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Characterization of antifreeze protein gene expression in summer spruce budworm larvae

Wensheng Qin[1], Michael G. Tyshenko[1], Daniel Doucet, Virginia K. Walker*

Department of Biology, Queen's University, Kingston, Ont., Canada K7L 3N6

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Abstract

Not surprisingly, in the spruce budworm, *Choristoneura fumiferana*, antifreeze protein (AFP) gene expression is most abundant in the second instar, overwintering stage. However, low level RNA and protein expression was also found in the sixth instar larvae, a summer stage. In situ hybridization further confirmed the presence of AFP mRNA in sixth instar midgut tissues. Sequencing of cDNAs corresponding to "summer-expressed" transcripts revealed an isoform that was not apparent in a cDNA library made to second instar larvae. Although similar to AFP cDNAs obtained from overwintering larvae, this AFP-like isoform (CfAFP6) has two Cys substitutions. Since AFPs from this species fold into a β-helix that is stabilized by disulfide bonds, it was of interest to determine if this summer-expressed isoform had AFP activity. No thermal hysteresis activity was found when CfAFP6 was cloned and expressed in *E. coli,* even after in vitro denaturation and refolding. As well, there was no activity detected when the sequence of a known, active isoform was changed to mimic the Cys substitutions in CfAFP6. Since CfAFP6 does not appear to contribute to freeze resistance, its apparent absence in the overwintering second instar should not in itself be considered curious.

Keywords: Spruce budworm; Antifreeze protein; Thermal hysteresis; AFP-like isoform; Summer larvae

1. Introduction

Physiological and biochemical responses keep insect development in phase with seasonal changes, initiated as a result of environmental clues such as photoperiod, temperature and humidity (Tauber et al., 1986; Denlinger et al., 1991). During the winter, larvae of the spruce budworm, *Choristoneura fumiferana* (Cf), synthesize cryoprotectants including glycerol, sorbitol, trehalose and antifreeze proteins (AFPs) prior to and throughout the second instar diapause stage (Han and Bauce, 1993; Walker et al., 2001). AFPs depress the freezing point of the hemolymph non-colligatively. This depression results in a difference between the freezing and melting points, termed thermal hysteresis (TH). CfAFP is a hyperactive AFP compared to AFPs from some other organisms (Tyshenko et al., 1997; Doucet et al., 2000), with recombinant protein showing up to 4 °C TH activity (2 mg/ml). CfAFP and other cryoprotectants help ensure larval survival at temperatures that can drop to −30 °C in the boreal forest. As might be expected, due to the role of AFPs in overwintering, CfAFP transcripts accumulate to high levels prior to and during diapause but drop dramatically after molting to the third instar in the spring (Doucet et al., 2002).

Short and long CfAFP isoforms of 9 and 12 kDa, respectively, are encoded by a small gene family (~17 copies). Combined nuclear magnetic resonance, X-ray crystallographic structural analysis and molecular modeling (Graether et al., 2000; Leinala et al., 2002a, b) revealed that both short and long isoforms have a left-handed β-helix fold with Thr–X–Thr (where X can be any amino acid) ice-adsorption motifs, arranged along a flat face. This repetitive structure is stabilized by parallel β-sheets, a hydrophobic core and disulfide bonds (Leinala et al., 2002a) allowing the "Thr buttons" to match the ice crystal lattice. These proteins are folded and stabilized by disulfide bonds when they are secreted into the hemolymph.

*Corresponding author. Tel.: +1 613 5336123; fax: +1 613 5336617.
E-mail address: walkervk@biology.queensu.ca (V.K. Walker).
[1]These authors contributed equally to this work.

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Hyperactive but evolutionarily distinct AFPs have also been characterized in two species of beetles. The yellow meal worm beetle, *Tenebrio molitor* (Tm), produces high levels of AFP during larval quiescence (Graham et al., 2000). In the fire-colored beetle, *Dendroides canadensis*, there is also a seasonal increase in AFP abundance but it appears that not all isoforms are identically regulated (Andorfer and Duman, 2000). As a group, the developmental expression of CfAFPs appears not to be strictly regulated since low, but detectable, levels of certain isoform transcripts persist in post-diapause larvae. It is unclear why it would be beneficial for late instars to produce AFPs for freezing point depression since at this developmental stage in late June and July, field temperatures are close to their yearly maximum. This conundrum prompted us to investigate CfAFP gene expression in summer larvae.

2. Results

2.1. AFP transcript and isoform accumulation in sixth instar larvae

Northern analysis showed that first and second instar larvae accumulate high levels of AFP mRNA (Doucet et al., 2002), but after breaking diapause, AFP transcript abundance decreased significantly. Nevertheless, low levels of transcripts corresponding to various isoforms were still detectable in fourth, fifth and sixth instars. It was estimated from these blots that sixth instar larvae retain less than 0.3% of the transcript level seen in diapausing second instar larvae (not shown). The same low transcript levels were observed throughout the sixth instar stage (Fig. 1A), with the duration of this stage at 23 °C ranging from 5 to 7 days with a peak in the number of insects reaching pupation at day 6. AFP transcripts were not found at pupation or throughout the pupal stage.

