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Type II Antifreeze Protein from a Mid-latitude Freshwater Fish, Japanese Smelt (*Hypomesus nipponensis*)

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A lot of reports of antifreeze protein (AFP) from fish have been published, but no report has mentioned of commercialized mid-latitude fresh water fish which producing AFP in its body fluid. We found that the AFP in the body fluid of Japanese smelt (*Hypomesus nipponensis*) from mid-latitude fresh water was purified and characterized. The N-terminal amino acid sequence of the Japanese smelt AFP was 75.0% identical to Type II AFP from herring. Results of EDTA treatment and ruthenium red staining suggested that the Japanese smelt AFP had at least one Ca^{2+}-binding domain. Interestingly, the antifreeze activity of the Japanese smelt AFP did not completely disappear when Ca^{2+} ions were removed. The molecular mass of the Japanese smelt AFP was calculated to be 16,756.8 by the TOF-mass analysis. The Open reading flame of the gene coding for the Japanese smelt AFP was 444 bp long and was 85.0% identical with the entire herring AFP gene. The cDNA and amino acid sequence of the Japanese smelt AFP were the same length as those of herring AFP.

Key words: antifreeze protein; Type II; Japanese smelt

Fish antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) form a group of structurally diverse macromolecules that confer freeze-resistance to teleost fishes living in ice-laden marine environments.[1–3] The fish AFPs are further classified into Types I, II, III, and IV. Type I AFP is a single α-helix with a molecular mass of 3,000 to 5,000.[4,5] Type III AFP is globular and has a molecular weight of 6,500 to 14,000; no one kind of amino acid residue is particularly abundant in it.[6–9] Type IV AFP is glutamate rich, has a molecular mass of 12,300 and a four-helix bundle structure.[10] AFGP has some Ala-Ala-Thr repeats and a molecular mass of 2,600 to 33,000.[11] Type II AFP, the largest AFP in fish with a molecular mass of 14,000 to 24,000, is globular and Cys rich.[12] A portion of the molecule resembles the carbohydrate-recognition domain of calcium-dependent C-type lectins in its one- to three-dimensional structures.[13] Type II AFP has disulfide bridges patterns, α-helices and β-sheets, and widespread coil construction. On the basis of the need for Ca^{2+} for antifreeze activity, Type II AFPs have been classified into two subtypes.[12,14] Both herring and smelt (''smelt'' alone refers to smelt other than the Japanese smelt) Type II AFPs need Ca^{2+} ions to have antifreeze activity.[14]

In this study, we purified and characterized AFP from the Japanese smelt (*Hypomesus nipponensis*), a popular food in Japan, especially in winter. The amino acids sequence of Japanese smelt AFP (jAFP) closely resembled that of herring AFP, but the relationship between its antifreeze activity and Ca^{2+} ions was different from that of other Type II AFPs.[14] Here we discuss the mechanism of the antifreeze effects of jAFP including its independence of Ca^{2+}.

Materials and Methods

Experimental animal. Japanese smelts were purchased from a supermarket. After removal of the skin, head, bones, and internal organs, the muscles of the fish were washed with 50 mM potassium phosphate buffer (KPB). Unless otherwise specified, the pH of the buffer was 7.0.

Thermal hysteresis and shapes of ice crystals. The antifreeze activity of purified sample of protein was assayed by the method of Meyer *et al.*[15] The assay is based on changes in the shape of seed ice crystal caused only by AFPs. One micro liter of a protein sample was put on the center of a temperature-controlled freezing stage (LK-600PM, Linkham Scientific Instruments, UK) on a circular glass cover. The freezing stage was fitted onto the stage of a

