the simplest and gives the fastest access to the protein of interest, but selection of the appropriate cell-free expression system is crucial for functionality. Here we describe cell-free expression methods for three ISPs that differ widely in structure and glycosylation status from three organisms: a fish (*Macrozoarces americanus*), an insect (*Dendroides canadensis*) and an alga (*Chlamydomonas* sp. CCMP681). We use both prokaryotic and eukaryotic expression systems for the production of ISPs. An ice recrystallization inhibition assay is used to test functionality. The techniques described here should improve the success of cell-free expression of ISPs in future applications.

1. Introduction

Ice structuring proteins (ISPs), also known as antifreeze proteins (AFPs), form a heterogeneous group of proteins, which help organisms to survive in ice-laden environments (Duman, 2001; Graether and Sykes, 2004; Kawahara, 2002). Some species use AFPs to prevent their body fluids from freezing (Duman et al., 1998; Hew et al., 1988). AFPs also inhibit the recrystallization of ice, a process in which the migration of ice grain boundaries creates larger ice crystals at the expense of smaller ones (Knight et al., 1984). Some species use ISPs to inhibit recrystallization to prevent freezing damage to their cell membranes rather than to lower the freezing point (Griffith and Yaish, 2004; Knight et al., 1995). Such proteins do lower the freezing point by a small amount, but not enough to be physiologically meaningful.

The study of ISPs goes back to 1969, when DeVries discovered resistance toward freezing in blood of some species of Antarctic fishes (DeVries and Wohlschlag, 1969). Since then, many additional vertebrate (Fletcher et al., 2001), invertebrate (Duman, 2001; Tyshenko et al., 1997), bacterial (Gilbert et al., 2004; Raymond et al., 2008; Sun et al., 1995; Yamashita et al., 2002), fungal (Duman and Olsen, 1993; Lee et al., 2010; Raymond and Janech, 2009), plant (Griffith et al., 1992; Smallwood et al., 1999) and algal (Raymond et al., 2009; Raymond and Kim, 2012) ISPs have been described. It is likely that nature has many others that have not yet been identified. Because of their remarkable structural diversity (Venketesh and Dayananda, 2008), ISPs have no common structural features or sequences by which they can be identified. When a candidate ISP gene is identified, the best proof that its product is functional is to express the recombinant protein and show that it has activity. Many expression systems using whole cells are available but they are time-consuming and often result in failure.

On the other hand, cell-free expression systems can give results in a matter of hours. With these systems, it is possible to synthesize proteins straight from a PCR product, thereby avoiding time-consuming cloning steps. In addition, protein functionality can be evaluated from the translation reaction mixture and hence no time-consuming purification steps are required. Several cell-free protein synthesis systems derived from prokaryotic and eukaryotic organisms have been previously developed (Endo and Sawasaki, 2003; Erickson and Blobel, 1983; Jackson and Hunt, 1983; Kubick et al., 2003; Madin et al., 2000; Pelham and Jackson, 1976). Each of these

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systems offers intrinsic advantages in terms of yield, proper protein folding, post-translational modifications, cost, speed and ease of use (Braun and LaBaer, 2003). Choosing the appropriate system for expressing a candidate ISP can save time and expense. A prokaryotic cell-free expression system was recently used to confirm the activity of a bacterial ISP (Raymond et al., 2008).

In the present study, we demonstrate the use of different cell-free expression systems (both prokaryotic and eukaryotic) to synthesize ISPs with differing structural requirements from three widely separate species: a fish (*Macrozoarces americanus*), an insect (*Dendroides canadensis*) and an alga (*Chlamydomonas* sp. CCMP681). The method of choice for analyzing functionality of ISPs is the ice recrystallization inhibition (IRI) assay (Knight et al., 1988), which requires only a few microliters of reaction mixture. We show that by choosing the appropriate cell-free expression system, one can quickly and inexpensively evaluate a candidate ISP.

2. Materials and methods

2.1. Materials

Complementary DNA (cDNA) encoding the ISP of an insect (*D. canadensis*) (DAFP-1; Uniprot accession no. O46351) was subcloned in the vector pBluescript SK and provided by one of the authors (J.D.). PCR-amplified cDNA encoding the ISP of an alga (*Chlamydomonas* sp. CCMP681) (CIBP-1; accession no. B1P0S8) was provided by another author (J.R.). For *M. americanus* type-III ISP HPLC 12 (MISP), we started with the amino acid sequence according to Uniprot accession no. P19614. Two nucleic acid sequences were designed and manufactured by GeneArt (Life Technologies GmbH, Darmstadt, Germany). DNA sequences were based on the most common codon usage in *E. coli* and insect cells (Supplementary Fig. 1).

2.2. DNA template design

Linear DNA templates were generated by one, two and three-step Expression-PCR (E-PCR) procedures, respectively (Merk et al., 2003).

2.2.1. One-step E-PCR

In the case of MISP (Supplementary Fig. 1), two codon-optimized constructs harboring regulatory sequences for enhanced expression in cell-free systems (RS 5′-MISP-RS 3′; RS: regulatory sequences for expression) were purchased from GeneArt. Subsequently, cDNA templates of MISP were amplified by PCR with primers T7-F and T7-R (Supplementary Table 1).