To determine if transcript levels could be environmentally manipulated, 2-day old sixth instars were transferred to 4 °C (16L:8D) or 4 °C and constant darkness. These larvae survived, but developed at a much slower rate than did controls maintained at 23 °C and 16L:8D. Even after 10 days, these two experimental treatment regimes did not result in higher AFP transcript accumulation than was observed in control larvae (Fig. 1B). Larvae at 23 °C pupated 4 days after transfer, so no CfAFP mRNAs were seen, as expected, 5 and 10 days after the transfer, when the insects had pupated (Fig. 1B).

When larvae from a nondiapause strain were compared to the wild, obligate diapause strain, real time RT-PCR demonstrated the presence of AFP transcripts in both

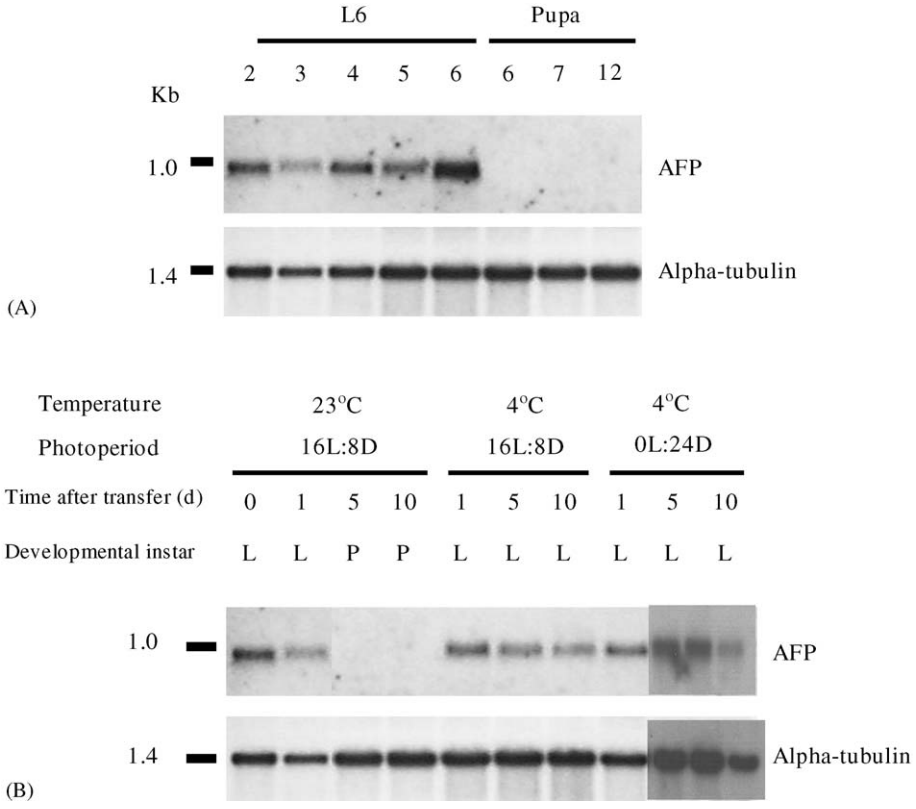

Fig. 1. Representative northern blots showing CfAFP transcripts in sixth instar larvae: (A) The accumulation of CfAFP10-like transcripts at 23 °C (16L:8D) during normal development after molting to sixth instar (L, day 2–6) and in pupae (P, day 6–12). (B) The effect of temperature and photoperiod on transcript accumulation. The transcript abundance was compared in larvae exposed to "summer" temperature and photoperiod (23 °C, 16L:8D), low temperature and "summer" photoperiod (4 °C, 16L:8D) and low temperature and continuous darkness (4 °C, 0L:24D) for 1, 5 and 10 days after transfer from "summer" photoperiod and temperature. Both (A) and (B) were reprobed with *Drosophila* α-tubulin as a RNA loading control. A composite is shown for (B) since another blot was used for the 4 °C, 0L:24D samples (the 0L:24D 1d L sample is present in duplicate, for comparison).

strains at both the sixth instar and second instar stage of development (not shown). However, CfAFP transcripts were consistently at lower levels in the sixth instar larvae than in second instars from both strains, and there was no significant difference between sixth instar larvae from either strain, including wild-type diapause larvae kept at 23 °C and the nondiapause, selected larvae kept at 4 °C.

The distribution of CfAFP transcripts in sixth instar larvae was examined in various tissues (fat body, gut, Malpighian tubules, integument and hemocytes) by northern blot analysis. Total RNA was isolated from individuals maintained under conditions of low temperature and constant darkness for 10 days and hybridized to the CfAFP probe. A 1 kb transcript was detected only in the gut (Fig. 2A) and PCR analysis showed this was in both the fore and midgut (Fig. 2B). In situ hybridization confirmed that the gut tissues expressed CfAFP transcripts (Fig. 2C).

