A Thaumatin-like Protein from Larvae of the Beetle *Dendroides canadensis* Enhances the Activity of Antifreeze Proteins†

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ABSTRACT: The levels of thermal hysteresis (antifreeze activity) produced by purified antifreeze proteins (DAFPs) from the larvae of the beetle *Dendroides canadensis* at endogenous concentrations are lower than what are present in the hemolymph of overwintering larvae. Thermal hysteresis activity of DAFPs is dependent not only on AFP concentration but also on the presence of enhancers that may be either proteins (including other hemolymph DAFPs) or low-molecular mass enhancers such as glycerol. The purpose of this study was to identify endogenous protein enhancers using yeast two-hybrid, co-immunoprecipitation, and finally the enhancement of antifreeze activity. Here we show that a thaumatin-like protein from *D. canadensis*, until recently known only from plants, significantly enhances the thermal hysteresis of DAFP-1 and -2. Glycerol can further this enhancement, presumably by promoting the interaction of the DAFPs and thaumatin-like protein.

Antifreeze proteins (AFPs)[1] lower the nonequilibrium freezing point of water while not affecting the melting point. This results in a difference between the freezing and melting points that is termed thermal hysteresis (*1*). According to the adsorption−inhibition mechanism of action (*2*), the AFPs depress the freezing point by adsorbing onto the surface of ice crystals at preferred growth sites (*2*−*4*), by means of hydrogen bonding (*5*, *6*), hydrophobic, and/or van der Waals interactions (*7*−*9*). This adsorption permits ice crystal growth to occur only between the AFPs in highly curved (high surface free energy) fronts rather than the preferred low-curvature (low surface free energy) fronts, and therefore, the temperature must be lowered below the equilibrium melting point before ice crystal growth can take place.

Overwintering larvae of the beetle *Dendroides canadensis* produce a family of antifreeze proteins (DAFPs) that function to inhibit inoculative freezing across the cuticle (*10*) and to promote supercooling by inhibiting ice nucleators (*11*−*13*). The group I DAFPs (1, 2, 4, and 6) are present in the hemolymph, while groups II and III DAFPs occur in the gut fluid (*14*). There are both low-molecular mass (*15*) and high-molecular mass antifreeze protein enhancers (*16*) present in the hemolymph, and these significantly increase the thermal hysteresis activity (THA). The protein enhancers interact with the DAFPs, forming complexes that, because they are larger than the DAFP alone, block a larger surface area of the potential seed ice crystal than does the DAFP alone. Therefore, THA is increased. Because the protein enhancers are thought to bind to the DAFPs, a yeast two-hybrid screen was used to identify *D. canadensis* proteins that interact with DAFPs and might therefore be potential enhancers. This screen previously demonstrated that certain group I DAFPs (1, 2, 4, and 6) found in the hemolymph interact with one another, and these were then shown to enhance the thermal hysteresis activities of one another (*17*). The study reported here demonstrates that a thaumatin-like protein (until recently known only from plants) was also identified by the yeast two-hybrid screen as interacting with DAFP-1 and -2. The recombinant thaumatin-like protein was subsequently shown to enhance DAFP-1 and -2 activities.

EXPERIMENTAL PROCEDURES

Rationale. This project used a three-step approach to identify *D. canadensis* proteins that interact with DAFPs and enhance DAFP activity. Initially, the yeast two-hybrid system was used to screen a *D. canadensis* cDNA library for "prey" proteins which interact with DAFP "bait". This was followed by co-immunoprecipitation to verify that proteins identified by the yeast two-hybrid screen interact with certain DAFPs. Finally, proteins identified by these two procedures as interacting with DAFPs were expressed in an *Escherichia coli* expression system, purified, and then tested for their ability to enhance certain DAFPs by comparing the thermal hysteresis of aqueous solutions of DAFPs with and without the potential enhancer.

Yeast Two Hybrid Screen. The Matchmaker Two-Hybrid System 3 (CLONETECH Laboratories, Inc.) used in this study is a GAL4-based two-hybrid system that provides a transcriptional assay for detecting protein−protein interaction in vivo in yeast cells. (See Matchmaker GAL4 Two-Hybrid System 3 & Libraries User Manual.) A bait gene (encoding

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[1] Abbreviations: AFP, antifreeze protein; DAFP, *D. canadensis* antifreeze protein; THA, thermal hysteresis activity; PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; IPTG, isopropyl β-D-thiogalactoside; OD, optical density; TLP-*Dcan*, *D. canadensis* thaumatin-like protein.

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certain DAFPs) was expressed as a fusion protein with the GAL4 DNA-binding domain, while a cDNA (encoding a possible prey protein) is expressed as a fusion with the GAL4 activation domain. When bait and library fusion prey proteins interact, the DNA-binding domain and the activation domains are brought into proximity, thereby activating transcription of four reporter genes.

