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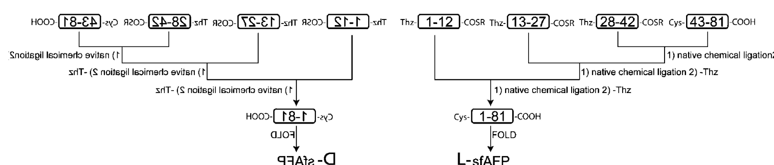

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Mirror Image Forms of Snow Flea Antifreeze Protein Prepared by Total Chemical Synthesis Have Identical Antifreeze Activities

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Abstract: The recently discovered glycine-rich snow flea antifreeze protein (sfAFP) has no sequence homology with any known proteins. No experimental structure has been reported for this interesting protein molecule. Here we report the total chemical synthesis of the mirror image forms of sfAFP (i.e., L-sfAFP, the native protein, and D-sfAFP, the native protein's enantiomer). The predicted 81 amino acid residue polypeptide chain of sfAFP contains Cys residues at positions 1, 13, 28, and 43 and was prepared from four synthetic peptide segments by sequential native chemical ligation. After purification, the full-length synthetic polypeptide was folded at 4 °C to form the sfAFP protein containing two disulfides. Chemically synthesized sfAFP had the expected antifreeze activity in an ice recrystallization inhibition assay. Mirror image D-sfAFP protein was prepared by the same synthetic strategy, using peptide segments made from D-amino acids, and had an identical but opposite-sign CD spectrum. As expected, D-sfAFP displays the same antifreeze properties as L-sfAFP, because ice presents an achiral surface for sfAFP binding. Facile synthetic access to sfAFP will enable determination of its molecular structure and systematic elucidation of the molecular basis of the antifreeze properties of this unique protein.

Introduction

A glycine-rich antifreeze protein (sfAFP) isolated from the Canadian snow flea has recently been described.[1] The prediced 81 amino acid residue polypeptide chain of sfAFP has no sequence homology with any known proteins, and no experimental structure has been reported for this interesting molecule. Scientific investigation has been limited by lack of material; difficulties have been reported both for recombinant expression and for isolation of the protein from natural sources.[2] The absence of an experimental structure has led researchers to propose a theoretical 3D model of the molecular structure of sfAFP.[2] It is of considerable interest to understand the molecular origins of the antifreeze activity of proteins such as sfAFP.[3] Mechanisms for the inhibition of ice crystal formation by antifreeze proteins have been proposed; typically, these involve a surface of the protein that is hydrophobic and has exposed backbone functional groups geometrically disposed to facilitate interaction with the surface of ice crystals.[4] It is believed that the adsorption of antifreeze protein molecules to the surface of the ice crystal interferes with the further ordered growth necessary for enlargement of the ice crystal.[5] An understanding of the molecular mechanism of action of antifreeze proteins could also have important practical applications; for example, in the storage of human tissue and organs for transplant.[6] For these reasons, it would be useful to have a reliable source of high-purity sfAFP in amounts (multiple tens of milligrams) useful for study by advanced physical techniques. It would also be important to have an experimental structure for the folded sfAFP molecule and to be able to systematically vary the covalent structure of the sfAFP molecule and measure the effects on folding, stability, and function.

Chemical synthesis is a useful and versatile way to prepare multiple-milligram quantities of highly pure protein.[7] Total synthesis of proteins by modern methods, most notably by native chemical ligation,[8] is robust, reproducible, and enables the facile production of protein analogues. We set out to develop an efficient total chemical synthesis of the sfAFP protein in order to provide high-purity material for structure−function studies. In this paper, we report the total chemical synthesis of the mirror

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image forms of sfAFP (i.e., L-sfAFP, the native protein, and D-sfAFP, the enantiomer of the native protein molecule). We show that chemically synthesized sfAFP has the expected antifreeze activity and that the mirror image D-sfAFP protein displays identical antifreeze properties.

Experimental Section

Materials. N^{α}-Boc-L-amino acids and N^{α}-Boc-D-amino acids were manufactured by Peptide Institute, Osaka, Japan, and were purchased from Peptides International, Louisville, KY. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Peptides International. Aminomethyl resin used in solid-phase peptide synthesis was prepared from Bio-Beads SX-1 (Bio-Rad Laboratories) by published procedures[9] or purchased from Rapp Polymere, Tübingen. Trifluoroacetic acid (TFA) was from Halocarbon. *N*,*N*-Diisopropylethylamine (DIEA) was obtained from Applied Biosystems. *N*,*N*-Dimethylformamide (DMF), dichloromethane, diethyl ether, HPLC-grade acetonitrile, and guanidine hydrochloride were purchased from Fisher. HF was purchased from Matheson. All other reagents were purchased from Sigma−Aldrich.