2.2. AFP accumulation in sixth instars

Given the low level of AFP transcripts, it might be expected that there would be insufficient protein in sixth instar larvae for TH activity, and when dissected tissues were assessed, no significant change in freezing point depression (>25 mOs) was observed (not shown). To determine if low levels of AFPs were detectable in hemolymph and gut fluid with a more sensitive assay, western blot analysis was undertaken. There was strong cross-reactivity of anti-AFP antibody with two bands, representing short and long protein isoforms, in the collected gut contents from sixth instar (day 2) larvae (Fig. 2D). Gut contents from sixth instars obtained on days 3–6, also contained cross-reacting material, but the bands were faint. No cross-reactivity was observed from sixth instar hemolymph at any time point (Fig. 2D and data not shown).

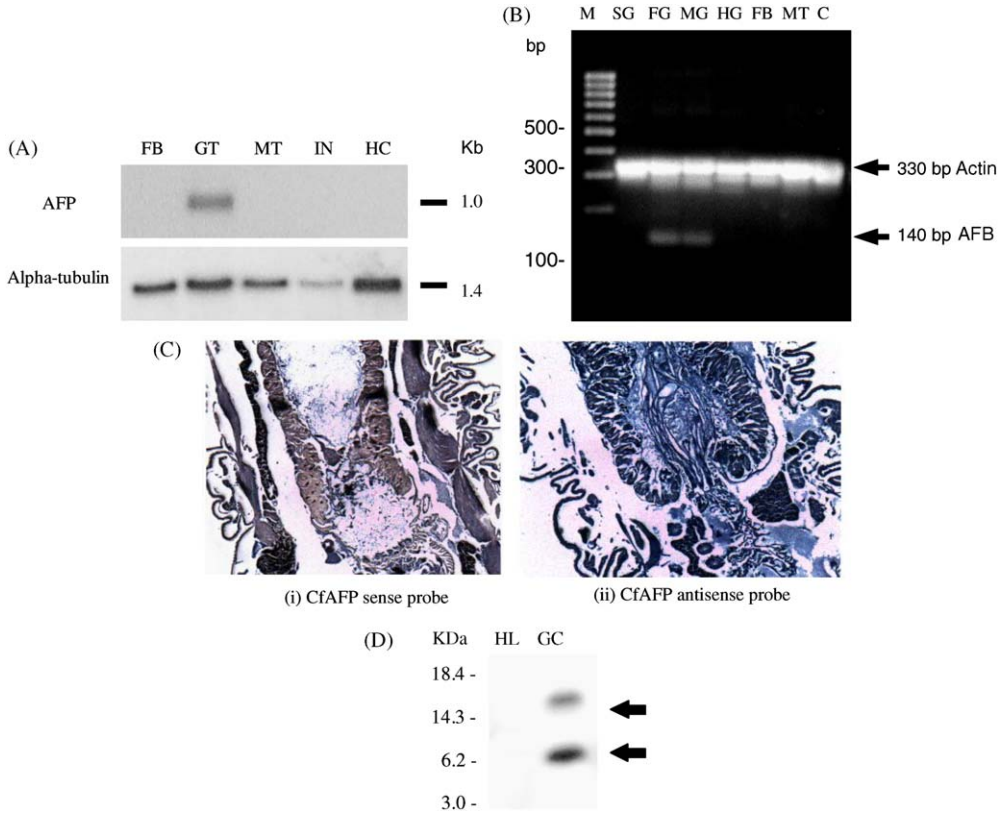

Fig. 2. Localization of CfAFP transcripts in sixth instar larvae. (A) Dissected tissues for northern analysis included fat body (FB), gut (GT), Malpighian tubules (MT), integument (IN) and hemocytes (HC) and these were hybridized to CfAFP10. *Drosophila* α-tubulin sequences were used as loading controls. (B) RT-PCR analysis of various tissues including those listed in above as well as salivary gland (SG), foregut (FG), midgut (MG) and hindgut (HG) along with a 100 bp size ladder (M). An amplified actin fragment was used as a control. (C) In situ hybridization of sixth instar whole larval sections, showing a longitudinal section through the fore and midgut with a CfAFP10 sense probe (i) and antisense probe (ii). The darkly colored cuticle, which surrounds the representative sections, shows no hybridization to either probe, and neither do the bands of muscles under the cuticle (more easily visualized in (i)). The gut dominates these sections with the foregut at the bottom of each. The cells of the midgut are stained darkly (dark blue in the slides) in (ii), representing hybridization to the antisense probe, whereas in (i) there is no hybridization shown as evidenced by the absence of staining (light brown colored in the slides) of the same region. (D) A representative western blot of sixth instar (day 2) gut contents and hemolymph with CfAFP antibody shows *C. fumiferana* hemolymph (HL), 10 μL, and gut contents (GC), 10 μL, separated on an 18.5% SDS-PAGE gel and transferred to PVDF membrane prior to chemiluminescence detection. Cross-reacting proteins corresponding to long (upper arrow) and short (lower arrow) isoforms are shown at the right. The position of protein size markers is shown to the left.

