

ENHANCEMENT OF INSECT ANTIFREEZE PROTEIN ACTIVITY BY SOLUTES OF LOW MOLECULAR MASS

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Summary

Antifreeze proteins (AFPs) lower the non-equilibrium freezing point of water (in the presence of ice) below the melting point, thereby producing a difference between the freezing and melting points that has been termed thermal hysteresis. In general, the magnitude of the thermal hysteresis depends upon the specific activity and concentration of the AFP. This study describes several low-molecular-mass solutes that enhance the thermal hysteresis activity of an AFP from overwintering larvae of the beetle *Dendroides canadensis*. The most active of these is citrate, which increases the thermal hysteresis nearly sixfold from 1.2 °C in its absence to 6.8 °C. Solute concentrations between 0.25 and 1 mol l⁻¹ were generally required to elicit optimal thermal hysteresis activity.

Glycerol is the only one of these enhancing solutes that is known to be present at these concentrations in overwintering *D. canadensis*, and therefore the physiological significance of most of these enhancers is unknown. The mechanism(s) of this enhancement is also unknown.

The AFP used in this study (DAFP-4) is nearly identical to previously described *D. canadensis* AFPs. The mature protein consists of 71 amino acid residues arranged in six 12- or 13-mer repeats with a consensus sequence consisting of Cys-Thr-X₃-Ser-X₅-X₆-Cys-X₈-X₉-Ala-X₁₁-Thr-X₁₃, where X₃ and X₁₁ tend to be charged residues, X₅ tends to be Thr or Ser, X₆ to be Asn or Asp, X₉ to be Asn or Lys and X₁₃ to be Ala in the 13-mers. DAFP-4 is shorter by one repeat than previously described *D. canadensis* AFPs.

Key words: antifreeze protein, thermal hysteresis activity, antifreeze protein activation, *Dendroides canadensis*, beetle, insect antifreeze protein.

Introduction

Antifreeze proteins (AFPs), also known as thermal hysteresis proteins, were first identified in polar marine fishes where they function to depress the freezing point of the body fluids of the hypo-osmoregulating fish below that of sea water (DeVries, 1971; DeVries and Cheng, 1992; Davies and Hew, 1990). AFPs are also present in certain terrestrial arthropods, including insects (Duman, 1977; Duman *et al.* 1993), spiders (Duman, 1979a; Husby and Zachariassen, 1980), mites (Block and Duman, 1989) and centipedes (Tursman *et al.* 1994; Tursman and Duman, 1995). More recently, thermal hysteresis proteins have been identified in many plants (Urrutia *et al.* 1992; Griffith *et al.* 1992a,b; Duman and Olsen, 1993; Duman, 1994), fungi and bacteria (Duman and Olsen, 1993). In overwintering larvae of the beetle *Dendroides canadensis*, the AFPs function to inhibit inoculative freezing across the cuticle by external ice (Olsen *et al.* 1998) and to inhibit ice nucleators both in the gut and in hemolymph (Olsen and Duman, 1997a,b).

AFPs depress the non-equilibrium freezing point of water by a non-colligative mechanism while not lowering the melting

point, except for a small colligative effect. This produces a difference between the freezing and melting points which has been termed thermal hysteresis (DeVries, 1971, 1986). While the structures of the various AFPs differ considerably, their general mechanism of freezing point depression depends on the ability to adsorb onto the surface of potential seed ice crystals, probably *via* hydrogen bonding. This forces crystal growth into highly curved (high free energy) fronts, rather than the preferred low-curvature (low free energy) fronts. Therefore, growth is halted by the Kelvin effect until the temperature is lowered sufficiently (Raymond *et al.* 1977, 1989; Knight *et al.* 1991; DeVries and Cheng, 1992). While details of the adsorption mechanism are somewhat uncertain, considerable amounts of data are now available on the fish AFPs (Sicheri and Yang, 1995), and a lattice match between polar side-groups of the AFP and water molecules in the ice crystal appears likely.

Four basic categories of fish AFPs have been identified, one glycoprotein type with considerable repeating structure and three protein (peptide) types with varying levels of

repeating structure, presumably to allow efficient hydrogen bonding to the crystal lattice of ice (for reviews, see DeVries and Cheng, 1992; Davies and Hew, 1990). Three of the AFPs from the larvae of the beetle *Dendroides canadensis* have been characterized (Duman *et al.* 1998). These are approximately 8.7 kDa in molecular mass and consist of seven 12- or 13-mer repeat units with a consensus sequence of C-T-X₃-S-X₅-X₆-C-X₈-X₉-A-X₁₁-T-X₁₃, where X₃ and X₁₁ tend to be charged residues, X₅ tends to be threonine or serine, X₆ to be asparagine or aspartate, X₉ to be asparagine or lysine and X₁₃ to be alanine in the 13-mers. Through most of the length of the proteins, every sixth residue is a cysteine, and all these residues are involved in disulfide bridges (Li *et al.* 1998). The AFPs from the larvae of the beetle *Tenebrio molitor* are quite similar to those of *D. canadensis*, including complete identity of the cysteine residues (Graham *et al.* 1997). Although the AFP from the spruce budworm *Choristoneura fumiferana* has 12-mer repeating units, this AFP is not similar to the *D. canadensis* AFPs (Tyshenko *et al.* 1997).