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phase-contrast light microscope and connected to a pressurized air supply cooled with liquid N_2. The stage temperature was controlled by a programming unit (LK-600PM). First, the stage was heated to 20°C and then cooled to −40°C at the rate of 100°C/min. The stage was then reheated at the same rate to 5°C. The temperature was increased further at the rate of 5°C/min to the temperature at which controlled thawing took place. Thawing was allowed to proceed until a single ice crystal was remained. At this point, the temperature was lowered at the rate of 1°C/min until slow growth of one ice crystal was observed. The temperature was then increased until the ice crystal slowly melted and this time from the start of heating to the start of melting of the ice crystal was measured. One-sixtieth of the rate of 1°C/min (60 sec) times the time (sec) was defined as thermal hysteresis (°C). Under these conditions, as for AFPs of some other fish, antifreeze activity was taken to be high when the ice crystal was multifaceted or bipyramidal,[17] and antifreeze activity was taken to be low when the ice crystal was flat and hexagonal.[16] With no antifreeze activity, ice crystals was spherical disc.

Purification and identification of AFP from Japanese smelt. Muscles of Japanese smelt (127 g) were crushed with 130 ml of 50 mM KPB for 2 min at room temperature in a Magnum blender (Hamilton Beach Co.), and the mixture was centrifuged (15,000 $\times g$, 4°C, 15 min). This supernatant (6310 mg of protein) after centrifugation was treated at 57.5°C for 6 min with a block heater (T4L-2G, Taitec) and then centrifuged at 27,700$\times g$ for 15 min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant to 35% saturation, and the mixture was incubated at 4°C for 30 min. After the precipitate that formed was removed, $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation, and the mixture was incubated at 4°C for 30 min and then centrifuged. The precipitate thus obtained was dissolved in 10 ml of 50 mM KPB. This was 11.2 ml of a crude AFP solution; it was put at the flow rate of 4.0 ml/min into a Superdex 200 column (2.6×60 cm, Acta Prime, Amersham Pharmacia) equilibrated with 5 mM KPB, and eluted with the same buffer. The active fractions were pooled and the partially purified AFP solution was put at the flow rate of 2 ml/min onto a Super Q column (1.6×10 cm, Amersham Pharmacia) previously equilibrated with 5 mM KPB. The adsorbed material was eluted with a linear 0 to 0.4 M NaCl gradient in an equilibration buffer. The active fractions were pooled and dialyzed several times against the 5 mM KPB to remove the NaCl. The dialysate solution was put onto the Mono Q column (1.0×10 cm, Amersham Pharmacia) equilibrated with 5 mM KPB. The adsorbed AFP was eluted with a linear gradient of 5 to 100 mM KPB.

SDS-PAGE and TOF-Mass analysis. In an estimation of the mass of the AFP, purified samples of AFP were treated by preparative SDS-PAGE (15×15×0.1 cm) by the method of Laemmli.[18] A 12.5% separating gel and a 4% stacking gel were used in the electrophoresis, done at a constant current of 40 mA for about 100 min. Mass analysis of AFP was done with TOF-mass spectrometer (Mariner, PerSeptive Biosystems). The evaporated AFP purified by Superdex 200, Super Q and Mono Q column was dissolved in 400 μl of a 1:1 (vol/vol) mixture of MeOH and H_2O, and then then mixed in 1% AcOH. Finally the AFP solution was injected into the LC/MS system in a positive ionization mode. Deconvolution of the AFP mass was done with a BioSpec Data Explorer Mariner Ver. 3.

Ca^{2+} binding and ruthenium red staining of Japanese smelt AFP. The binding of ruthenium red with Japanese smelt AFP was examined by the method of Evert *et al.*[19] The AFP samples which dialyzed against KPB and KPB containing 0.1 M EDTA (4 μg/lane) were run on SDS-PAGE under non-reducing conditions and then the band was transferred electrophoretically to nitrocellulose membrane as recommended by the supplier (Mini Protean II, Bio Rad). Three identical blotting membranes were incubated in 50 mM KPB with 0.1 mg/ml ruthenium red for 15 min at 4°C. Each membrane was washed at 4°C the 50 mM KPB without dye but containing 50 mM $CaCl_2$ or $MgCl_2$.

