

## CRYSTALLIZATION NOTE

## Crystallization and Preliminary X-Ray Crystallographic Analysis of Spruce Budworm Antifreeze Protein

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**Antifreeze proteins have the ability to bind to ice with high affinity and inhibit further crystal growth. The insect antifreeze protein from spruce budworm exhibits very high thermal hysteresis activity and is implicated in the protection of overwintering larvae from freezing. This protein has been crystallized in 20–25% polyethylene glycol ( $M_r$  6000), 0.4 M NaCl, 0.1 M Tris-HCl, pH 8.5, by vapor diffusion using the hanging drop method. The resulting crystals are very thin (typically  $<0.01$  mm in the shortest dimension), and only after repeated seeding could crystals be grown large enough for data collection using synchrotron radiation. The crystals belong to the monoclinic space group  $C2$ , with cell dimensions  $a = 82.28$  Å,  $b = 62.29$  Å,  $c = 63.63$  Å, and  $\beta = 113.7^\circ$ . Molecules in the asymmetric unit are related by a twofold axis of symmetry with two molecules present. Native data to a resolution of 2.6 Å have been collected with 90.3% completeness and a  $R_{\text{sym}}$  of 6.9%. © 1999 Academic Press**

**Key Words:** spruce budworm; antifreeze protein; adsorption inhibition; crystallization; thermal hysteresis.

Organisms have evolved various strategies to survive in cold environments. For example, spruce budworm (sbw) overwinter as early stage (second instar) larvae in the tips of tree branches where the temperatures reach  $-30^\circ\text{C}$  or lower (Sanders, 1991), and yet they resist freezing. During the winter there is an increase in glycerol concentration in the hemolymph (Han and Bause, 1993) and the production of antifreeze proteins (AFPs), which are also known as thermal hysteresis proteins (THPs), that inhibit the growth of ice crystals (for a review, see Hew and Yang, 1992). Increased levels of glycerol decrease

both the freezing and the melting points in a concentration-dependent (colligative) manner, but AFPs lower the freezing point below the melting point in a noncolligative way. The difference between the temperatures of freezing and melting points is termed thermal hysteresis (TH) and is used as an indicator of AFP activity. AFPs act by adsorbing to the ice surface, causing it to grow with a submicroscopically curved surface (Raymond and DeVries, 1977; Knight *et al.*, 1991). Addition of water to the ice lattice becomes energetically unfavorable and further ice growth is prevented.

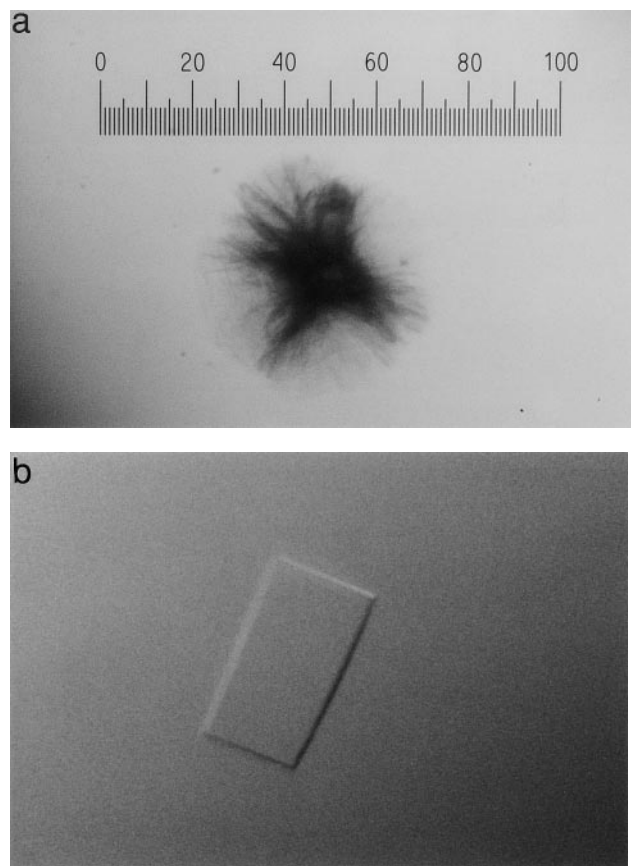
The maximum TH activity of the well-characterized fish AFPs is approximately  $1.5^\circ\text{C}$  at 3 mM. In contrast, the TH activity of hemolymph and crude extracts from spruce budworm larvae is  $\sim 5^\circ\text{C}$ , demonstrating that this antifreeze potentially offers superior protection to the organism. Similarly, the recombinant protein showed exceptionally high TH activity (Tyshenko *et al.*, 1997). Biochemical analysis has shown that this 9.1-kDa protein is a monomer. It commonly has been thought that the interactions between polar side chains and ice are most important for ice binding by AFPs. However, other interactions may also have a role as has been shown by more recent studies where the substitution of threonine residues by valine in the Type I AFP resulted in a relatively small loss of activity, while a "conservative" change to serine caused a large loss in TH activity (Chao *et al.*, 1997; Haymet *et al.*, 1998). Indeed, given that a third of the 91 residues in spruce budworm antifreeze protein (sbwAFP) are threonine or serine, it would be very difficult to envisage that these polar residues would not play a dominant role in protein-ice binding. A tertiary structure in conjunction with modeling of the protein to ice would help reveal the structural basis for sbwAFP binding to ice and explain its high activity compared to fish AFPs. The primary sequence of