In general, the magnitude of the thermal hysteresis activity of AFPs depends upon the specific activity of the particular AFP and the AFP concentration, up to a point where the activity plateaus. While the blood of AFP-producing fish may exhibit up to approximately 2 °C of thermal hysteresis activity, a value sufficient to prevent freezing in ice-laden sea water, it is common for the hemolymph of insects to have considerably greater activity. Overwintering larvae of *D. canadensis* routinely have a mean thermal hysteresis activity of 5–6 °C in the hemolymph, and some individuals may have an activity of 8–9 °C (Duman, 1980). Therefore, it was perplexing when purified *D. canadensis* AFP, even at very high protein concentrations, produced much less activity than that seen in the hemolymph (Wu *et al.* 1991a). However, an endogenous activator (enhancer) protein was later purified (Wu and Duman, 1991). Addition of the activator protein to a solution containing 4 mg ml⁻¹ of *D. canadensis* AFP raised the thermal hysteresis activity from 1.60 °C to 5.24 °C. This activator appears to function by binding to the AFP, or *vice versa*, such that the AFP–activator complex can still hydrogen bond to ice but now blocks a larger surface area of the potential seed ice crystal and/or is more difficult to overgrow, so that the hysteretic freezing point is further depressed (Wu and Duman, 1991; Wu *et al.* 1991b). Studies reported here identify certain low-molecular-mass solutes which demonstrate unusual abilities to increase the thermal hysteresis activity of a *D. canadensis* AFP. Since the AFP used in this study has not been described previously, information on the sequence and stability of this *D. canadensis* AFP (DAFP-4) is also presented.

Materials and methods

Purification of *Dendroides canadensis* AFP-4

Dendroides canadensis AFP was purified as described previously (Li *et al.* 1998). The *D. canadensis* AFP-4 (DAFP-

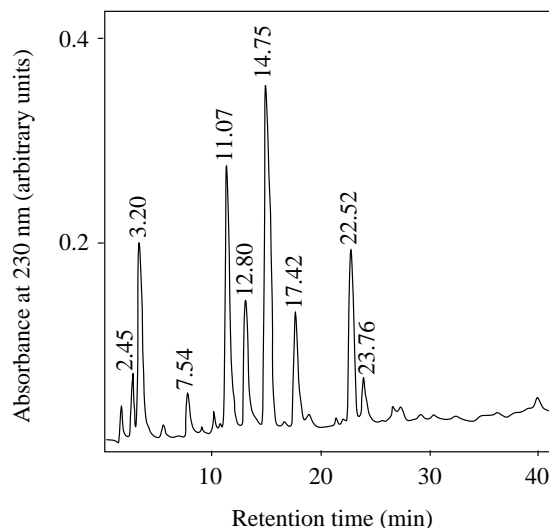


Fig. 1. Reverse-phase HPLC of the peptide mixture resulting from trypsin digestion of DAFP-4. Conditions used were as stated in the text. DAFP-4 elutes at 22.46 min.

4) used in this study elutes from the reverse-phase high-performance chromatography (RP-HPLC) column used in the final step of the purification at 22.46 min.

Peptide sequencing of DAFP-4

DAFP-4 (200 µg) was digested with 8 µg of trypsin (13 000 international units mg⁻¹; Sigma Chemical Co.) in 80 µl of 50 mmol l⁻¹ Tris–HCl, pH 7.0 at 37 °C, for 24 h. The digestion mixture of peptides was separated using a Rainin reverse-phase C₁₈ HPLC column (4.6 mm × 100 mm) with a 0.1% trifluoroacetic acid/acetonitrile gradient of 5% to 30% in 40 min (Fig. 1). Peptides 3.20, 11.07, 14.75 and 22.52 were each fully reduced in 0.1 mol l⁻¹ sodium citrate, pH 3.0, with 15 mmol l⁻¹ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 60 °C for 20 min. The reduced peptides were separated again by RP-HPLC and sequenced on a Beckman LF 3000 protein sequencer.

Peptide 14.75 had a blocked N terminus which was deblocked by treatment with pyroglutamyl peptidase (Tsunasawa and Hirano, 1993) and then sequenced.

Mass spectrometry analysis

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were obtained on a PerSeptive Biosystems/Voyager-DE MALDI-TOF mass spectrometer. Matrix (α -cyano-4-hydroxycinnamic acid) was prepared as a saturated solution in 1:1 (v/v) water:acetonitrile with 0.1% trifluoroacetic acid (TFA). The sample was dissolved in double-distilled H₂O–TFA at pH 3.0. A 0.5 µl portion of sample solution was then mixed with an equal volume of the saturated matrix solution in a sample plate and allowed to air-dry before analysis. All samples were run in linear mode with 20 kV accelerating voltage, 18.6 kV secondary grid voltage and 15 V guide wire voltage.