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CfAFP6          DGSCVNTNSQVSENSWCVRSTLTFRNIDNSQLEST-TCTRSQYNGAYVVSSRTTNYNIRN 59
CfAFP10         DGSCVNTNSQVSENSWCVRSTLTFCNIDNSQLEST-TCTRSQYNGAYVVSSRTTNCNIRN 59
CfAFP501RY      DGTCVNTNSQITANSQCVKSTATNRYIDNSQLVDTSICTRSQYSDANVKKSVTTDYNIDK 60
                **:*******:: ** **:** *   ****** .*  ******..* * .* **: ** :

CfAFP6          SQLHTTTCTNSQYDGVYITSSTTTSTRISGPACSISRCTITRGVAAPSAACRISGCTLRA 119
CfAFP10         SQLHTTTCTNSQYDGVYITSSTTTNTRISGPACSISRCTITRGVAAPSAACRISGCTLRA 119
CfAFP501RY      SQVYLTTCTGSQYNGIYIRSSTTTGTSISGPGCSISTCTITRGVATPAAACKISGCSLSA 120
                **:: ****.***:*:** *****.* ****.**** ********:*:***:****:* *

CfAFP6          N 120
CfAFP10         N 120
CfAFP501RY      M 121
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Fig. 3. Comparison of the amino acid sequence of the sixth instar-specific CfAFP6 isoform with those of CfAFP10 and the in vitro mutagenized CfAFP501 isoform, CfAFP501RY. Asterisk (*) indicates the consensus sequences and residues substituted in the wild type CfAFP501 are shown as shaded boxes.

2.3. A novel AFP-like protein

Cloning and sequencing of 30 cDNAs derived from sixth instar midgut RNAs revealed 2–3 copies of cDNAs representing specific isoforms including long isoforms CfAFP10, 18, 104 and 501, as well as short isoforms CfAFP337, 4, 2.7a and Lu-1 (not shown). Transcripts corresponding to other isoforms found in second instar lavae (CfAFP14, 17, 1, 333 and 339) were not recovered from sixth instars, suggesting either that only a subset of AFP genes are expressed in the summer period, or that the recovered isoforms were more abundant at this stage. Only one cDNA, independently isolated three times from sixth instars, was not recovered from second instars even after extensive sequencing of second instar cDNAs (Doucet et al., 2000), and thus it was named CfAFP6. Sequence analysis of both strands of all three cDNAs revealed identical sequence and showed that CfAFP6 was very similar to the long isoform, CfAFP10, with 97.5% amino acid identity (Fig. 3). Conserved Cys residues in long isoforms include the disulfide-bonded pairs Cys4 & 17, Cys25 & 37, Cys55 & 67, Cys92 & 115 and Cys97 & 110 (Leinala et al., 2002a; Gauthier et al., 1998). Two Cys residues, which presumably contribute to protein stability, were absent in the CfAFP6 isoform with substitutions of Arg and Tyr at positions 25 and 55, respectively (Fig. 3). In addition, a third amino acid change was found at CfAFP6 position 84 with a Ser substitution for Asn.

To more easily visualize the effects of the residue substitutions, the amino acid changes in CfAFP6 were substituted for those in the CfAFP10 isoform model (Doucet et al., 2000) with the unpaired Cys residues at 37 and 67 purposely forming a disulfide bridge. After minimization, the model (Fig. 4) showed that Thr at positions 7, 38, 53 and 68 in adjacent Thr–X–Thr motifs were pulled away from the center, as compared to CfAFP10, and it was difficult to assess if CfAFP6 would retain TH activity despite the loss of two Cys residues.

To determine if the CfAFP6 isoform had TH activity, the cDNA encoding this new isoform, as well as CfAFP10, the isoform with the closest amino acid identity (Fig. 3), were bacterially expressed. After purification and subsequent refolding, only a small proportion (~4%) of both proteins were soluble. However, from a large quantity of starting protein (5 mg) enough refolded, concentrated, soluble protein was recovered for TH activity measurements. TH activity from refolded CfAFP6 and CfAFP10 was quite different; CfAFP6 (1 mg/ml) had no TH activity while CfAFP10 (0.9 mg/ml) had an average of 230 mOs. Since recombinant CfAFP10 itself is more difficult to extract from inclusion bodies than more soluble AFP isoforms, CfAFP501 was used as a template to make a "new" isoform with the Cys substitutions found in CfAFP6. In vitro mutagenesis resulted in the recovery of such a sequence that encoded a protein, dubbed CfAFP501RY. After bacterial expression, both CfAFP501 and CfAFP501RY proteins were isolated from the inclusion bodies, solubilized and folded in vitro. Isoform CfAFP501 had TH activity (185 mOs at 1.2 mg/ml), but CfAFP501RY (1.2 mg/ml) had no detectable activity. As well, there was no ice recrystallization inhibition activity with CfAFP501RY, but CfAFP501 showed AFP activity in this assay. CfAFP501 and CfAFP501RY proteins were further purified by FPLC and HPLC. As has been previously observed (Gauthier et al., 1998), the elution profile of CfAFP501 showed two peaks, corresponding to active and non-active forms of the AFP, as assessed by TH assays. CfAFP501RY had a broader elution profile with no peaks corresponding to the active form (not shown). Similarly, when the two column-purified proteins were again tested for AFP activity, the CfAFP501 protein inhibited ice recrystallization, but the CfAFP501RY did not.