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sbwAFP has no apparent homology to any other protein. Because of this, it is expected that the tertiary structure of sbwAFP would not resemble any of the known fish AFP structures (Davies and Sykes, 1997). Comparison to the first globular AFP crystal structure, type III AFP (Jia *et al.*, 1996; Yang *et al.*, 1998), may reveal common structural features of globular antifreeze proteins.

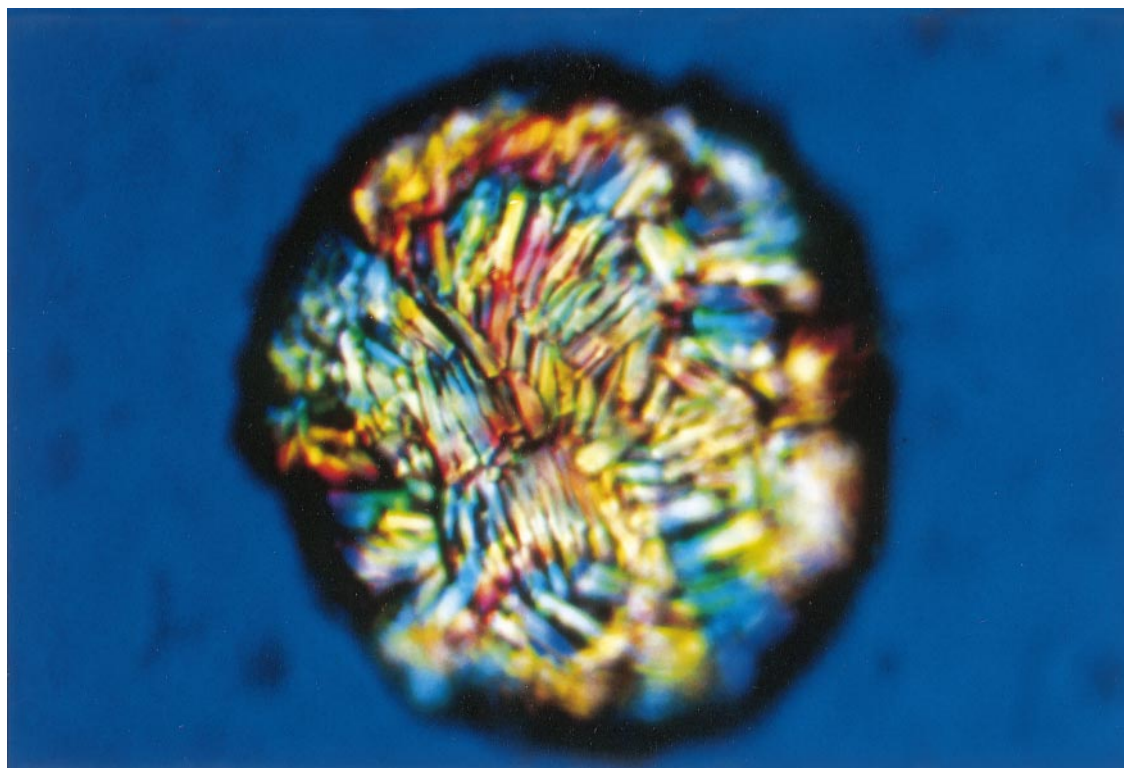
Recombinant sbwAFP was purified as previously described (Gauthier *et al.*, 1998). Sparse matrix screening was carried out using the hanging drop method at room temperature (Jancarik and Kim, 1991). Several conditions resulted in small crystals in the presence of medium to heavy precipitate. After routine optimization, the best condition for obtaining crystals was determined to be 100 mM Tris-HCl, pH 8.5, 20–25% polyethylene glycol ( $M_r$  6000), 400 mM NaCl using 2  $\mu$ L of 10 mg/mL protein and 2  $\mu$ L of crystallization solution. Despite exhaustive screening of this condition at various temperatures (0–37°C), with various additives (Hampton additives kit 1 and 2, several polyalcohol compounds and several chloride salts), and by several different crystallization methods (including hanging drop, batch method, gel method, dialysis method, and sitting drop), no apparent improvements were seen in the very small, hair-like, multicrystals (Fig. 1a). In order to obtain crystals suitable for diffraction, several rounds of seeding were performed. Using a fine hair, a multicrystal was touched and the hair was subsequently allowed to touch a fresh drop of protein and crystallization solution. The solution was identical to that in the source drop, but the protein concentration was decreased to a concentration of 2.5 mg/mL in the drop. Often, needle-like crystal clusters formed at this stage. Individual needles that appeared to be a single crystal were broken off of the cluster and washed sequentially in crystallization buffer containing 2, 4, and 6% less polyethylene glycol ( $M_r$  6000) than in the original crystallization solution, which may remove surface impurities and allow new growth. Generally, the crystals at this stage were about  $0.1 \times 0.1 \times <0.01$  mm in size. They were then placed in another fresh drop, which allowed the crystal to grow somewhat in the long dimensions but not in the thin dimension. This was repeated from one to four times; however, in approximately 95% of the trials the seed would become an unusable multicrystal. Eventually, some seeds grew to a size of about  $0.2 \times 0.15 \times <0.02$  mm (Fig. 1b) and were just large enough for data collection using synchrotron radiation.