Construction of Bait Plasmids. As described previously (*17*), the cDNA encoding the mature proteins of *D. canadensis* DAFP-1 was obtained by PCR from the pBluescript plasmid containing the appropriate sequence using the following primers: 5′ primers, 5′ GCTGAATTCCAATG-TACTGGTGGTTCCGAT 3′; and 3′ primer, 5′ GCAG-GATCCTGGACATCCCGTGGAATCGGT 3′. The sequence was designed to include an EcoRI restriction site in the 5′ primer and a BamHI restriction site in the 3′ primer. Amplified DNA was cleaved with the restriction site enzymes and cloned into pGBKT7 that had been previously linearized with the same enzymes. The resulting plasmids, which contain the DAFP-1 coding sequences in frame with the DNA-binding domain of Gal4, were designated pGBKT7-DAFP-1. These constructs were used as baits for screening a *Dendroides* whole-body cDNA library.

Western Blot Analysis. To verify that DAFP-1 is expressed well in yeast strain AH109, pGBKT7-DAFP-1 was transformed into strain AH109, and the protein extracts were prepared from yeast by the urea−SDS method (Clontech yeast protocols handbook). The Western blot was carried out with a polyclonal antibody (made using DAFP-1 and -2 as antigens) to DAFPs using purified DAFP-1 as a positive control and plain yeast strain AH109 protein extracts as a negative control.

Construction of the Prey cDNA Library [As Described Previously (17)]. The *D. canadensis* larvae were collected in late October. Total RNA was extracted from 3 g of *D. canadensis* whole body, and part of it was used to extract mRNA and then synthesize the cDNA (Stratagene cDNA synthesis kit) with a designed EcoRI restriction site in the 5′ primer and an XhoI restriction site in the 3′ primer. These cDNAs were then cloned into the pGADT7 vector that was lineanized with the same enzymes. The primary titer of the constructed cDNA library was 1.5×10^7 pfu/mL, and the titer of the amplified library was 2.4×10^{10} pfu/mL. The average size of inserts was 1.2 kb. The resulting plasmids, which contained the cDNAs of *D. canadensis* whole body with the DNA activation domain of Gal4, were designated the pGADT7 library.

Yeast Two-Hybrid Screen [As Described Previously (17)]. The AH109 yeast strain was simultaneously transformed using large-scale transformation with pGBKT7-DAFP-1 and the pGADT7 library using the lithium acetate method (Clontech BD Matchmaker Two-Hybrid System 3). The transformants were plated on synthetic medium lacking adenine, histidine, leucine, and tryptophan, and with X-αGal. Colonies were picked at times from 4 to 8 days and replated on the second-generation plates for 6−10 days with synthetic medium lacking histidine, leucine, and tryptophan, and with X-αGal. The blue colonies that were well-isolated were picked up and replated on synthetic medium lacking adenine, histidine, leucine, and tryptophan to verify that they maintained the correct phenotype. The yeast plasmids were purified using the lyticase method (Clontech yeast protocols handbook), transformed into *E. coli* using electroporation, and purified using a Miniprep kit (Qiagen). The purified pGAD plasmids were retransformed into strain AH109 carrying pGBKT7-DAFP-1 to verify positive colonies. The positives were sequenced by automated DNA sequencing (ABI 3700).

In Vitro Transcription/Translation and Immunoprecipitation. As described previously (*17*), the pGBKT7-DAFP-1 and pGAD-TLP-*Dcan*1 plasmids from the yeast two-hybrid system were transcribed and translated using Promega's TNT T7 Coupled Reticulocyte Lysate System in vitro to prepare [^{35}S]Met-labeled bait and prey proteins. The interaction between DAFP-1 and TLP-*Dcan*1 was confirmed and visualized by in vitro co-immunoprecipitation (Co-IP) using the BD Matchmaker Co-IP kit (Clontech) which is compatible with the BD Matchmaker Two-Hybrid System 3. The co-immunoprecipitates were separated by SDS−PAGE and analyzed by autoradiography.

5′ Rapid Amplificaiton of cDNA Ends (RACE). 5′ RACE, followed by automated sequencing, was used to determine the full-length sequence of cDNAs encoding proteins that were shown by yeast two-hybrid and co-immunoprecipitation to interact with DAFPs. The RNA Oligo ligated to full-length mRNA was made from the same total RNA with which the pGADT7 library was made. Then the RACE-ready cDNA was made by reverse-transcribing the mRNA; this was used as a template for 5′ RACE (Invitrogen). Two approaches were used to amplify and identify the 5′ end of TLP-*Dcan*1. In the first approach, the primary amplification was performed on 0.3 μL of cDNA template using GeneRacer 5′ primers (5′ CGACTGGAGCACGAGGACACTGA 3′) and TLP-*Dcan*1 gene-specific 3′ primers (5′ GCGAAAGCTTG-GCACGTGAAGGTACTCTTGT 3′). For secondary amplification, 1 μL of reactants was taken from the first amplification product and amplified using GeneRacer 5′ Nested primers (5′ GGACACTGACATGGACTGAAGGAGTA 3′) and TLP-*Dcan*1 gene-specific 3′ primers (5′ GCGAAAGCT-TGGCACGTGAAGGTACTCTTGT 3′), and PCR products were identified by automated sequencing (ABI 3700).