Peptide Synthesis. Both D- and L-peptides were prepared by manual Boc chemistry stepwise solid-phase peptide synthesis (SPPS) using in situ neutralization protocols.[10] Peptides were synthesized on a 0.4 mmol scale, on -OCH$_2$-phenyl-CH$_2$CONHCH$_2$ (Pam) resins,[9] (α-carboxyl peptides), or on HSCH$_2$CH$_2$CO-Xaa-OCH$_2$-Pam-resin (α-thioester peptides).[11] Side-chain protection for amino acids was as follows: Arg(Tos), Asn(Xan), Asp(OcHex), Cys(4-CH$_3$Bzl), His(Bom), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl). Where appropriate (i.e., for the three peptide-thioester segments), N-terminal cysteine was incorporated as 1,3-thiazolidine-4-*R*-carboxylic acid (Thz).[12] After completion of the chain assembly, the N$^{\alpha}$-Boc group was removed by treatment with trifluoroacetic acid (TFA); the N$^{\alpha}$-deprotected peptide−resin was thoroughly washed with DMF and dichloromethane and dried under a stream of nitrogen; and the peptides were then cleaved from the resin support, and side-chain protecting groups were simultaneously removed, by treatment with anhydrous HF containing *p*-cresol (90:10 v/v) for 1 h at 0 °C. After complete evaporation of the HF under reduced pressure, crude peptide products were precipitated and triturated with chilled diethyl ether, and the peptide products were dissolved in 50% aqueous acetonitrile containing 0.1% TFA and lyophilized.

Analytical LCMS. Peptide compositions were confirmed by analytical reverse-phase high-pressure liquid chromatography−mass spectrometry (LCMS) with a gradient of acetonitrile versus 0.1% TFA in water. For all the work reported, unless otherwise noted, analytical HPLC was carried out as follows: Vydac C4 2.1 × 150 mm column with a linear gradient of 1−61% buffer B over 15 min with a flow rate of 0.5 mL/min (buffer A = 0.1% TFA in H$_2$O; buffer B = 0.08% TFA in acetonitrile) at 40 °C. The eluent was monitored at 214 nm by online ion trap electrospray mass spectrometry.

Preparative HPLC. Peptides were purified on C4, C8, or C18 silica with columns of dimension 22 × 250 mm, 10 × 250 mm, or 10 × 100 mm. The silica used was TP Vydac or self-packed Varian Microsorb or Agilent Zorbax. Crude peptides (50−300 mg) were dissolved in 1−5% acetonitrile/95% (0.1% TFA in water) to a concentration of ~20 mg/mL and loaded onto the prep column by pumping at a flow rate of 5−10 mL/min. After the nonpeptidic material had eluted, as judged by the re-establishment of the 214 nm baseline, the peptidic components were eluted at a flow rate of 10 mL/min with a shallow gradient (e.g., 20%−40% B over 60 min) of increasing concentrations of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water). The exact gradient used was determined by the elution behavior of the desired peptide, as assessed by prior analytical HPLC and confirmed by preliminary runs at low loading on the preparative column being used. Fractions containing the pure target peptide were identified by analytical LCMS or by MALDI MS and were combined and lyophilized.

Native Chemical Ligation. Ligation reactions of purified synthetic peptide segments were carried out as previously described: sodium phosphate buffer (200 mM) containing 6 M guanidine hydrochloride; 20 mM tris(carboxyethyl)phosphine·HCl, pH = 6.8, at a concentration of 5−10 mM for each peptide segment; with 10−30 mM 4-(carboxymethyl)thiophenol (mercaptophenylacetic acid, MPAA) as catalyst.[13] The ligation buffer had previously been purged with helium and the ligation reaction was carried out under argon. After the completion of each ligation, as judged by LCMS analysis of aliquots, methoxyamine hydrochloride (0.2 M) was directly added to the reaction mixture; the pH was lowered to 4.0. This chemical step converts the N-terminal Thz- to Cys- and was essentially complete in 2−4 h, as judged by analytical LCMS of aliquots. Intermediate ligation products were either isolated by solid-phase extraction or purified by reverse-phase HPLC prior to subsequent ligations, in order to avoid potential methoxyaminolysis of thioester peptides.

Representative Synthesis of L-sfAFP. The synthesis described below was carried out on a 9.9 μmol scale of each peptide segment; after folding/disulfide formation and purification, 1.54 μmol (10.0 mg) of the final product was isolated (16% yield).