Effects of pH on thermal hysteresis activity of DAFP-4

The thermal hysteresis activity of DAFP-4 was measured at pH values ranging from 1 to 12 by capillary freezing-melting point techniques (DeVries, 1986). Universal buffer was made from a mixture of 100 mmol⁻¹ sodium citrate, 100 mmol⁻¹ sodium phosphate and 100 mmol⁻¹ sodium borate, and the pH was adjusted by adding either acid or base. A sample of DAFP-4 (20.0 mg ml⁻¹) was added to a pH-adjusted buffer so that the final concentration of protein was 10.0 mg ml⁻¹ and the final concentration of each of the buffers was 50 mmol⁻¹. For pH 1, thermal hysteresis of DAFP-4 was measured in 100 mmol⁻¹ HCl and 150 mmol⁻¹ NaCl.

Effects of solute on thermal hysteresis activity of DAFP-4

The thermal hysteresis activity (DeVries, 1986) of DAFP-4 was measured in the presence of a number of low-molecular-mass solutes at varying concentrations, and these activities were compared with that of DAFP-4 without the solutes. The following solutes were tested (values in parentheses give the melting point of a 1 mol⁻¹ solution of the salt in the presence of the protein): citrate, trisodium salt, Sigma Chemical Co. (-3.40 °C); malate, disodium salt, Sigma (-3.35 °C); sodium acetate, Fisher Scientific (-3.80 °C); malonic acid, disodium salt, Sigma (-3.35 °C); proline, Sigma (-2.72 °C); glycine, Sigma (-2.43 °C); glutamic acid, monosodium salt, Sigma (-2.55 °C); aspartic acid, monosodium salt, Sigma (-3.12 °C); alanine, Sigma (-2.10 °C); sucrose, Fisher (-1.96 °C); sorbitol, Fisher (-1.82 °C); glycerol, Sigma (-1.85 °C); ethylene glycol, Fisher (-1.79 °C); sodium sulfate, Mallinckrodt Chemicals (-4.98 °C); sodium chloride, Fisher (-3.80 °C); trimethylamine hydrochloride, Sigma (-4.12 °C); trimethylamine *N*-oxide, Sigma (-3.20 °C); ammonium sulfate, Fisher (-4.00 °C); ammonium bicarbonate, Sigma (0.4 mmol⁻¹, -2.7 °C); ammonium chloride, Fisher (-4.3 °C); and succinic acid, disodium salt, Sigma (0.4 mol⁻¹, -2.35 °C). The melting points of these solutes in the presence of DAFP-4 are very similar to the expected values taken from the *Handbook of Chemistry and Physics*. All chemicals were of reagent grade or higher.

Thermal stability of DAFP-4

Thermal hysteresis activity of DAFP-4 was measured by capillary freezing-melting point techniques (DeVries, 1986) after incubation at different temperatures ranging from 25 to 100 °C for 5 min. A thermal unfolding curve was also generated by measuring the mean residue ellipticity at 222 nm on an Aviv model 60DS CD spectrometer at a protein concentration of 0.4 mg ml⁻¹ in 10 mmol⁻¹ sodium phosphate (pH 7.2) using a cell with a path length of 0.2 cm.

RNA isolation

D. canadensis larvae were collected from Warren Woods, in southwestern lower Michigan, in December 1995. A Polytron was used to homogenize 1 g of larvae (approximately 20 individuals in Tri-Reagent; Molecular Research Center, Inc.), and total RNA was extracted following the manufacturer's

instructions. Total RNA was resuspended in an appropriate volume of Formazol (Molecular Research Center, Inc.) and stored at -20 °C. Total RNA was enriched for poly(A)+ RNA by passing it twice over oligo(dT) columns (Promega).

cDNA library construction and screening

A cDNA library was generated from the December pool of poly(A)-enriched RNA with a ZAP Express cDNA synthesis kit (Stratagene), blunt-ended, ligated into a *Sma*I-cut pUC18 plasmid vector and transformed into *Escherichia coli* TOP10F'. The resulting library was screened with [³²P]dCTP-labelled *D. canadensis* antifreeze protein 1A (DAFP-1A) cDNA probe (radiolabelled by random priming using Klenow (BRL). The blot was incubated in a final wash of 2×SSC/0.5% SDS (1×SSC is 150 mmol⁻¹ NaCl, 15 mmol⁻¹ sodium citrate) at 55 °C and exposed to a DuPont NEF-496 film for 48 h at -70 °C. Hybridization conditions were chosen to encourage binding to heterologous sequences.

DNA sequencing and sequence analysis

Cloned DNA nucleotide sequences were determined using the dideoxy chain-termination method on an automated liquid fluorescence sequencer (Pharmacia ALF Express). Clones were sequenced in both the forward and reverse directions.

Sequence analysis and alignment were performed with Geneworks 2.2 (Intelligenetics).