Expression of Recombinant DAFPs and TLP-Dcan1. Recombinant DAFP-1, -2, -4, and -6 were expressed in *E. coli* BL21(DES) pLysS competent cells as described previously (*17*). The DNA encoding the mature TLP-*Dcan*1 was amplified by PCR using the 5′ primer (5′ TCGGATCCT-TCGTGCTCCTAGTC 3′) and the 3′ primer (5′ TAGTGCG-GCCGCGCACGTGAAGGTACTCTTGTGGTC 3′). Sequences were designed to include a *Bam*HI restriction site in the 5′ primer and an *Not*I restriction site in the 3′ primer. Amplified DNA was digested and cloned into pET-20b (Novagen), previously linearized with the same enzymes. The resulting plasmids were sequenced (ABI 3700). They were then transformed into *E. coli* BL21(DE3) pLysS competent cells. The cells were grown in LB broth containing carbenicillin, ampicillin, and chloramphenicol. IPTG was added to the culture to a final concentration of 0.4 mM for pet-20b(+) when the OD_{600} reached 0.6. Cells were grown at 37 °C for 3 h in the induction medium and collected by centrifugation at 6000 rpm for 20 min. The extracted crude protein was purified using the His-bind purification kit (Novagen). The purified protein was dialyzed, freeze-dried, and redissolved in sterilized distilled water.

RT-PCR of TLP-*Dcan*1. RT-PCR was used to determine when the transcript encoding TLP-*Dcan*1 was produced by the larvae. Total RNA was isolated from both fat body cells and gut epithelium cells of winter and summer *D. canadensis*. First-strand cDNA was synthesized using the First Strand cDNA synthesis kit (Novagen). The cDNA products were amplified using the TLP-*Dcan*1-specific 5′ primer (5′ CCA TCT CAG CAA CGG TGG TCT CAT CCT CGA CC 3′) and the 3′ primer (5′ GGT AGT CGA CGG GCC AGT CCG AAG ACT TGC AG 3′).

Thermal Hysteresis Activity. The capillary technique was used to measure thermal hysteresis activity (*1*). The sample (~4 μL) was placed in a sealed capillary tube; a small (~0.25 mm) seed crystal was spray-frozen in the sample, and the capillary was placed into a refrigerated alcohol bath which was equipped with a viewing chamber through which the crystal could be observed with a microscope. The bath temperature was controlled to ±0.01 °C. The temperature was slowly increased (0.02 °C/5 min) until the crystal disappeared. This temperature was taken as the melting point of the sample. Another crystal was spray-frozen in the sample, the capillary again placed in the chamber, and the bath temperature lowered (0.1 °C/2.5 min) until the crystal grew. The temperature at which the seed crystal began to grow was taken as the hysteretic freezing point. Aqueous solutions, which do not contain thermal hysteresis antifreeze proteins, exhibit identical freezing and melting points. However, in the presence of these proteins, the freezing point is often depressed several degrees below the melting point. This difference between the melting and freezing points is termed thermal hysteresis. Generally, at least two freezing/melting point determinations were made for each sample, and the two measurements were usually within ≤0.4 °C of one another. If the difference was greater, additional measurements were taken.

RESULTS

To search for cDNA clones encoding proteins that interact with DAFP-1, the coding sequence for *D. canadensis* hemolymph DAFP-1 was fused with the Gal4 DNA-binding domain and used to screen a *D. canadensis* whole-body library expressed from the pGADT7 vector. Nineteen clones were isolated that displayed an Ade+His+LacZ+ phenotype, representing 15 different cDNAs as judged by cDNA sequencing. However, only three turned out to be positives on the basis of co-immunoprecipitation results. As reported previously (*17*), two of these were other DAFPs (DAFP-2 and DAFP-4). The interaction of DAFP-1 with these proteins was specific, as no interaction was observed between the identified proteins and the Gal4 DNA-binding domain (BD) alone. The interaction was confirmed by co-immunoprecipitation (Figure 1).

One positive yeast clone contained the partial cDNA sequence of a thaumatin-like protein. Using a RACE approach, the complete gene was sequenced. The open reading frame encoded a 253-amino acid protein (Figure 2) with a predicted molecular mass of 28 kDa. The N-terminal region of the protein presents a putative signal peptide of 15 amino acids.

The 28 kDa mature protein consists of 253 amino acids. Its sequence is very similar to those of thaumatin-like proteins in insects and plants (Figure 3). It is 65% identical and 76% positive with TLP of *Schistocercus gregaria* (TLP-*Sgre*1, GenBank accession number AY512591) and 47% identical and 61% positive with TLP of *Arabidopsis thaliana* (TLP-*Atal*, GenBank accession number NP177641).