3. Discussion

It is curious that although high levels of AFP transcripts accumulate prior to, and during the overwintering stage of spruce budworms, AFP mRNAs are also present in sixth instar larvae. This stage corresponds to a period of high summer temperatures in the boreal forest, with presumably little need to depress the freezing point of hemolymph, and

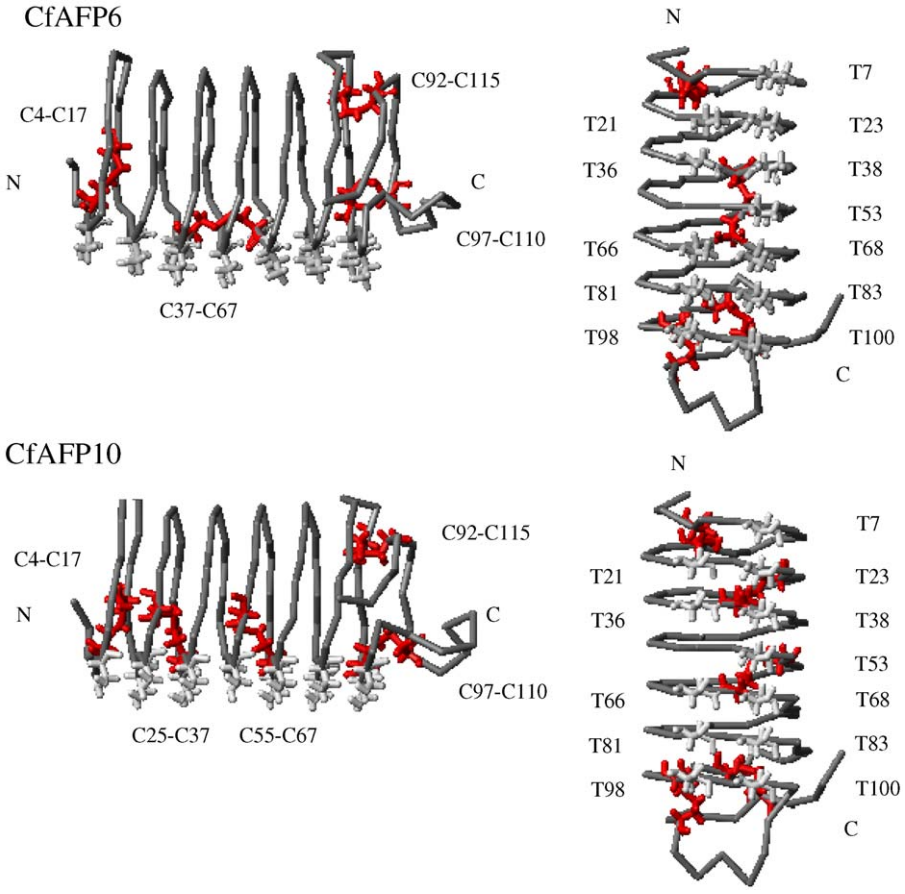

Fig. 4. Theoretical model of CfAFP6 in comparison with CfAFP10 showing minimization of a potential, alternate disulfide bond pattern for CfAFP6. Side view with Cys disulfide pairs (red) and front view with Thr (light gray) are shown.

indeed, no TH activity was found at this stage. In an effort to characterize this expression, summer larvae were examined for the presence of AFP transcripts and proteins using northern and western blots, and by in situ hybridization (Figs. 1–3). In sixth instar budworms, AFP transcripts were observed and localized to the fore and midguts. Similarly, the proteins were found in the gut lumen, with no AFPs detected in the hemolymph. As well, no AFP transcripts were found at pupation or in the pupal stage, a time when the Lepidopteran midgut undergoes histolysis (Chapman, 1985).

AFP expression is better known in the *D. canadensis* and *T. molitor* beetles that overwinter at various larval stages. In beetles, transcripts are present at low levels in younger larval instars, increase during the last instar, and can be induced to accumulate at even higher levels (Graham et al., 2000; Andorfer and Duman, 2000; Duman, 1982; Horwath and Duman, 1983). In contrast to these insects, AFP transcript levels in spruce budworm were not changed by transfer of the last instar larvae to low temperatures or constant darkness (Fig. 1). As well, transcripts that had hitherto been found in all tissues of second instar larvae (unpublished observations) were only detected in the sixth instar midgut. Again in beetles, the fat body, the site of most hemolymph-borne proteins (Keeley, 1985), is also the tissue that accumulates AFP mRNAs (Duman et al., 1991). However, autumn-collected *D. canadensis* larvae have AFPs in both hemolymph and midgut fluids (Duman, 1984), suggesting to some workers that gut AFPs could prevent inoculative freezing from remaining food particles or other ice nucleators. This function may not be applicable in summer spruce budworm larvae since records show no temperatures below 0 °C in the boreal forest adjacent to the collection site for the last 45 years (www.climate.weatheroffice.ec.gc.ca).