Synthesis of Peptide Segments. Full experimental details and yields for each synthesis are given in the Supporting Information. The peptide building blocks (and corresponding masses) used in this synthesis were as follows: Thz^{1}-Lys-Gly-Ala-Asp-Gly-Ala-His-Gly-Val-Asn-Gly^{12}-CO-S-CH$_2$-CH$_2$-CO-Ile-Pro-COOH [observed (ob) 1395.3 ± 0.5 Da, calculated (ca) 1395.4 Da (average isotopes)], Thz^{13}-Pro-Gly-Thr-Ala-Gly-Ala-Ala-Gly-Ser-Val-Gly-Gly-Pro-Gly^{27}-CO-S-CH$_2$-CH$_2$-CO-Leu-Pro-COOH (ob = 1468.4 ± 0.5 Da, ca = 1468.5 Da), Thz^{28}-Asp-Gly-Gly-His-Gly-Gly-Asn-Gly-Gly-Asn-Gly-Asn-Pro-Gly^{42}-CO-S-CH$_2$-CH$_2$-CO-Ile-COOH (ob = 1481.9 ± 0.5 Da, ca = 1482.4 Da), and Cys^{43}-Ala-Gly-Gly-Val-Gly-Gly-Ala-Gly-Gly-Ala-Ser-Gly-Gly-Thr-Gly-Val-Gly-Gly-Arg-Gly-Gly-Lys-Gly-Gly-Ser-Gly-Thr-Pro-Lys-Gly-Ala-Asp-Gly-Ala-Pro-Gly-Ala-Pro^{81}-COOH (ob = 3025.0 ± 0.5 Da, ca = 3025.2 Da).

Ligation of [Thz^{28}−Gly^{42}]-α-thioester and [Cys^{43}−Pro^{81}]-COOH. Reaction was carried out at room temperature, with concentrations of 5 mM for each peptide, at pH 6.8 and 10 mM MPAA thiol catalyst. After overnight reaction at room temperature, the crude products were treated with 0.2 M methoxyamine hydrochloride for 2 h to give [Cys^{28}−Pro^{81}]-COOH, which was purified from nonpeptidic materials by solid-phase extraction.

Ligation of [Thz^{13}−Gly^{27}]-α-thioester and [Cys^{28}−Pro^{81}]-COOH. Ligation was carried out as described above. After overnight reaction at room temperature, the crude products were treated with 0.2 M methoxyamine hydrochloride for 2 h to give [Cys^{13}−Pro^{81}]-COOH. The product of this reaction was purified by reverse-phase HPLC; in other syntheses we continued without isolation of this intermediate, thereby increasing overall yields.

Ligation of [Thz^{1}−Gly^{12}]-α-thioester and [Cys^{13}−Pro^{81}]-COOH. Ligation was carried out as described above. After overnight reaction at room temperature, the crude products were treated with 0.2 M methoxyamine hydrochloride for 2 h to give [Cys^{1}−Pro^{81}]-COOH. The full-length reduced polypeptide was purified by reverse-phase HPLC.

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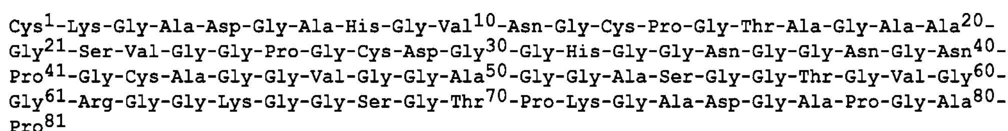
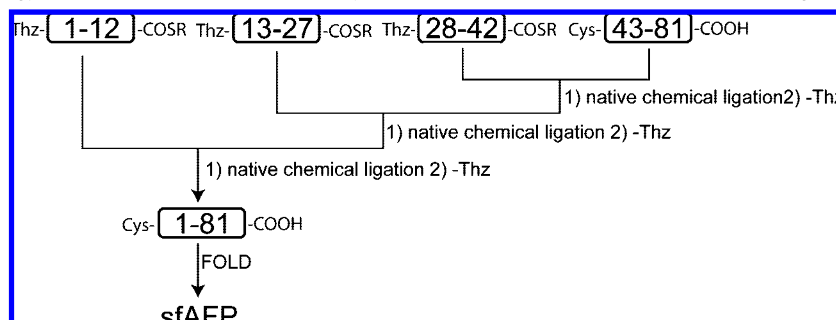
Scheme 1. Predicted 81-Residue Glycine-Rich Amino Acid Sequence of sfAFP[1]



Cys^{1}-Lys-Gly-Ala-Asp-Gly-Ala-His-Gly-Val^{10}-Asn-Gly-Cys-Pro-Gly-Thr-Ala-Gly-Ala-Ala^{20}-
Gly^{21}-Ser-Val-Gly-Gly-Pro-Gly-Cys-Asp-Gly^{30}-Gly-His-Gly-Gly-Asn-Gly-Gly-Asn-Gly-Asn^{40}-
Pro^{41}-Gly-Cys-Ala-Gly-Gly-Val-Gly-Gly-Ala^{50}-Gly-Gly-Ala-Ser-Gly-Gly-Thr-Gly-Val-Gly^{60}-
Gly^{61}-Arg-Gly-Gly-Lys-Gly-Gly-Ser-Gly-Thr^{70}-Pro-Lys-Gly-Ala-Asp-Gly-Ala-Pro-Gly-Ala^{80}-
Pro^{81}

Scheme 2. Synthetic Strategy Used for Preparation of sfAFP by Sequential Condensation of Four Peptide Segments[a]



[a] Thz- is 1,3-thiazolidine-4-*R*-carboxylic acid; -COSR is a thioester moiety. The exact structure of the thioester moiety varies for each peptide and is given in the Experimental Section.