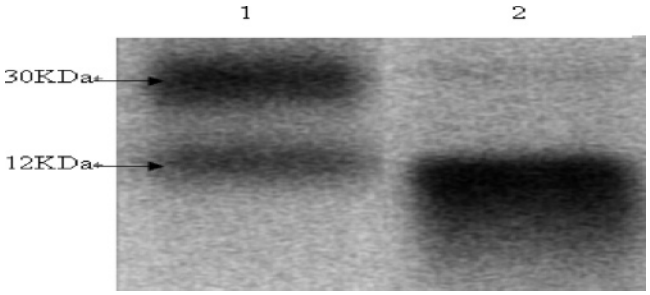

FIGURE 1: Interaction between DAFP-1 and TLP-*Dcan*1 was confirmed by co-immunoprecipitation (Co-IP). The pGBKT7-DAFP-1 and pGAD-TLP-*Dcan*1 plasmids from the yeast two-hybrid system were transcribed and translated as [^{35}S]Met-labeled Myc-tagged DAFP-1 and [^{35}S]Met-labeled HA-tagged TLP-*Dcan*1. Either Myc monoclonal or HA-Tag polyclonal antibodies were used to precipitate the mixture of [^{35}S]Met-labeled Myc-taged DAFP-1 and [^{35}S]Met-labeled HA-tagged TLP-*Dcan*1. The co-immunoprecipitates were separated by SDS−PAGE and analyzed by autoradiography: lane 1, [^{35}S]Met-labeled Myc-tagged DAFP-1 and [^{35}S]Met-labeled HA-tagged TLP-*Dcan*1 mixture precipitated by HA-Tag polyclonal antibodies; lane 2, [^{35}S]Met-labeled Myc-tagged DAFP-1 precipitated by Myc monoclonal antibodies.

Reverse transcription using TLP-*Dcan*1-specific primers was done to confirm the expression of the TLP-*Dcan*1 gene. The expected amplification products were present in both the fat body cells and gut epithelial cells, in both winter and summer (Figure 4). However, in summer the fat-body amplification product was larger than expected (Figure 4, lane 3), and by sequencing all the amplification products, we identified another TLP in *D. canadensis*, TLP-*Dcan*2. Although the TLP-*Dcan*2 transcript is larger than that encoding TLP-*Dcan*1 (Figure 4), the former contains a stop codon at residue 525 and consequently TLP-*Dcan*2 has a lower molecular mass than TLP-*Dcan*1. The open reading frame encodes a 177-amino acid protein (Figure 5) with a predicted molecular mass of 19 kDa, including a putative signal peptide of 15 amino acids.

To determine if the interaction between DAFP-1 and TLP-*Dcan*1 can enhance the thermal hysteresis activity of DAFP-1, mature DAFP-1 and TLP-*Dcan*1 were expressed in the bacterial expression system and purified and the THA of DAFP-1 alone was compared with that of DAFP-1 to which various amounts of purified TLP-*Dcan*1 were added. Figure 6 shows that the thermal hysteresis activities of the 0.5 and 1.0 mg/mL DAFP-1 solution were enhanced by addition of TLP-*Dcan*1, even at very low TLP-*Dcan*1 concentrations. For example, at a concentration of 0.5 mg/mL TLP-*Dcan*1, the THA of the 0.5 mg/mL DAFP solution was increased from 2.7 to ~7 °C. At TLP-*Dcan*1 concentrations above 0.5 mg/mL, the THA of the 0.5 mg/mL DAFP solution did not increase further. In similar experiments, the addition of TLP-D*can*1 to DAFP-2 resulted in enhancement of THA; however, no enhancement was seen with addition of TLP-D*can*1 to the other two hemolymph DAFPs (4 and 6) (data not shown). TLP-*Dcan*1 alone does not exhibit THA.

As shown in Figure 7, the enhancement of DAFP-1 by TLP-*Dcan*1 is further enhanced by glycerol. Glycerol, which