Results*Sequence of DAFP-4*

The sequences of the peptide fragments resulting from the trypsin treatment of DAFP-4 (Fig. 1) are summarized in Table 1. Peptide 14.75 was the blocked N-terminal fragment, and sequencing was completed after deblocking with

Table 1. Peptide sequencing of DAFP-4

	Sequences	
Peptide 3.20	63	71
	TCTDSTGCP	
Peptide 11.07		
Peptide 3.12	55	60
	STNCYK	
Peptide 10.10	39	54
	TCTDSHDCHNAETCTR	
Peptide 14.75	1	25
	pQCTGGSDCQSCTVSTDCQNCNAR	
Peptide 22.52		
Peptide 3.16	55	60
	STNCYK	
Peptide 25.66	26	54
	TACTGSSNCINALTCTDSHDCHNAETCTR	

Separated peptides (Fig. 1) were reduced by TCEP and purified by RP-HPLC before sequencing (see Materials and methods). For peptide 11.07, two fragments (A and B) were obtained after reduction.

Fig. 2. The amino acid sequence of DAFP-4 and the complete sequence of the cDNA clone from which this sequence was derived. The sequence was confirmed by peptide sequencing (see Table 1). The amino-terminal glutamine (Q) residue at position 26 (actually pyroglutamine) is identified by an asterisk, while the residues prior to this constitute the signal peptide. Numbers identify the nucleic acid sequence of the cDNA and the amino acid residues of the mature DAFP-4. This sequence has been deposited in GenBank under accession number AF046862.

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ATGGTTGGGTTTGCAAAAGTTCGTTGTTAGTAATTAGTGTAGTTCTCATGTTTGTATGTCATGAGTGTATGGC 75
M V W V C K S S L L V I S V V L M F V C H E C Y G 25

CAATGTA CTGGAGGTTCCGATTGTCAATCGTGTACAGTATCTTGTACTGACTGCCAGAAGTCCCAAATGCACGT 150
*Q C T G G S D C Q S C T V S C T D C Q N C P N A R 50

ACAGCATGTACAGGCTCTTCAAAC TGCATTAACGCGTTAACCTGTACGGATTGCGATGATTGCCACAATGCCGAA 225
T A C T G S S N C I N A L T C T D S H D C H N A E 75

ACCTGTA CTAGATCAACCAATTGTTATAAAGCCAAGACCTGTACCGATTCTACGGATGTCCAtga 291
T C T R S T N C Y K A K T C T D S T G C P . 96

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pyroglutamyl peptidase. This peptide sequence matched the deduced amino acid sequence of one of the cDNA clones isolated (Fig. 2).

The nucleic acid sequence of this cDNA clone and its deduced amino acid sequence are shown in Fig. 2. As noted above, the sequence of the mature DAFP-4 was confirmed by peptide sequencing. The cDNA clone for DAFP-4 is 291 base pairs, with a presumed open reading frame of 291 base pairs that encodes 96 amino acid residues, the initial 25 of which comprise a signal peptide. The mature protein is 71 amino acid residues in length, and its sequence is very similar to those of previously sequenced *D. canadensis* AFPs (Duman *et al.* 1998), except that DAFP-4 is shorter by 13 residues (one repeat unit).

MALDI-TOF mass data shows that DAFP-4 has a relative molecular mass of 7324.95, which is identical to the relative molecular mass calculated on the basis of the above sequence, with the N terminus being pyroglutamine and all the cysteine residues being involved in disulfide bridges.

Fig. 3 compares the thermal hysteresis activity of DAFP-4 with that of a mixture of DAFP-1 and DAFP-2. At very high concentrations of AFP, DAFP-4 has slightly less activity than the mixture of DAFP-1 and DAFP-2. However, at physiological concentrations, their activities are essentially

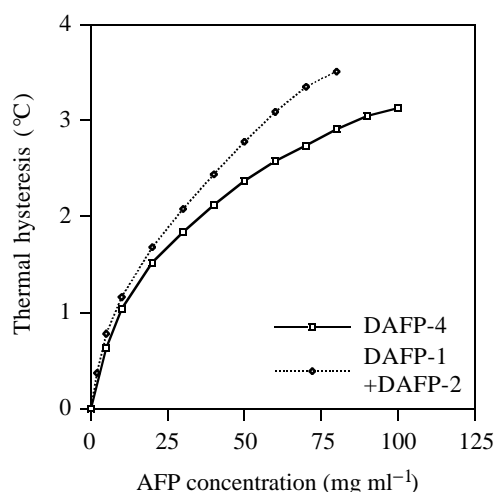


Fig. 3. Thermal hysteresis activity of DAFP-4 compared with that of a mixture of DAFP-1 and DAFP-2 at various protein concentrations.

identical (the concentrations of combined DAFPs in January hemolymph are approximately 22 mg ml⁻¹).

Effects of pH on thermal hysteresis activity

The influence of pH on the activity of DAFP-4 was studied to determine whether different ionization states of charged side chains affect the ability of the AFP to bind onto ice. It was found that thermal hysteresis activity was unchanged from pH 2 to pH 11 (results not shown). At pH 1, the thermal hysteresis activity was decreased by 25%, but this decrease could result from the presence of a low solute concentration since it was measured in HCl-adjusted H₂O. These data indicate that the protein folding is stable over a wide range of pH. They also indicate that the ionization states of charged amino acid side-chains (Arg, Lys, Asp, Glu) in the protein do not affect its ice-binding abilities.