Folding/Disulfide Formation of Synthetic L-sfAFP(Cys^{1}−Pro^{81}). Purified [Cys^{1}−Pro^{81}]-COOH polypeptide was folded by dissolving 2.2 μmol (14.6 mg) in 30 mL of 50 mM phosphate folding buffer, pH = 7.8, containing 8 mM cysteine, 1 mM cystine·2HCl, at 4 °C. A single product containing two disulfide bonds was formed within approximately 24 h as confirmed by LCMS and an observed mass loss of 4 Da. After completion of the folding reaction, dialysis or HPLC was used to isolate the product. For the case reported here, the folding buffer was added to a 3500 MW cutoff dialysis bag, dialyzed extensively against water at 4 °C, and then lyophilized to give 1.54 μmol (10 mg) of material. If HPLC was used, standard purifications were carried out on a Vydac C18 10 × 250 mm column as described above.

Circular Dichroism. CD spectra were recorded on an Aviv model 202 instrument at room temperature by dissolving 0.03 mg (prepared from a stock solution) of D- or L-sfAFP protein in 300 μL of 50 mM phosphate buffer, pH = 6.9. A 1 mm path length cell was used.

Ice Recrystallization Inhibition Activity Assays. The procedure used was based upon the method of Knight et al.[14a] Samples were loaded into 25 μL microcapillary tubes (Drummond Microcaps, Drummond Scientific Co., Broomall, PA) and each end was flame-sealed. Then samples were flash-frozen for about 10 s in 2,2,4-trimethylpentane cooled with dry ice and immediately placed in a bath of the same solvent cooled to −6 °C by a jacketed beaker connected to a Fisher Isotemp 1016S circulating bath. Images were taken at 40× total magnification by use of a Nikon SMZ-2B microscope (Melville, NY) and a DCM35 digital microscope camera (Hangzhou Huaxin IC Technology, Silicon Valley, CA) utilizing the software ScopePhoto 1.0 (Scopetek). Authentic antifreeze protein type 1 (AFP I) (A/F Protein, Waltham, MA) was used as a positive control.

Results

Synthetic Design. The predicted 81-residue sfAFP polypeptide (Scheme 1) contains three Gly-Cys sites, each of which represents a potential retrosynthetic disconnection to a set of unprotected peptide segments for assembly by native chemical ligation. We initially set out to assemble the sfAFP polypeptide from three peptide building blocks. However, this strategy was precluded by chronic side reactions in the synthesis, by stepwise Boc chemistry solid-phase peptide synthesis (SPPS),[10] of segments containing Asp-Gly and Asn-Gly sites. Such Asx-Gly sites are abundant in the sfAFP sequence, and the observed level of aspartimide formation,[15] with consequent 18 or 17 Da lower observed masses (for an example of a crude synthetic product containing these byproducts, see Figure S3 in Supporting Information), complicated the preparation of larger peptide segments of acceptable purity. As a consequence, a four-segment sequential ligation approach (Scheme 2) was used in order to give reasonable yields of purified peptide segments to be used as building blocks for the preparation of sfAFP. Sequential native chemical ligation requires the use of a temporary protecting group for peptide-thioester segments that have an N-terminal Cys residue. Here we used the Thz form of Cys, as previously described.[12]

Assembly of Polypeptide Chain. Analytical data for the ligation reactions in a representative synthesis of L-sfAFP are given in Figure 1.

Folding/Disulfide Formation. The full-length reduced 81-residue polypeptide chain was purified and then subjected to folding with concomitant formation of disulfide bonds. We screened approximately 10 sets of conditions to optimize folding and oxidation of the reduced sfAFP polypeptide. Folding parameters screened included temperature, redox systems, and concentration of the chaotrope guanidine hydrochloride. The best folding conditions that we found were to treat the purified, full-length polypeptide at 0.5 mg/mL with a redox couple consisting of 8 mM cysteine/1 mM cystine in pH 7.8 buffer at 4 °C for ∼24 h. These conditions reproducibly gave a good yield of a single product that coeluted with the reduced polypeptide on reversed-phase HPLC but that had a mass 4 Da lower, consistent with the formation of two intramolecular disulfide bonds (Figure 2).[1]

Characterization. The folded, disulfide-containing synthetic sfAFP was purified by preparative reverse phase HPLC. Overall synthetic yields in several syntheses ranged from 15% to 30%, depending on the number of intermediate purifications/isolations performed. This range of overall yields corresponds to an average of 80% ± 4% yield per chemical transformation, for the seven chemical steps shown in Scheme 2 (above). Typical amounts of sfAFP prepared in a single synthesis were 35−100 mg. LCMS analyses of folded, purified L-sfAFP (prepared by