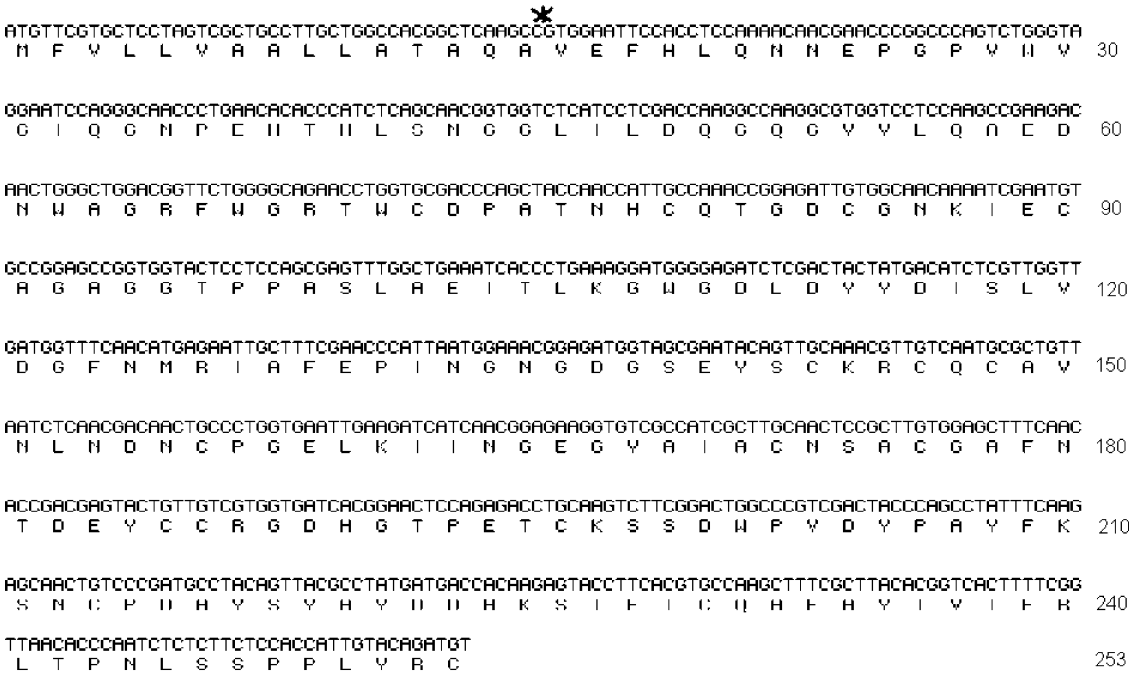

FIGURE 2: Nucleotide and deduced amino acid sequence of the 28 kDa TLP-*Dcan*1. The asterisk indicates the predicted signal peptide cleavage site (between residues 15 and 16).

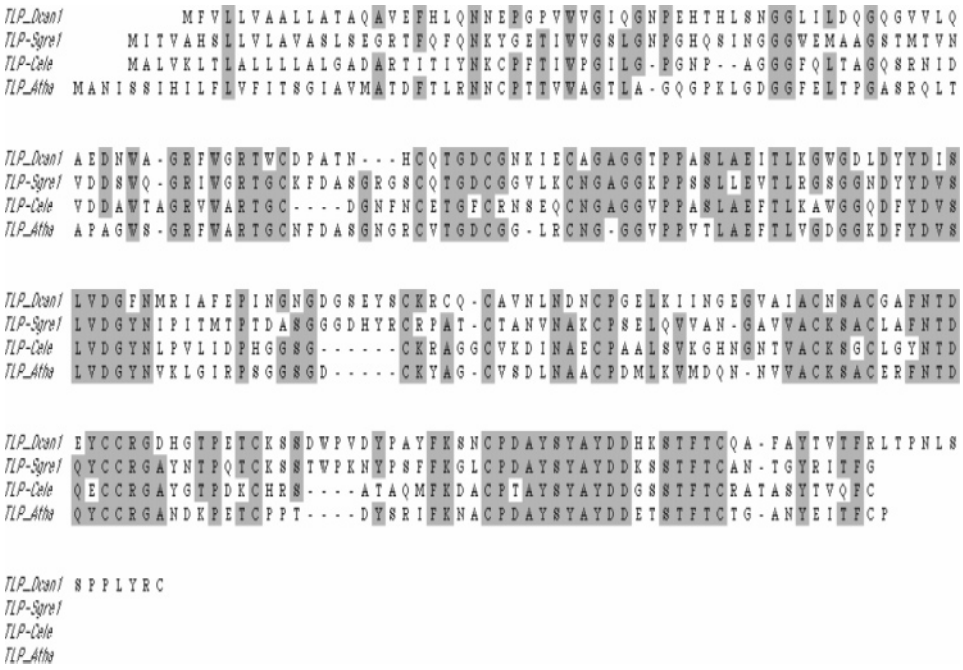

FIGURE 3: Complete amino acid sequence of the thaumatin-like protein (TLP) of *D. canadensis* (TLP-*Dcan*1, GenBank accession number DQ023319), aligned with representative TLP of *S. gregaria* (TLP-*Sgre*1, GenBank accession number AY512591), *C. elegans* (TLP-*Cele*, GenBank accession number NP502362), and *A. thaliana* (TLP-*Atal*, GenBank accession number NP177641). Identical residues are shaded.

is present in the hemolymph of *D. canadensis* in winter at concentrations of 0.5−1.0 M, was especially effective at low TLP-*Dcan*1 concentrations. For example, at 0.25 mg/mL TLP-*Dcan*1, the THA of 0.5 mg/mL DAFP-1 increased from 3.5 to 8.7 °C. However, there was much less enhancement at the higher TLP-*Dcan*1 concentrations.

It was previously shown that DAFP-1 and -2 enhance one another and that glycerol provides further enhancement (*17*). Therefore, 1 mg/mL TLP-*Dcan*1 was added to a solution containing DAFP-1 and -2 in equal concentrations with 0.5 M glycerol. As demonstrated in Figure 8, the addition of TLP-*Dcan*1 greatly increased THA, at both high and low DAFP concentrations. However, comparison of Figures 7 and 8 indicates that the addition of DAFP-2 (Figure 8) to the combination of DAFP-1, TLP-*Dcan*1, and glycerol (Figure 7) did not increase the maximal THA.