Effects of solutes on thermal hysteresis activity

The effects of addition of various low-molecular-mass solutes on the thermal hysteresis activity of DAFP-4 are shown in Figs 4–7. In these studies, the activity of the DAFP-4 alone in aqueous solution with 50 mmol l⁻¹ Tris buffer was 1.0–1.3 °C.

Fig. 4 demonstrates the increases in thermal hysteresis activity resulting from the addition of various concentrations of the sodium salts of five organic acids (succinate, citrate, malate, malonate and acetate). Citrate had the largest enhancing effect of all the solutes tested in this study, increasing the activity of DAFP-4 from 1.3 °C in its absence to 6.8 °C at a citrate concentration of 1 mol l⁻¹. Succinate, at a concentration of 0.4 mol l⁻¹, also had a significant effect, increasing the activity nearly fourfold but, owing to solubility problems, succinate could not be tested at a higher concentration. Malate at concentrations of 1.0 and 1.5 mol l⁻¹ also increased the activity nearly fourfold, while malonate was slightly less effective. Acetate was more effective than malate or malonate at 0.5 mol l⁻¹, but at 1 mol l⁻¹ it was slightly less effective than malate and equal in effect to malonate. The high concentrations of the organic acids used in these studies are obviously non-physiological.

Fig. 5 shows the effects of addition of various concentrations of polyhydroxy alcohols and sucrose. In contrast to the organic acids depicted in Fig. 4, the polyols used in these studies can be found at high concentrations in the

Fig. 4. The effect of the addition of the sodium salts of various organic acids on the thermal hysteresis activity of DAFP-4. Values represent the means of a minimum of three thermal hysteresis measurements. Standard deviations ranged between 0.25 and 0.35 °C.

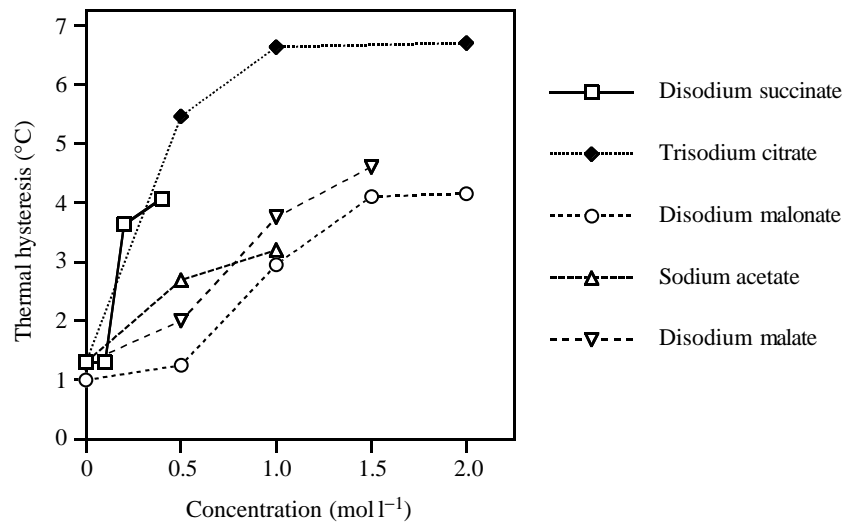
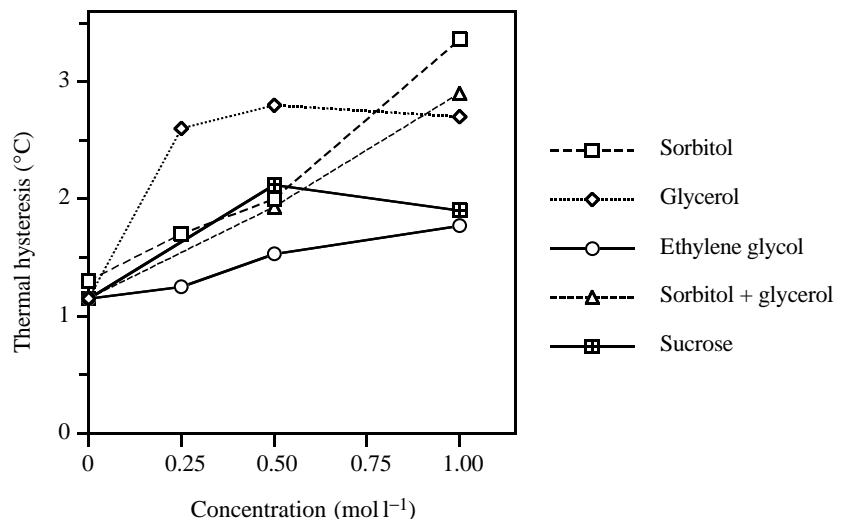


Fig. 5. The effect of the addition of various polyhydroxy alcohols and sucrose on the thermal hysteresis activity of DAFP-4. Values represent the means of a minimum of three thermal hysteresis measurements. Standard deviations ranged between 0.15 and 0.22 °C.