2.2.2. Two-step E-PCR

In a first PCR step, cDNAs of ISPs were amplified by PCR, using the following gene-specific primers: DAFP-1-SP-F, DAFP-1-F, DAFP-1-R, CIBP-1-SP-F, CIBP-1-F, CIBP-1-R (Supplementary Table 1). In each case, two constructs from DAFP-1 as well as from CIBP-1 were amplified. Constructs amplified with DAFP-1-SP-F or CIBP-1-SP-F, harbor a native N-terminal cleavable signal sequence whereas constructs which have been amplified with DAFP-1-F or CIBP-1-F did not exhibit any signal sequence. Potential signal peptide cleavage sites were previously analyzed by the software Signal P 4.0 (Petersen et al., 2011). In the second PCR step adapter primers were used to add regulatory sequences (RS 5′, RS 3′; Supplementary Table 1) for cell-free expression. Resulting E-PCR products were designed in the following way: RS 5′-ISP open reading frame-RS 3′ (RS: regulatory sequences; see also Supplementary Fig. 2). For purification of His-tagged MISP, RS 5′-MISP was amplified (T7-F, MISP-His-R) in the first step and the coding sequence of the His-tag was added by an adapter primer in the second PCR step (T7-F, RS-His 3′).

2.2.3. Three-step E-PCR

cDNA of enhanced yellow fluorescent protein (EYFP) was fused to cDNAs of DAFP-1 and CIBP-1 by a three-step overlap extension (oe) E-PCR. In the first PCR step, the coding sequences of the individual ISP and the coding sequence of EYFP were amplified separately (gene-specific primers: oe-EYFP-F, EYFP-R, DAFP-1-SP-F, DAFP-1-oe-EYFP-R, CIBP-1-SP-F, CIBP-1-oe-EYFP-R; Supplementary Table 1). In the second PCR step, gene-specific primers (DAFP-1-SP-F, CIBP-1-SP-F, EYFP-R; see also Supplementary Table 1) were used to fuse the two genes (ISP and EYFP). The resulting gene fusion constructs were used as a template in PCR step three. In this PCR step, regulatory sequences were added at the 5′ and 3′ non-coding regions of the final template (RS 5′, RS 3′; Supplementary Table 1).

For each PCR, a DNA polymerase with proofreading ability (HotStar HiFidelity Polymerase, Qiagen) was used to minimize mutations. Detailed PCR conditions are listed in Supplementary Table 2. Amplification products were analyzed in an ethidium bromide stained 1% agarose gel. The following linear DNA template sizes were calculated *in silico*: MISP (394 bp), MISP-His (418 bp), DAFP-1 (445 bp), DAFP-1-SP (517 bp), CIBP-1 (1186 bp), CIBP-1-SP (1252 bp), DAFP-1-SP-EYFP (1230 bp), CIBP-1-SP-EYFP (1965 bp). All PCR products were detected as homogeneous bands showing the expected size (data not shown). An aliquot of the E-PCR was directly pipetted to the cell-free protein synthesis reaction. The Expression-PCR system originally developed by RiNA GmbH (Berlin, Germany) is commercially available (EasyXpress Linear Template Kit PLUS, Qiagen; Linear Template Kit Signal Peptide, RiNA GmbH).

2.3. E. coli lysate preparation procedure

2.3.1. Standard E. coli lysate

Standard *E. coli* lysate was prepared according to the method of Nirenberg with slight modifications (Nirenberg and Matthaei, 1961). *E. coli* cells were grown at 37 °C to the early log phase. Cells were harvested and lysed using a French press. Fractionation of the resulting S30 extract and downstream processing of the translationally active lysate was performed by dialysis and centrifugation steps according to Nirenberg et al. and Merk et al. (Merk et al., 1999; Nirenberg and Matthaei, 1961). Aliquots of the lysate were immediately shock-frozen in liquid nitrogen and stored at -80 °C to preserve maximum activity. *In vitro* translation systems based on standard *E. coli* lysates are commercially available (EasyXpress Protein Synthesis Kit, Qiagen).

2.3.2. Redox-optimized E. coli lysate

To facilitate the formation of disulfide bonds, *E. coli* lysates were redox-optimized (Kim and Swartz, 2004) by adding chaperones (DnaK, DnaJ), protein disulfide isomerase (PDI) and oxidized and reduced forms of glutathione. *In vitro* translation systems based on redox-optimized *E. coli* lysates are commercially available (EasyXpress Disulfide *E. coli* Kit, Qiagen).

2.4. Insect lysate preparation procedure

2.4.1. Standard insect lysate

Fall army worm (*Spodoptera frugiperda 21*, *Sf 21*) cells which were grown exponentially in well-controlled fermenters at 27 °C in an animal component free insect cell medium were harvested at a density of approximately 4×10^6 cells/ml. *Sf 21* cells were collected by centrifugation at $200 \times g$ for 10 min, washed once with a HEPES-based [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer consisting of 40 mM HEPES-KOH (pH 7.5), 100 mM KOAc, 4 mM DTT and the pellet was resuspended to reach a final