All four group I DAFPs (1, 2, 4, and 6) are normally present in the hemolymph. Consequently, TLP-*Dcan*1 was added to a mixture of equal concentrations of DAFP-1, -2, -4, and -6 with glycerol (Figure 9). As seen previously (*17*), these four DAFPs enhance one another at higher, but not lower, DAFP concentrations (>1 mg/mL). In contrast, the

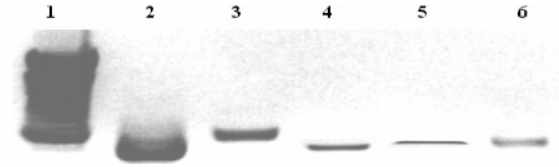

FIGURE 4: RT-PCR showing the presence of the TLP-*Dcan*1 transcript in fat body cells and gut epithelium cells in both winter and summer. First-strand cDNA synthesized from total RNA of winter and summer fat body cells and gut epithelium cells were amplified using TLP-*Dcan*1-specific primers: lane 1, 100 bp marker (Promega); lane 2, winter whole body; lane 3, summer fat body; lane 4, winter fat body; lane 5, summer gut; and lane 6, winter gut. Note the slower migration of the transcript in lane 3.

addition of TLP-*Dcan*1 resulted in a significant enhancement at the lower, but not the higher, DAFP concentrations. In fact, at the highest DAFP concentration that was tested (3 mg/mL), the addition of TLP-*Dcan*1 and glycerol to the mixture of the four DAFPs actually resulted in less THA.

DISCUSSION

Thaumatin, a sweet-tasting 207-residue protein, was first isolated from the fruits of the West African rain forest shrub *Thaumatococcus daniellii*; however, proteins with similar amino acid sequences have been identified in all plant species that have been examined (*18*−*20*). Pathogenesis-related (PR) proteins are produced by plants in response to environmental stress signals such as pathogen invasion, drought, and wounding. Members of family 5 of the PR proteins (PR-5 proteins) consist of thaumatin-like proteins (TLPs) and osmotin-like proteins. All PR-5 proteins have considerable sequence similarities, and they all have antifungal and/or antibacterial functions, whether they are classified as thaumatin-like or osmotin-like proteins. (Osmotin is a salt-stress-induced protein originally described from tobacco.) The structure of thaumatin has been determined (*21*, *22*). The TLPs are generally resistant to proteases and pH- or heat-induced denaturation. This is likely due to the presence of 16 conserved cysteines that form eight disulfide bridges. Their action against pathogenic microorganisms has been related in some cases to their endo-β-1,3-glucanase activity (*23*, *24*) and to α-amylase inhibiting properties (*25*). Also, PR-5 proteins have been reported to have membrane-permeabilizing activity (*26*).

On the basis of genome information, thaumatin-like proteins were predicted to exist in the nematode *Caenorhabditis elegans* (*27*). Recently, for the first time in animal species, genes encoding members of the thaumatin-like protein family were reported in the desert locust *S. gregaria* and in the related *Locusta migratoria* (*28*). The three-dimensional model of the *S. gregaria* thaumatin-like protein suggests a glucanase function, but this has not been confirmed experimentally.

It has been shown that endogenous protein and low-molecular mass enhancers (glycerol) are present and play important roles in increasing the thermal hysteresis activity in the hemolymph of overwintering *D. canadensis* larvae (*16*). Since protein enhancers of DAFPs, including polyclonal antibodies specific for DAFPs, are known to bind to the DAFPs (*29*, *30*), the yeast two-hybrid system was used to identify endogenous *D. canadensis* proteins which interact with certain DAFPs and which therefore might be endogenous DAFP enhancers. Among the proteins identified by the yeast two-hybrid screen were other DAFPs (*17*) as well as a thaumatin-like protein (TLP-*Dcan*1). Binding between DAFP-1 and TLP-*Dcan*1 was confirmed by co-immunoprecipitation. The interaction was further confirmed by adding TLP-*Dcan*1 to DAFP-1, resulting in enhanced THA (Figure 6). For example, with addition of 0.5 mg/mL TLP-*Dcan*1, the THA of a 0.5 mg/mL DAFP-1 solution was increased from 2.7 to ~7 °C. Our interpretation is that the DAFP-1−TLP-*Dcan*1 complex can still bind to ice, but the complexes are able to block a larger surface area of the ice and/or are more difficult for the ice to overgrow; consequently, the THA is increased. However, at TLP-*Dcan*1 concentrations above 0.5 mg/mL, the enhancement did not increase further (Figure 6). This could be a result of saturated binding of TLP-*Dcan*1 to DAFP-1. TLP-*Dcan*1 had a similar enhancing effect on DAFP-2.

Glycerol was previously shown to enhance the activity of DAFPs (*15*), presumably by stimulating enhanced DAFP interactions with one another (*17*). Glycerol also appears to promote the interaction between DAFP-1 and TLP-*Dcan*1, which consequently further improved the ability of TLP-*Dcan*1 to enhance the activity of DAFP-1 (Figure 7).

Figure 10 demonstrates the interactions of the four hemolymph (group I) DAFPs. DAFP-1 interacts with DAFP-2 and -4 but not DAFP-6, even though DAFP-4 and -6 are very similar (sequence differences at just four positions).