A native data set was collected at the A1 station of the Cornell High Energy Synchrotron Source. For cryo-protection, 25% glycerol was added to the crystallization buffer. A total 100° scan was collected



**FIG. 1.** Crystals of sbwAFP from the first condition. (a) Very small, hair-like multicrystals from initial crystallization. The ruler bar represents 1 mm length. (b) Crystal obtained after repeated seeding (approximately  $0.21 \times 0.12 \times 0.02$  mm).

with a 1° oscillation and a 20-s exposure per frame. During data collection, some reflections appeared distorted in the horizontal direction when the X-ray beam passed through the very thin sections of the crystal. However, in other sections the diffraction spots were of much better quality and could be easily resolved. This anisotropic diffraction pattern is not unusual for very thin crystals, presumably due to bending or distortion of the crystal lattice. The integration box parameters in the DENZO software package (Otwinowski and Minor, 1997) were adjusted so that reasonable compensation for the anisotropic diffraction could be achieved. While reflections were found at 1.9 Å, reasonable statistics were obtained only when the data were processed to 2.6 Å with 9208 unique reflections. The data set was 90.3% complete, with a  $R_{\text{sym}}$  of 6.9%. Using DENZO (Otwinowski and Minor, 1997) and careful examination of systematically absent reflections, the space group was determined to be  $C2$ , with unit cell parameters of  $a = 82.28$  Å,  $b = 62.29$  Å,  $c = 63.83$  Å,  $\beta = 113.7^\circ$ . The Collaborative Computing Project No.



**FIG. 2.** Multicrystal of sbwAFP from the second condition. Multiple colors are generated by the illumination of the crystals with crossed polarized light.

4 (CCP4, 1994) program “matthews\_coeff” was used to calculate the Matthew’s coefficient  $V_M$  and the solvent content of the unit cell. On the assumption that there are two molecules in the asymmetric unit (ASU), a  $V_M$  of  $4.3 \text{ \AA}^3/\text{Da}$  was calculated, with a solvent content of 71%. Assuming there are three molecules in the ASU, a  $V_M$  of  $2.9 \text{ \AA}^3/\text{Da}$ , with a solvent content of 56% was calculated, which are values more in agreement with those commonly observed (Matthews, 1968). Four molecules per ASU would give a  $V_M$  of  $2.1 \text{ \AA}^3/\text{Da}$  and 42% solvent content. However, the self-rotation function (Crowther, 1972) indicates only one twofold axis of symmetry, and no additional peaks were found. Using “polarfn” (CCP4, 1994), a single peak with 74% of the height of the origin peak was found in the  $\kappa = 180^\circ$  section. The twofold axis of symmetry is in the  $a$ - $c$  plane and makes an angle of  $12^\circ$  with the  $a$  axis. This makes three molecules per ASU unlikely, since one copy would need to be located on the axis of symmetry and have good twofold symmetry about itself. Therefore there must be two molecules in the ASU, with a unit cell solvent content of 71%.

Phasing attempts have been initiated. Due to the degree of difficulty in obtaining large single crystals, the phase problem will be approached by using seleno-methionyl protein and a multiwavelength

anomalous dispersion method. SbwAFP does not contain any internal methionine residues, so isoforms of sequences were examined for positions where aliphatic amino acid substitutions could be tolerated. Currently, two single (L11M, I57M) and two double methionine mutant proteins (L11M/L22M, L22M/I57M) have been successfully expressed by recombinant bacteria on a small scale and crystals can be expected to grow under similar conditions as wild-type sbwAFP.

A second condition that resulted in crystals with a rather unusual morphology was found (Fig. 2). The crystals were grown at room temperature using the hanging drop method in the presence of 25–35% polyethylene glycol ( $M_r$  5000) monomethyl ether, 100 mM morpholinoethanesulfonic acid, pH 6.0–7.0, 200 mM  $(\text{NH}_4)_2\text{SO}_4$ . When the “crystal ball” was broken apart, however, the resulting small crystals appeared to be twisted and did not result in improved crystals even after extensive optimization attempts and seeding.

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