hemolymph of overwintering insects. *D. canadensis* concentrates both glycerol and, to a lesser extent, sorbitol in winter (Duman, 1979b). The relative enhancing capabilities of these solutes varied somewhat depending upon their concentration. At 0.25 and 0.50 mol l⁻¹, the enhancing capability was glycerol>sorbitol=sucrose>ethylene glycol. However, at 1 mol l⁻¹, it was sorbitol>glycerol>sucrose=ethylene glycol. It is interesting that a mixture of equal molar parts of 0.25 mol l⁻¹ glycerol and 0.25 mol l⁻¹ sorbitol was less effective than either 0.25 or 0.50 mol l⁻¹ glycerol alone and showed the same activity as 0.5 mol l⁻¹ sorbitol alone. However, the activity of 0.5 mol l⁻¹ glycerol and 0.5 mol l⁻¹ sorbitol combined was approximately equal to that of 1 mol l⁻¹ glycerol alone, but less than that of 1 mol l⁻¹ sorbitol alone. Note that none of these compounds was as effective as succinate or citrate (Fig. 4).

Fig. 6 demonstrates the enhancing effect of various amino acids on the thermal hysteresis activity of DAFP-4. At 0.25 mol l⁻¹, alanine had the greatest effect, but this effect appeared to

plateau at a thermal hysteresis activity of approximately 3 °C. In contrast, at 0.5 mol l⁻¹, aspartate was noticeably more effective than alanine or glutamate. Proline and glycine had little effect at any concentration. At 1 mol l⁻¹, glutamate was nearly as effective as aspartate. While free amino acids are concentrated in the hemolymph of some insects in winter, the higher concentrations used in these studies are probably non-physiological.

Fig. 7 shows the effects of various amine compounds, sodium chloride and sodium sulfate on the activity of DAFP-4. At 0.5 mol l⁻¹, the most effective compound was ammonium bicarbonate, which was not soluble at 1 mol l⁻¹ and therefore could not be tested at this concentration. At 1 mol l⁻¹, the effects of the amine compounds were approximately equal, except for ammonium chloride which was slightly less effective; however, ammonium sulfate (which is soluble at quite high concentrations) was quite effective at 2 and 3 mol l⁻¹. Ammonium chloride, like sodium chloride, had only a minor enhancing effect, thereby demonstrating that the

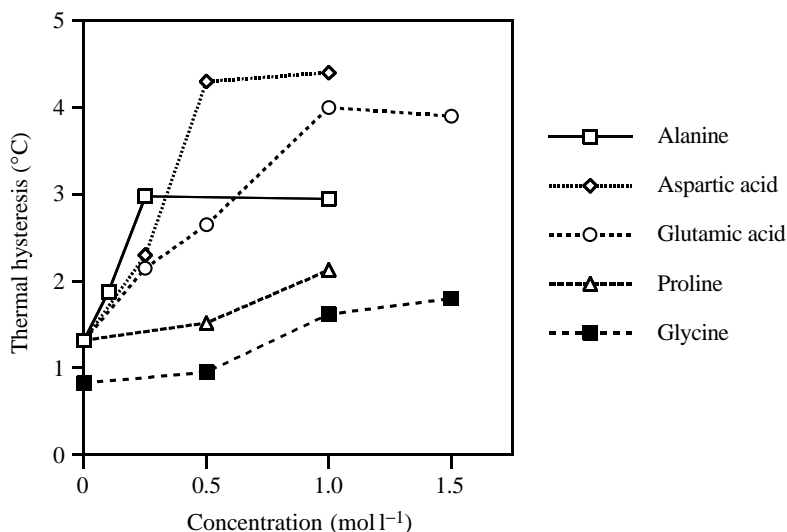


Fig. 6. The effect of the addition of various amino acids on the thermal hysteresis activity of DAFP-4. Values represent the means of a minimum of three thermal hysteresis measurements. Standard deviations ranged between 0.17 and 0.25 °C.

ammonium ion alone is not responsible for the positive effect of ammonium sulfate or ammonium bicarbonate. Interestingly, sodium sulfate was the only compound tested that inhibited the thermal hysteresis of DAFP-4, eliminating it entirely. Addition of a combination of 0.5 mol l⁻¹ sodium sulfate plus 0.5 mol l⁻¹ sodium citrate (which alone induces a considerable increase in DAFP-4 activity; see Fig. 4) resulted in a slight enhancement of DAFP-4 activity from 1.28 °C to 2.63 °C.

To determine whether DAFP-1 and DAFP-2 were affected by low-molecular-mass solutes in a fashion similar to that seen with DAFP-4, the effects of the addition of malonate (1 mol l⁻¹), succinate (0.5 mol l⁻¹) and ammonium sulfate (1 mol l⁻¹) were tested. These solutes, at the concentrations noted, enhanced thermal hysteresis activity of DAFP-1 and DAFP-2 to approximately the same extent as with DAFP-4 (Figs 4, 7) (results not shown).

Thermal stability of DAFP-4

The thermal hysteresis activity of DAFP-4 did not change

after incubation for 5 min at temperatures between 20 and 100 °C. The thermal denaturation curve (Fig. 8) shows that the unfolding temperature is approximately 84 °C, which is unusually high for proteins. Since the protein regains thermal hysteresis activity after thermal denaturation, the denaturation is obviously reversible. This remarkably high thermal stability of AFP-4 can be attributed to the seven disulfide bridges present in this protein.