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TLP-Dcan1     MFVLLVAALLATAQAVEFHLQNNEPGPVWVGIQGNPEHTHLSNGGLILDQ     50
TLP-Dcan2     MFVLLVAALLATAQAVEFHLQNNEPGPVWVGIQGNPEHTHLSNGGLILDQ     50

TLP-Dcan1     GQGVVLQAEDNWAGRFWGRTWCDPATNHCQTGDCGNKIECAGAGGTPPAS    100
TLP-Dcan2     GQGVVLQAEDNWAGRFWGRTWCDPATNHCQTGDCGNKIECAGAGGTPPAS    100

TLP-Dcan1     LAEITLKGWGDLDYYDISLVDGFNMRIAFEPINGNGDGSEYSCKRCQCAV    150
TLP-Dcan2     LAEITLKGWGDLDYYDISLVDGFNMRIAvsgfvvNfDsSltfvsdssnpl    150

TLP-Dcan1     NLNDNCPGELKIINGEGVAIACNSACGAFNTDEYCCRGDHGTPETCKSSD    200
TLP-Dcan2     metemvantvanvvn...AllistttalvN.....................    177

TLP-Dcan1     WPVDYPAYFKSNCPDAYSYAYDDHKSTFTCQAFAYTVTFRLTPNLSSPPL    250
TLP-Dcan2     ..................................................    177

TLP-Dcan1     YRC                                                   253
TLP-Dcan2     ...                                                   177
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FIGURE 5: Complete amino acid sequence of the thaumatin-like protein (TLP) of *D. canadensis* (TLP-*Dcan*1, GenBank accession number DQ023319), aligned with TLP-*Dcan*2 (GenBank accession number DQ112346). Different amino acids are shown in TLP-*Dcan*2 using lowercase letters.

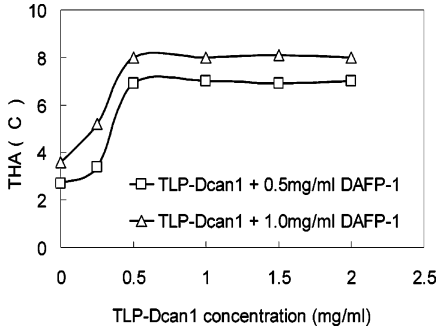

FIGURE 6: Effect of TLP-*Dcan*1 on THA of a 0.5 or 1.0 mg/mL DAFP-1 solution. The thermal hysteresis activities of the 0.5 and 1.0 mg/mL DAFP-1 solutions were enhanced by addition of TLP-*Dcan*1: (□) TLP-*Dcan*1 and 0.5 mg/mL DAFP-1 and (△) TLP-*Dcan*1 and 1.0 mg/mL DAFP-1. Values are means of two or three measurements of the same sample. The standard deviation ranged from 0.00 to 0.21. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

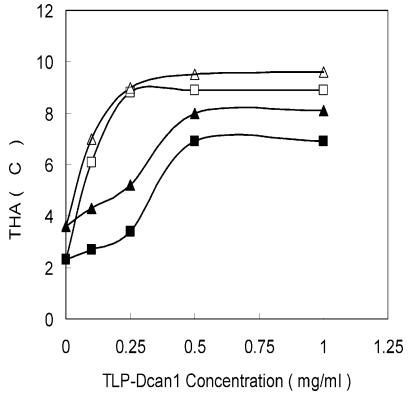

FIGURE 7: Enhancement of DAFP-1 by TLP-*Dcan*1 is further promoted by glycerol, especially at the lower TLP-*Dcan*1 concentrations. TLP-*Dcan*1 from 0 to 1 mg/mL was added to the solution with 0.5 mg/mL DAFP-1 and 0.5 M glycerol or with 1.0 mg/mL DAFP-1 and 0.5 M glycerol: (□) 0.5 mg/mL DAFP-1, TLP-*Dcan*1, and 0.5 M glycerol, (■) TLP-*Dcan*1 and 0.5 mg/mL DAFP-1, (▲) 1.0 mg/mL DAFP-1 and TLP-*Dcan*1, and (△) 1.0 mg/mL DAFP-1, TLP-*Dcan*1, and 0.5 M glycerol. Values are means of two or three measurements of the same sample. The standard deviation ranged from 0.00 to 0.28. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

DAFP-4 interacts with DAFP-1 and -6 but not DAFP-2, even though DAFP-1 and -2 are different at only two positions. Although DAFP-1 and -2 enhance one another, and glycerol provides further enhancement (*17*), comparison of Figures 7 and 8 indicates that the combination of DAFP-1 and DAFP-2 with TLP-*Dcan*1 did not increase the THA over that of TLP-*Dcan*1 with DAFP-1 alone, either with or without glycerol. This is interesting since TLP-*Dcan*1 is capable of enhancing either DAFP-1 or -2 alone. Also, TLP-*Dcan*1 has no effect on the THA of either DAFP-4 or DAFP-6 (data not shown). However, as shown in Figure 9, the combination of all four group I hemolymph DAFPs, with glycerol, was considerably enhanced by TLP-*Dcan*1 at lower DAFP concentrations (up to 1 mg/mL). The composition(s), size(s), and shape(s) of these complexes are not known at this time. Also, the composition, size, and stability of the complexes could vary with protein concentration (as suggested by Figure 9), protein ratios, or other factors. Consequently, this information must be gathered before an informed theory of the mechanism of increased freezing point depression of these complexes can be put forward. However, these data are likely to have important implications for the understanding of the mechanism of action of AFPs.