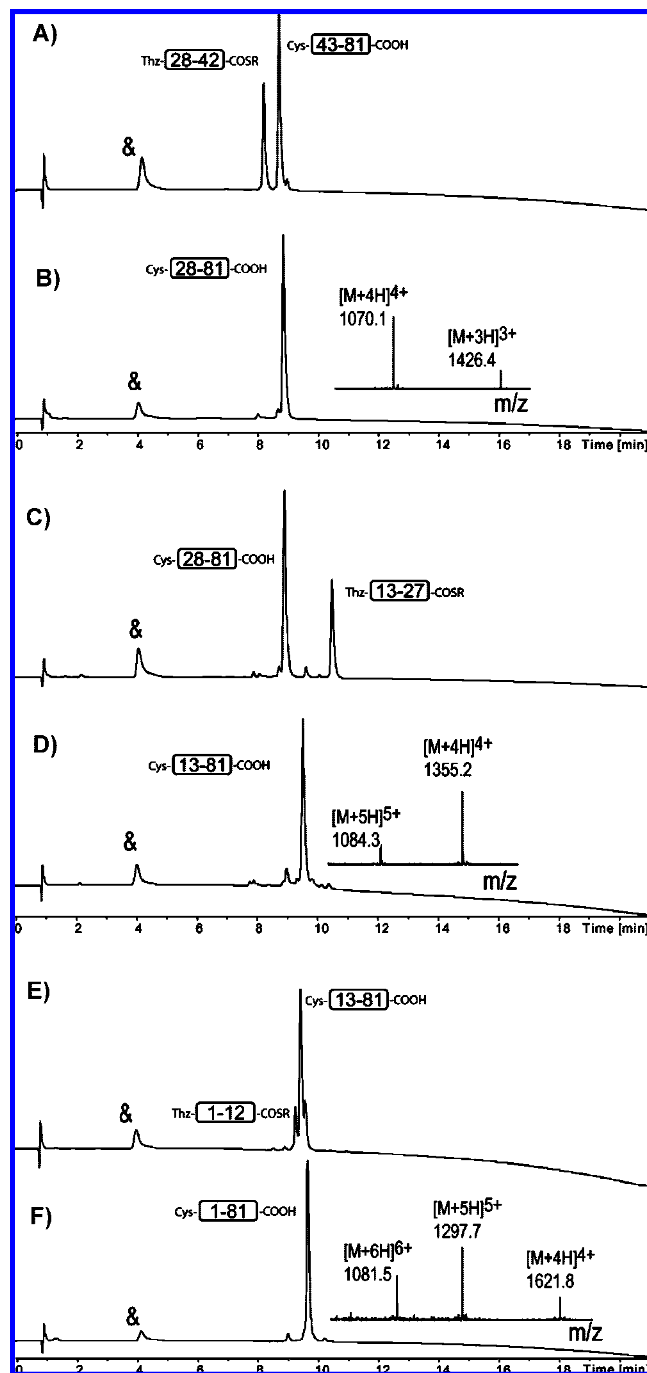


Figure 1. LCMS data for native chemical ligation reactions in the synthesis of sfAFP(1−81). (A, B) Reaction of [Thz[28]−Gly[42]]-α-thioester and [Cys[43]−Pro[81]]-COOH: (A) $t = 0$ and (B) $t =$ overnight. In panel B, the product shown was obtained after treatment with 0.2 M $CH_3ONH_2 \cdot HCl$ to give [Cys[28]−Pro[81]]-COOH [ob = 4276.3 ± 0.8 Da, ca = 4276.4 Da (average isotopes)]. (C, D) Reaction of [Thz[13]−Gly[27]]-α-thioester and [Cys[28]−Pro[81]]-COOH: (C) $t = 0$ and (D) $t =$ overnight. In panel D, the product shown was obtained after treatment with 0.2 M $CH_3ONH_2 \cdot HCl$ to give [Cys[13]−Pro[81]]-COOH [ob = 5416.6 ± 0.5 Da, ca = 5416.8 Da (average isotopes)]. (E, F) Reaction of [Thz[1]−Gly[12]]-α-thioester and [Cys[13]−Pro[81]]-COOH: (E) $t = 0$ and (F) $t =$ overnight. In panel F, the product shown was obtained after treatment with 0.2 M $CH_3ONH_2 \cdot HCl$ to give [Cys[1]−Pro[81]]-COOH [ob = 6483.2 ± 0.8 Da, ca = 6483.8 Da (average isotopes)]. The peak labeled with & is the ligation catalyst MPAA.

ligation of peptides synthesized from L-amino acids) and D-sfAFP (prepared by ligation of peptides synthesized from D-amino acids) are shown in Figure 3.