Discussion

As shown in Fig. 9, the sequence of DAFP-4 is quite similar to those of DAFP-1 and DAFP-2, as previously described (Duman *et al.* 1998). The protein consists of 12- or 13-mer repeats, and every sixth residue is cysteine. It is likely that these cysteine residues are all disulfide-bridged, as is the case with DAFP-1 and DAFP-2 (Li *et al.* 1998). The unusually high thermal stability, as well as the readily reversible nature of thermal denaturation, probably results from the stability

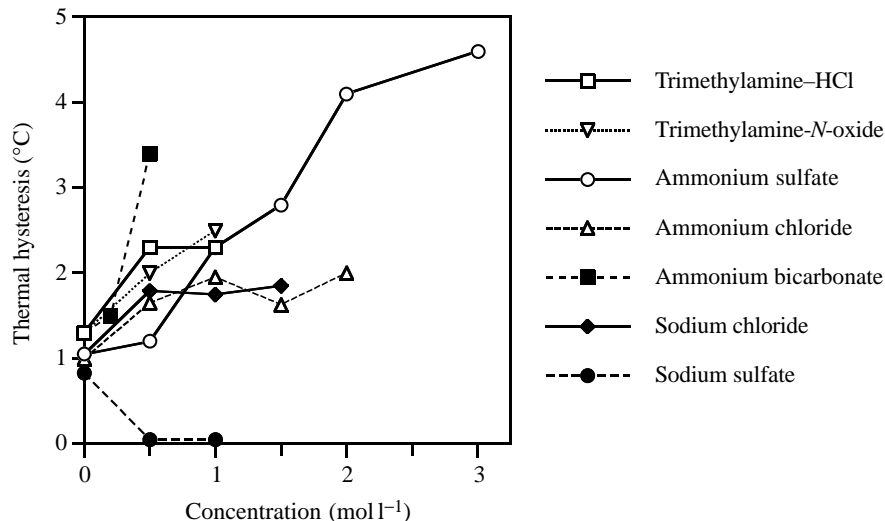


Fig. 7. The effect of the addition of various amine and sodium compounds on the activity of DAFP-4. Values represent the means of a minimum of three thermal hysteresis measurements. Standard deviations ranged between 0.19 and 0.25 °C.

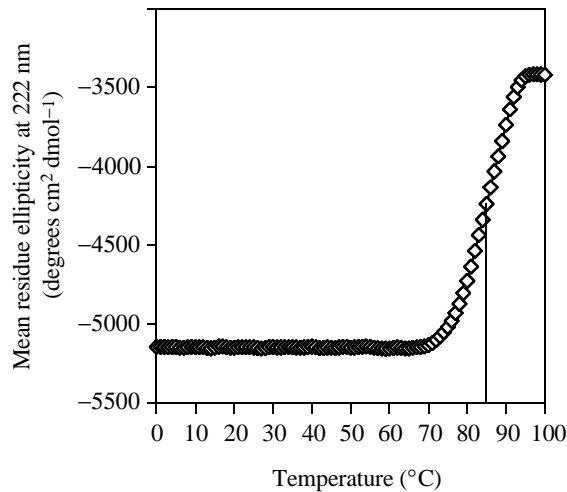


Fig. 8. The thermal unfolding curve of DAFP-4 (0.4 mg ml^{-1}) in 10 mmol l^{-1} sodium phosphate (pH 7.2). The melting temperature was 84°C .

Repeat		Position													
		1	2	3	4	5	6	7	8	9	10	11	12	13	
A	DAFP-1	pQ	C	T	G	G	S	D	C	R	S	C	T	V	S
	DAFP-2	pQ	C	T	G	G	S	D	C	R	S	C	T	V	S
	DAFP-4	pQ	C	T	G	G	S	D	C	Q	S	C	T	V	S
B	DAFP-1		C	T	D	C	Q	N	C	P	N	A	R	T	A
	DAFP-2		C	T	D	C	Q	N	C	P	N	A	R	T	A
	DAFP-4		C	T	D	C	Q	N	C	P	N	A	R	T	A
C	DAFP-1		C	T	R	S	S	N	C	I	N	A	L	T	-
	DAFP-2		C	T	R	S	S	N	C	N	N	A	L	T	-
	DAFP-4		C	T	G	S	S	N	C	I	N	A	L	T	-
D	DAFP-1		C	T	D	S	Y	D	C	H	N	A	E	T	-
	DAFP-2		C	T	D	S	Y	D	C	H	N	A	E	T	-
	DAFP-4		C	T	D	S	H	D	C	H	N	A	E	T	-
E	DAFP-1		C	T	R	S	T	N	C	Y	K	A	K	T	-
	DAFP-2		C	T	R	S	T	N	C	Y	K	A	K	T	-
	DAFP-4		C	T	R	S	T	N	C	Y	K	A	K	T	-
F	DAFP-1		C	T	G	S	T	N	C	Y	E	A	-	T	A
	DAFP-2		C	T	G	S	T	N	C	Y	E	A	T	T	A
	DAFP-4		-	-	-	-	-	-	-	-	-	-	-	-	-
G	DAFP-1		C	T	D	S	T	G	C	P					
	DAFP-2		C	T	D	S	T	G	C	P					
	DAFP-4		C	T	D	S	T	G	C	P					