The majority of the transcripts expressed in summer *C. fumiferana* larvae are the same as those found in winter larvae. Indeed, RT-PCR and subsequent cloning and sequencing of cDNAs, derived from sixth instar mRNA, confirmed the presence of transcripts for both long and short isoforms known to be expressed in second instar larvae. However, it also revealed another AFP-like isoform, CfAFP6, cloned from mRNA isolated from gut tissues, which was not recovered from the second instar cDNA library. When CfAFP6 was conceptually translated, it showed 97.5% amino acid identity to a previously cloned long isoform, CfAFP10, but with only eight of the 10 Cys residues. Thus if the two Cys residues that form disulfide bonds with other partners, formed bonds with each other, this isoform would have four of the five disulfide bonds

found in other long isoforms. Coincidentially, four disulfide bondages are found in the small isoforms, and these bonds are crucial for the proper folding of CfAFP (Gauthier et al., 1998).

Molecular modeling of CfAFP6 (Fig. 4), based on the model of CfAFP10, suggested that if the unpaired Cys25 paired with unpartnered Cys55, then the right register of "Thr buttons" in the repetitive Thr-X-Thr ice-adsorption motif would be slightly disrupted. It is these Thr resides that we had previously suggested could be crucially important for the initial contact with ice (Doucet et al., 2000). If the modeled pairing occurs, it is apparently not tolerated in the AFP helix structure of CfAFP6, as reflected by the lack of recoverable TH activity when compared to CfAFP10. Since recombinant CfAFP10 is a relatively insoluble isoform, in vitro mutagenesis of the more easily folded recombinant isoform, CfAFP501, was used to confirm that the lack of TH activity was due to the substitution of two Cys residues. There was no evidence of active protein after chromatography with either TH or ice recrystallization inhibition assays, indicating that AFP activity as assessed by these criteria depend on the appropriate disulfide bond formation.

Despite this finding, the long isoforms with their additional 30-residue insertion appear to be more tolerant of Cys substitutions compared to the short isoforms. The individual Cys residues that are substituted in CfAFP6 are also altered in CfAFP17 (with a Ser rather than a Cys at position 25) and in an AFP isoform from *C. conflictana* (the large aspen tortrix moth), CcAFP5 (with a Ser, rather than a Cys at position 55) (Tyshenko et al., 2005). It is possible that these two long isoforms, as well as other CfAFPs in sixth instar larvae have TH activity, but this work shows that the substitution of two Cys residues in the AFP-like protein, CfAFP6, can seriously disrupt ice-binding activity. In *D. canadensis,* there are seasonal increases in AFP transcripts and proteins, but there is a high expression of one isoform (Dafp-7), in summer-collected insects (Andorfer and Duman, 2000; Duman et al., 1982). It has been suggested that this isoform may be important, not for AFP activity, but for survival under conditions of low humidity. In this regard it has been shown that AFPs or antifreeze glycoproteins from polar fish protect various lipid membrane components during cellular dehydration (Rubinsky et al., 1990; Tomczak et al., 2001), protect ion transport (Negulescu et al., 1992), and offer hypothermic protection at low (but not freezing) temperatures (Wu et al., 2001), making this suggestion plausible. Alternatively, it has been postulated (Griffith and Yaish, 2004) that certain AFPs, similar to plant pathogenesis-related proteins, could provide protection against pathogens. We have tested this hypothesis by examining possible growth inhibition of several Gram positive or negative bacteria by CfAFP501 and CfAFP501RY. No inhibition was observed (not shown).

In summary, this study has demonstrated that spruce budworms express multiple AFP isoforms, not only in overwintering early instar larvae, but also in late instar, summer larvae. However, both the transcript and protein diversity appears somewhat more restrictive and overall, the transcript levels are considerably lower in these later stages. It is possible that others (Rubinsky et al., 1990; Tomczak et al., 2001) could be correct in that summer-expressed isoforms have another purpose in the fore and midgut, rather than TH activity, but the elucidation of this function awaits further study. Certainly it appears clear that an AFP-like protein, CfAFP6, which has not been found in overwintering insects, would not able to lower the freezing point of the gut contents in summer larvae.

4. Materials and methods

4.1. Insect rearing and sampling

C. fumiferana were purchased from the Canadian Forest Service (Sault Ste-Marie, Ontario). After breaking diapause, larvae were kept at 23 °C under a 16L:8D photoperiod and fed an artificial diet (McMorran, 1965). Sixth instars were sacrificed from days 2 to 6 and pupae at days 6, 7 and 12. For some experiments, larvae were subjected to different temperature and photoperiod regimes 2 days after the molt. A control group was allowed to complete development at the initial temperature and photoperiod (23 °C, 16L:8D). To determine if temperature affected expression, some larvae were transferred to 4 °C (16L:8D), while others were used to determine if temperature and photoperiod affected expression and were transferred to 4 °C and 0L:24D (temperature and light change). Insects from each regime were sacrificed 1, 5 and 10 days after transfer. Sixth instar larvae from a selected "nondiapause strain" were kept at 4 °C for 5 days in 0L:24D. All insects were frozen in liquid nitrogen and stored at −80 °C before RNA isolation.