As expected, the sfAFP enantiomers had equal and opposite CD spectra, within experimental error (Figure 4). The CD spectrum obtained for synthetic L-sfAFP corresponded to that reported for the sfAFP isolated from natural sources.[1,2] These CD data suggest that the synthetic sfAFP consists of either random coil or polyproline type II helices (PP-II).[2] We have since determined the X-ray structure of sfAFP and found that the protein contains only PP-II secondary structure.

Antifreeze Activity. The antifreeze activity of our folded, synthetic materials was verified by an ice recrystallization inhibition assay (Figure 5).[14] This assay distinguishes antifreeze proteins from the rest of nature's proteins by their unique ability to bind to ice surfaces and prevent the grain migration that causes ice recrystallization.[3] Chemically synthesized sfAFP showed full activity in this assay. Authentic AFP 1 was used as a positive control. Reduced sfAFP(Cys[1]−Pro[81])$(SH)_4$ polypeptide was devoid of antifreeze activity in this assay; thus, the folded, tertiary structure of sfAFP is essential for antifreeze activity.

As would be expected for an effect that depends on the sfAFP binding to the achiral surface of ice and as has been previously demonstrated for mirror image antifreeze peptides,[16] native L-sfAFP and its mirror image D-sfAFP display identical ice recrystallization inhibition activity (Figure 6).

Discussion

The total chemical synthesis of sfAFP reported here is experimental confirmation that a protein, with the polypeptide chain predicted from cDNA sequencing,[1] has the expected antifreeze activity. The synthesis according to the initial successful strategy was carried out a number of times over a 12-month period, in order to supply material for crystallization trials and to make analogues. We had encountered difficulty in obtaining useful crystals of L-sfAFP; consequently, we wanted to increase the possibility of obtaining useful crystals by the use of a racemic protein mixture, as suggested by Yeates and co-workers.[17] To that end, we undertook the preparation of the protein enantiomers D- and L-sfAFP, which is possible only by total chemical synthesis. As much as 100 mg of folded, purified sfAFP was prepared from a single synthesis. Efficient, reproducible chemical access to a protein molecule is a critical prerequisite for the cost-effective preparation of mirror image proteins (D-amino acids are ∼4 times more expensive than L-amino acids, for a protein of typical composition).

Efficient chemical access to sfAFP analogues, combined with knowledge of sfAFP's crystal structure, will enable the systematic study of the molecular basis of sfAFP antifreeze activity. In addition to the preparation of D-sfAFP, we have successfully applied our total synthesis to the preparation of a selenium-containing sfAFP analogue, for use in anomalous dispersion X-ray crystallography experiments, and to the preparation of two distinct sets of site-specifically isotope-labeled sfAFP

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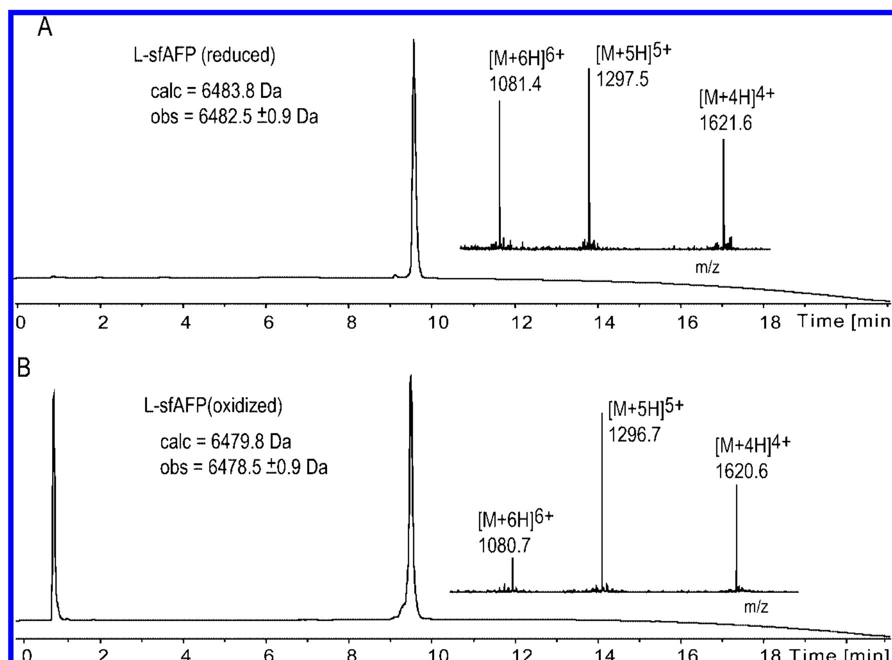

Figure 2. LCMS data for folding/disulfide formation for synthetic sfAFP(Cys[1]−Pro[81]). (A) $T = 0$, purified sfAFP(Cys[1]−Pro[81])(SH)$_4$. (Inset) Electrospray MS of the main component. (B) $T = 24$ h, crude sfAFP folding product. (Inset) Electrospray MS of the main component. Reduced and oxidized sfAFP coeluted under these analytical conditions; however, the observed mass loss of 4 Da for the oxidized sfAFP after purification confirms the presence of two disulfides in the folded protein molecule.