Fig. 9. The sequence of DAFP-4 showing the 12- or 13-mer repeating units comprising the mature protein. The sequences of DAFP-1 and DAFP-2 (Duman *et al.* 1998) are shown for comparison. Note both the similarities between the three proteins and the conservation at certain positions between the various repeats. DAFP-4 differs from the other two DAFPs primarily in the deletion of repeat F.

provided by the seven disulfide bonds. The major difference between DAFP-4 and the previously described DAFPs is that DAFP-4 is 13 residues shorter than the others (i.e. repeat F is lacking). Otherwise, DAFP-4 differs at only three positions from DAFP-1 (Q for R at position 9; G for R at position 30, H for Y at position 44). Likewise, the thermal hysteresis

activities of DAFP-1, DAFP-2 and DAFP-4 are quite similar. Therefore, it was not surprising that the enhancement of DAFP-4 activity by certain low-molecular-mass solutes described in this study was also seen with DAFP-1 and DAFP-2. However, more recent sequencing studies on additional DAFPs have identified proteins with as little as 65% sequence homology to DAFP-1 (C. A. Andorfer and J. G. Duman, unpublished). It remains to be seen whether these DAFPs respond to thermal hysteresis enhancers in the manner described here for DAFP-4.

The 70 kDa endogenous activator (enhancer) protein, or other proteins known to enhance DAFP activity, functions by binding to the DAFPs (or *vice versa*), thereby increasing the surface of the potential seed ice crystal blocked by the DAFP and thus lowering the hysteretic freezing point (Wu and Duman, 1991). However, it is highly unlikely that a similar mechanism of action is operative with the low-molecular-mass enhancers described in the present study. There are no obvious trends in the efficacy of the various enhancers which might suggest a mechanism. Some of the enhancers (glycerol, ammonium sulfate, etc.) are well known for their abilities, at appropriate concentrations, to stabilize protein structure and therefore biological activity, often by preferential exclusion of the stabilizing solute from the surface of the protein, thus leading to preferential hydration of the protein surface (Timasheff, 1992). Although the seven disulfide bridges (eight in the other DAFPs) provide considerable stabilization to the structure of the DAFPs, it is possible that the enhancing solutes provide more subtle stabilization, thereby optimizing the higher-order structure of the AFPs for binding to ice. However, the mechanism(s) of the enhancement by low-molecular-mass solutes is unknown.

Citrate was the best enhancer identified in this study, increasing thermal hysteresis activity from 1.2°C in its absence to approximately 6.8°C (approximately sixfold). Succinate, malate, aspartate, glutamate and ammonium sulfate were also quite effective, increasing activity approximately fourfold. Glycerol, sorbitol, alanine and ammonium bicarbonate were less effective, but still increased activity by approximately threefold.

An earlier study found that certain low-molecular-mass solutes such as glycerol, sorbitol and inorganic ions, which are known to be present in the hemolymph in winter, failed to produce a large increase in activity when added to an aqueous solution of mixed *D. canadensis* AFPs (Duman *et al.* 1993). However, activity did increase slightly, but significantly (from approximately 0.80°C to approximately 1.35°C). In the earlier study, only 100 mmol l^{-1} sorbitol was tested. As shown in Fig. 5, this concentration is insufficient to produce a large enhancement. However, 500 mmol l^{-1} glycerol was tested in the earlier study, but activity was only increased from 0.80 to 1.32°C . As seen in Fig. 5, in this study, 0.5 mol l^{-1} glycerol raised the activity from 1.20 to 2.7°C . The reason for this difference is unknown. The results from the addition of inorganic ions (i.e. NaCl) were consistent between the two studies.

Do low-molecular-mass enhancers play a physiological role in enhancing the activity of DAFPs in overwintering *D. canadensis*? Among the enhancers identified in this study, only glycerol is likely to be present at sufficiently high concentrations (approximately 1.3–1.5 mmol l⁻¹) in *D. canadensis* to enhance activity (Duman, 1979b). However, the mean melting points of the hemolymph often approach -4.5 to -5.0 °C (2.4–2.7 osmol) in overwintering *D. canadensis*. Glycerol and sorbitol (40–100 mmol l⁻¹) are the only polyols concentrated in winter, and these only account for approximately 50 % of the solute required for such low melting points. Consequently, it is possible that other small solutes capable of enhancing thermal hysteresis activity of the DAFPs are present. Free amino acids are sometimes concentrated in the hemolymph of overwintering insects (up to a few hundred millimolar), but not at the levels required for maximal enhancement. The combined effect of normal (i.e. low) winter physiological concentrations of several different enhancers is as yet unknown and therefore whether the small enhancers, other than glycerol, have a physiological effect in *D. canadensis* has yet to be determined.

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