4.2. Tissue dissection

Larvae were dissected in cold Pringle's saline (9 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 4 g glucose, per liter) (Pringle, 1938) for tissue collection. Fat body and Malpighian tubules were removed with forceps. Fat body and tracheal remains were removed from the rest of the carcass that was composed primarily of the epidermis and attached muscles; this was designated the integument. Tissues were washed briefly in saline and stored in liquid nitrogen. Hemocytes were pelleted from collected hemolymph at 1000*g* (10 min) in a refrigerated benchtop centrifuge with a crystal of phenylthiourea (PTU) added to each tube to inhibit phenoloxidase activity, before storage in liquid nitrogen.

4.3. RNA isolation, transcript analysis and real time PCR

RNAs from larval instars and tissues were isolated using Trizol reagent (Gibco-BRL, Mississauga, Canada). Midgut RNAs from sixth instar larvae (20–30) were used to make

first strand cDNAs and subsequently cloned and sequenced as previously described (Doucet et al., 2000). For northern analysis, 10 μg total RNA was loaded onto a 1.2% agarose-formaldehyde gel, electrophoresed and transferred to Hybond N nylon membrane (Amersham-Pharmacia, Baie d'Urfe, Canada). A pool of RNA, isolated from a minimum of 10 individuals was used for each time point. Probes specific for the cDNA CfAFP10 isoform (GenBank accession number AY004172; (Doucet et al., 2000)) were synthesized by PCR, using a consensus AFP forward primer (Saup F): 5′-GCTCGTGTAIAAACACGAACTC and reverse primer (Saup R): 5′-TGGAAATITTGCATC CAGGGCC. Northern blots were hybridized at 42 °C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100–150 μg/mL of sheared salmon sperm DNA (Gibco-BRL) for a minimum of 16 h (Sambrook et al., 1989). Blots were re-probed with a *Drosophila melanogaster* α-tubulin fragment to control for RNA loading in each lane (Genbank accession number M14643). High stringency washes were done in 0.1X SSC, 0.5% SDS at 65 °C for a minimum of 20 min. After washing, blots were exposed to Biomax MS film (Eastman Kodak, Rochester, USA) with intensifying screens at −80 °C for 4 days prior to developing. Densitometry was carried out using ImageQuant software v5.2 (Amersham-Pharmacia), using volume calculation by pixel density summation with local average background correction on scanned northern autoradiographs. Total RNA loadings were corrected by using alpha-tubulin hybridizing bands to standardize densitometry values.

For real time PCR analysis, RNAs were isolated as previously described and re-purified with the RNeasy mini protocol (Qiagen, Mississauga, Canada). Degenerate AFP primers (AFPm1: 5′-ATA ASG WCT TCA ACA WCT ACY RRT WC and AFPm2: 5′-TYA RTY TMR GAC GAC TTT CAT GSC CT; S = G + C, W = A + T, Y = C + T, R = A + G, M = A + C), with or without QuantiTect RT mix (Qiagen) were used for amplification. Actin primers (CfActin1: 5′-CTT CTA CAA TGA GCT GCG TGT GGC and CfActin2: 5′-CTC GAA CAT GAT CTG TGT CAT CTT C) were used to amplify the *C. fumiferana* actin sequence, as a control. Serial dilutions of all templates were made and 5, 10, 25, 50, 100, or 250 ng were used in each 25 μl reaction with each set of primers for 50 °C 30 min; 95 °C 15 min; 94 °C 15 s, 50 °C 30 s, 72 °C 30 s, 79 °C 15 s, 45 cycles. Amplification cycles were monitored and transcript quantification was done according to the manufacturer's protocol (Cepheid, Sunnyvale, CA, USA).

4.4. Transcript in situ hybridization

Hybridization probes were designed to detect many CfAFP isoform transcripts (including CfAFP10 and 337). Cf10NotI and Cf10SalI primers (5′-ATA AGA ATG CGG CCG CGT GAC GTC ACA CCG TGA G; 5′-ACG CGT CGA CAG AGT ATC AGA AAA TAT TGC AC) were used to amplify a 148 bp fragment from CfAFP10 cDNA. The reaction was performed at 94 °C 5 min; 94 °C 1 min, 55 °C 40 s, 72 °C 45 s, 30 cycles; 72 °C 10 min. The amplification products were digested with NotI and SalI, cloned into pBluescript SK II, and designated Cf10NS. The sense riboprobe was transcribed from SalI-linearized Cf10NS plasmid DNA using T3 RNA ploymerase and the anti-sense probe was transcribed from NotI-linearized Cf10NS using T7 RNA polymerase (DIG-RNA labeling kit; Roche, Laval, Canada). Sixth instar larvae were fixed in formalin and embedded in paraffin and 10 μm tissue sections were made. Sections were hybridized with the sense and antisense probes, following the manufacturer's protocol. Briefly, after treating the slides with xylene, 4% paraformaldehyde–PBS and equilibrating in 5x SSC, the slides were prehybridized (2 h) at 58 °C in hybridization buffer (50% formamide, 5x SSC, 40 μg salmon sperm DNA). The slides were hybridized overnight at 58 °C in hybridization buffer with either the sense or antisense riboprobes (2 ng/μl). They were then washed 30 min in 2x SSC at room temperature, 60 min in 2x SSC, and at increasingly high stringency to 60 min in 0.1x SSC, 65 °C. After blocking, an anti-digoxingenin-AP (Fab fragments) antibody was diluted to 1:250 in 1x blocking buffer (Roche) and incubated with the slides for 2 h at room temperature. Subsequently they were washed and stained with 200 μl 50x diluted NBT/BCIP stock solution overnight and then washed according to standard protocols and mounted in AquaPerm Mounting medium (Immunon™ Thermoshandon, Pittsburgh, USA). The slides were examined at a magnification of (4× or 10×) using Olympus BX51 camera system.