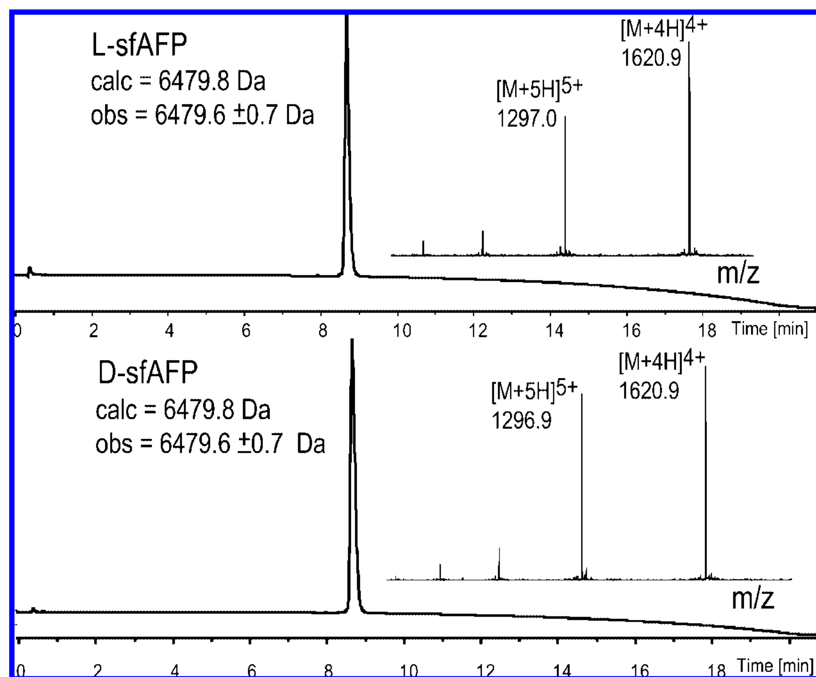

Figure 3. Analytical LCMS data for synthetic L- and D-sfAFP. The chromatographic separations were carried out on a self-packed Varian microsorb C_4 2.1 × 50 mm column with a linear gradient of 1−61% buffer B over 15 min (buffer A = 0.1% TFA in H_2O; buffer B = 0.08% TFA in acetonitrile). Flow rate was 0.5 mL/min with detection at 214 nm. (Inset) Online electrospray MS summed over the entire LC peak in each chromatogram. Calculated masses were based on average isotope composition.

preparations, for use in NMR experiments (manuscript in preparation). Preparation of multiple sfAFP analogues is facilitated by the modular nature of the synthesis reported above. Thus, the selenium analogue was prepared by ligation of a nonnative N-terminal peptide to the Cys[13]−Pro[81] polypeptide, eliminating the need to resynthesize unchanged portions of the sequence in the preparation of the analogue.

Finally, D-sfAFP antifreeze activity may have important practical applications because D-proteins are expected to be nonimmunogenic and resistant to degradation by natural proteases.[18] Therefore, D-AFPs could potentially be more effective

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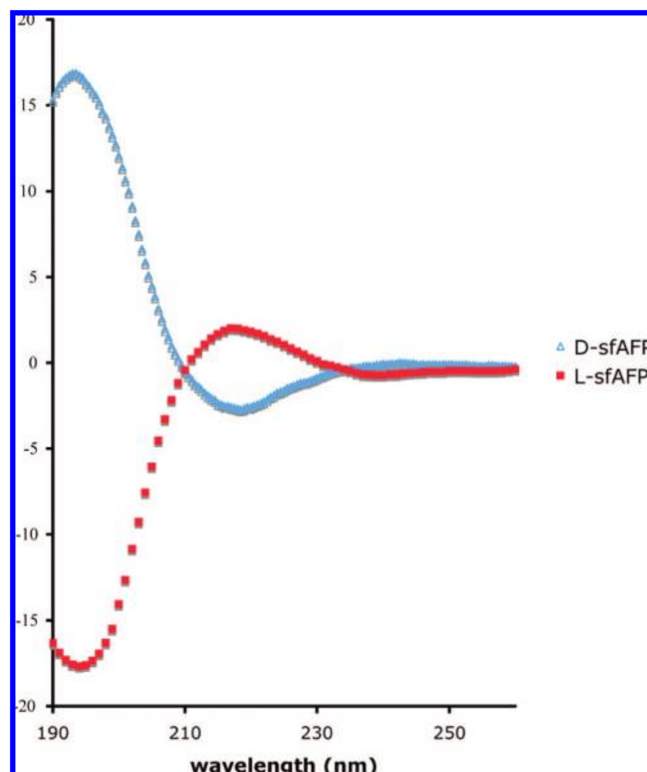

Figure 4. CD spectra of the protein enantiomers D- and L-sfAFP. CD spectra were recorded on an Aviv model 202 instrument at room temperature by dissolving 0.03 mg (prepared from a stock solution) of D- or L-sfAFP protein in 300 μL of 50 mM phosphate buffer, pH = 6.9. A 1 mm path length cell was used.