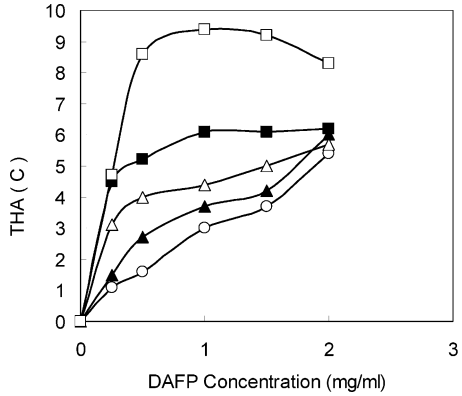

FIGURE 8: Effect of TLP-D*can*1 on the thermal hysteresis activity of the combination of DAFP-1 and -2: (▲) DAFP-1, (○) DAFP-2, (△) DAFP-1 and DAFP-2, (■) DAFP-1, DAFP-2, and 0.5 M glycerol, and (□) DAFP-1, DAFP-2, 0.5 M glycerol, and 1.0 mg/mL TLP-*Dcan*1. Values are means of two or three measurements of the same sample. The standard deviation ranged from 0.00 to 0.28. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

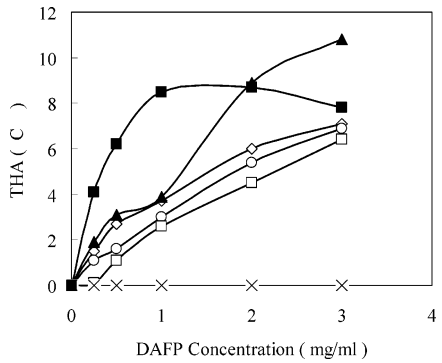

FIGURE 9: Effect of TLP-*Dcan*1 on the thermal hystresis activity of a combination of DAFP-1, -2, -4, and -6: (◇) DAFP-1, (○) DAFP-2, (□) DAFP-4, (×) DAFP-6, (▲) DAFP-1, DAFP-2, DAFP-4, DAFP-6, and 0.5 M glycerol, and (■) DAFP-1, DAFP-2, DAFP-4, DAFP-6, 1.0 mg/mL TLP-*Dcan*1, and 0.5 M glycerol. Values are means of two or three measurements of the same sample. The standard deviation ranged from 0.00 to 0.28. Computer curve fitting (Excel) was used to connect points in the graph to guide the eye.

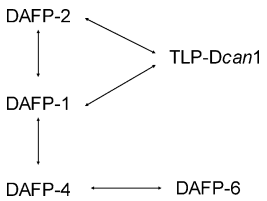

FIGURE 10: Interactions of the four hemolymph (group I) DAFPs (summarized from ref *17* and this study).

Group I DAFPs (1, 2, 4, and 6) are produced by fat body cells and secreted into the hemolymph (*14*). TLP-*Dcan*1 is also produced by the fat body, and since TLP-*Dcan*1 has a signal peptide, it is probably also secreted into the hemolymph. RT-PCR demonstrated that gut epithelial cells produce TLP-*Dcan*1 in the winter and TLP-*Dcan*2 in the summer (Figure 4). Because of the presence of a signal peptide, both are apparently secreted into the midgut fluid. While DAFP-1 is not present in the midgut fluid, group II and III DAFPs are produced in the gut epithelia and secreted into the gut fluid

(*14*). At this time, we do not know if TLP-*Dcan*1 enhances any of the gut DAFPs; however, when DAFP-8 (group III) was used as bait in a yeast two-hybrid screen, neither of the TLP-*Dcan* proteins was identified as a positive (L. Wang and J. G. Duman, unpublished observations). Despite this, it is possible that TLP-*Dcan*1, which is produced by gut epithelia in winter (Figure 4), enhances one or more of the gut DAFPs. However, at this time, the function of TLP-*Dcan*1 in the gut fluid remains unknown. Also, since TLP-*Dcan*1 is produced in the fat body in summer as well as in winter (Figure 4), it is possible that it has another function (i.e., antimicrobial activity) in addition to enhancing DAFPs. Multiple functions of plant PR proteins are not uncommon. In fact, a number of plant antifreeze proteins are PR proteins (including some TLPs), having a sequence similar to those of PR proteins and providing protection against psychrophilic microbial pathogens (*31*). Since the plant AFPs produce only minimal thermal hysteresis (a few tenths of a degree, at most) and appear to function to inhibit the recrystallization of ice in intracellular spaces of the freeze tolerant plants that produce them, it is perhaps preferable to refer to them as ice-binding proteins, rather than AFPs. In any event, although as mentioned earlier, TLP-*Dcan*1 lacks AFP activity, it is interesting that TLP-*Dcan* 1 has evolved to enhance DAFPs.

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