4.5. cDNA cloning, in vitro mutagenesis and expression

To isolate isoforms expressed in dissected foregut tissue, total RNA from 20 to 30 larvae was used for reverse transcription PCR (RT-PCR) and performed using an oligo(dT)CSX primer to generate first strand cDNA (at 50 °C for 60 min). Full-length AFP was amplified using the conserved AFP forward primer to the signal sequence (AFPsignalXho): 5′-GGCTCGAGATGAAGTGTTTAA TGCTGATCATG and the reverse primer (OligodTCSX): 5′-GACTCGAGTCGACATCGA$(T)_{17}$ directly following amplification: 94 °C for 5 min, then 94 °C for 1 min, 64 °C 1 min, 68 °C 1 min for 32 cycles with a final extension at 68 °C for 5 min.

Full length fragments (~1.0 kb) containing sequences corresponding to selected isoforms were directionally subcloned in pET 102 and transformed into TOP10 *E.* coli (Invitrogen, Burlington, Canada) by reamplification using a pET102-Topo AFP specific forward primer: 5′-ACCATGGATGGCTCGTGTGTAAACACG and reverse primer: 5′-TTAATTAGCACTCAATGAACATCC to obtain the mature coding region. After sequencing, plasmid corresponding to AFP isoform CfAFP6 was transformed into host cells (Origami-DE3; Novagen, San Diego, USA) for expression. Isoform CfAFP10 (GenBank

accession number AY004172; (Doucet et al., 2000)) was subcloned into the pET 102 analogously to create a thioredoxin–AFP fusion construct for western blots.

In vitro mutagenesis experiments used CfAFP501 cDNA (accession number AY004173) template, cloned to pET-20b (Novagen) using HindIII/NdeI. Nucleotide sequences corresponding to two Cys residues at position 62 and 83 were changed to Arg and Tyr by in vitro mutagenesis (Quick Change Site-directed Mutagenesis kit, Stratagene, La Jolla, USA). Two sets of primers (C62Rforward, 5′-TCGAC GGCGACCAACCGCTACATCGATAACA and C62Rreverse, 5′-TGTTATCGATGTAGCGGTTGGTCGCCGTC GA; C83Yforward, 5′-CAGTAACGACCGACTACAA CATCGATAAAAG and C83Yreverse, 5′-CTTTTATC GATGTTGTAGTCGGTCGTTACTG) were used in the PCR reactions. Mutations were confirmed by double-strand sequencing and the construct was designated CfAFP501RY.

Both CfAFP isoform 501 and the synthesized mutant 501RY were expressed in *E. coli* BL21(DE3) cells after induction with 1 mM IPTG and proteins were refolded in a manner similar to that for CfAFP337 (Gauthier et al., 1998). Recombinant proteins were subsequently purified using FPLC and HPLC column chromatography as reported in the same study.

4.6. AFP activity measurements

TH activity was assessed in whole or dissected larvae as well as for recombinant proteins using a nanoliter osmometer as previously described (Chakrabartty and Hew, 1991). An ice recrystallization inhibition assay (Tomczak et al., 2003), which can be used to indicate AFP activity, was also used for recombinant AFPs.

4.7. Western blot analysis

Gut contents and hemolymph were collected from individual sixth instar larva (day 2) and homogenized in an equal volume of 1X PBS, 2 mM phenylmethylsulphonylfluoride (PMSF), 1 mM PTU. Samples were centrifuged for 5 min at 13,000*g* prior to loading on 18.0% SDS-PAGE gels (Sambrook et al., 1989). The gels were electroblotted onto Immobilon PVDF membranes at 30 V for 3 h. Membranes were blocked in 5% skim milk buffer for 8 h and incubated with rabbit anti-CfAFP antiserum for 2.5 h, washed in 1X PBS, 0.05% Tween-20 and incubated for 60 min with goat anti-rabbit IgG antibody–horseradish peroxidase conjugate (Pierce Chemical, Rockford, IL, USA) and then washed to remove excess unbound antibody. The membrane was incubated for 2 min in Enhanced Chemiluminescence reagent (Amersham-Pharmacia), blotted dry and exposed to Kodak Biomax MS film for 10–120 s.

4.8. Molecular modeling of CfAFP6 and CfAFP10

Molecular modeling was performed using a Silicon Graphics 02 workstation running SYBYL software (version 6.8, Tripos, St. Louis, USA). Previously modeled isoforms (Doucet et al., 2000) were used for the template by manually substituting residues and minimized using Gasteiger–Marsili energy parameters.

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