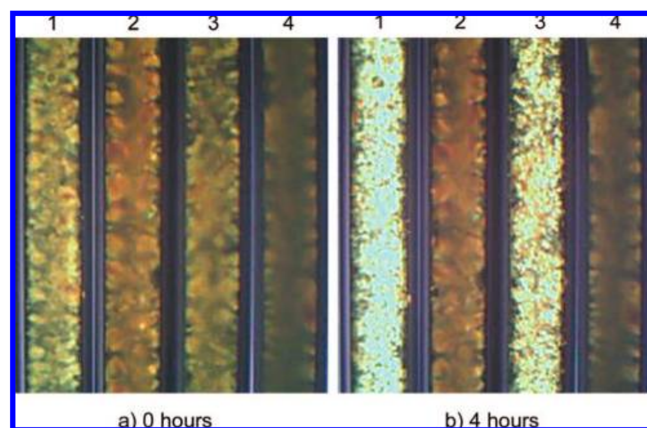

Figure 5. Ice recrystallization inhibition assays at (a) 0 and (b) 4 h. Samples from left to right are (1) 10 mM sodium phosphate, pH 7.5, with 100 mM NaCl; (2) approximately 25 mg/mL AFP I; (3) approximately 25 mg/mL sfAFP, reduced form; and (4) approximately 25 mg/mL sfAFP, oxidized form.

in preventing tissue damage that occurs during the freezing of organs for long-term storage.[14c,19]

Conclusion

Efficient preparation of sfAFP by total chemical synthesis enabled us to confirm the antifreeze properties of the protein having the predicted amino acid sequence. The utility of chemical synthesis for preparing protein analogues was exemplified by the synthesis of the enantiomers D- and L-sfAFP. These mirror image proteins had identical antifreeze activity. Mirror image D-proteins are currently accessible only through total chemical synthesis, and to date only a handful of D-proteins have been prepared.[18,20–22] The present work is the first synthesis of a D-protein to utilize modern chemical ligation methods.[7,8]

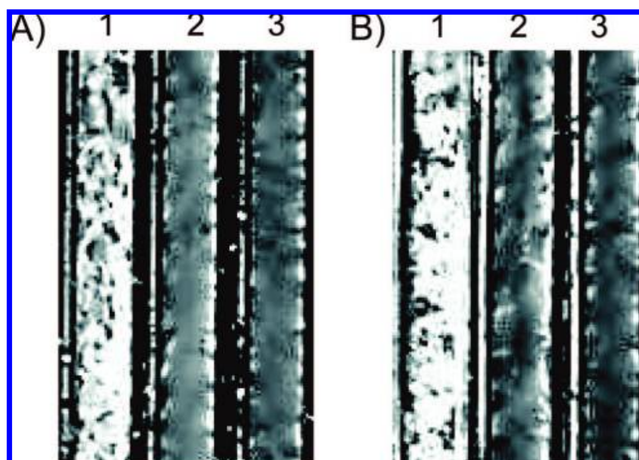

Figure 6. Ice recrystallization inhibition assay (for details see Experimental Section) of D- and L-sfAFP at (A) $t = 0$ and (B) $t = 20$ h. The lanes are as follows: lane 1, 100 mM NaCl and 10 mM phosphate, pH = 7.5; lane 2, 25 mg/mL L-sfAFP, 100 mM NaCl, and 10 mM phosphate, pH = 7.5; lane 3, 25 mg/mL D-sfAFP, 100 mM NaCl, and 10 mM phosphate, pH = 7.5.

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Supporting Information Available: Synthetic protocols, yield, and analytical data for the peptides L-[Thz−Gly^{12}]-thioester (Figure S1), L-[Thz^{13}−Gly^{27}]-thioester (Figure S2), L-[Thz^{28}−Gly^{42}]-thioester (Figure S3), and L-[Cys^{43}−Pro^{81}]COOH (Figure S4) and for the peptides D-[Thz−Gly^{12}]-thioester (Figure S5), L-[Thz^{13}−Gly^{27}]- thioester (Figure S6), L-[Thz^{28}−Gly^{42}]-thioester (Figure S7), and L-[Cys^{43}−Pro^{81}]COOH (Figure S8). This information is available free of charge via the Internet at http://pubs.acs.org.

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