N-terminal analysis of purified Japanese smelt AFP. The N-terminal sequence of AFP was analyzed with a PPSQ-21 sequencer (Shimadzu Biotech). The repetitive yield was 96.7% per cycle. The AFP was treated by 20 cycles of Edman degradation.

Isolation of Japanese smelt mRNA and construction of cDNA. Total RNA of Japanese smelt was isolated with an EASYPrep RNA (Takara Shuzo) from 1.0 g of liver, and poly(A) mRNA was isolated with an Oligotex-dt30 Super mRNA purification kit (Takara Bio Inc.) from total RNA. cDNA was constructed from the mRNA with a cDNA synthesis kit (Takara Bio Inc.).

Plasmid construction. The UA cloning vector pDrive containing the *lac*Z gene, and kanamycin and ampicillin resistance genes for selection on *Escherichia coli* was used. A 5′-primer, GF75 (5′-GGTCAAAGTCATCTCCACTACCAGG-3′), and a 3′-primer, GB535 (5′-GACTTGATACATGAAA-AACACAGAAGAG-3′), designed on the basis of the sequence of the gene coding for herring AFP were used to amplify mature cDNA of Japanese smelt AFP by PCR. The amplified AFP cDNA was ligated into the UA cloning vector (pDrive, Qiagen), and

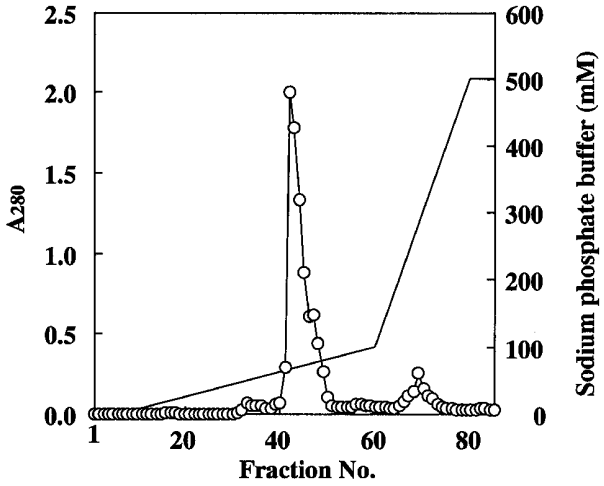

Fig. 1. Anion-exchange Chromatography of Japanese Smelt AFP on Mono Q Column.

After anion-exchange on a Super Q column, fractions with antifreeze activity were dialyzed and put on a Mono Q column equilibrated with 5 mM KPB (pH 7.0). The antifreeze protein was eluted with a linear gradient of KPB (pH 7.0). Fractions 41 to 44 were collected as the purified Japanese smelt AFP.

then used to transform competent *E. coli* JM109 cells (Wako Pure chemical Industries).

DNA sequencing. After the JM109 competent cells were transformed, white colonies were selected and cultured with nutrient broth (Difco) containing 100 μg/ml ampicillin at 37°C for 8 h. The plasmid containing the AFP gene was extracted from the JM109 cells with a Plasmid Miniprep kit (Bio-Rad). DNA was sequenced by the dideoxy-chain termination method[19] with a DNA sequencer (SQ5500E, Hitachi Ltd.). T7 and M13 were used as the dye primers. Sequence data were analyzed with the software program Mac Vector system (Ver. 7.1, Accelrys), and alignment was done in a search for similar sequences in the SCBI Data Bank.

Results

Purification and characterization of Japanese smelt AFP

When the supernatant obtained after centrifugation of the crushed minced fish meal was incubated at temperatures from 60 to 100°C for 5 min, the antifreeze activity of the supernatant disappeared at 65°C. For that reason, we treated such supernatants at 57.5°C for 6 min to remove impurities from the muscle. Starting with 6310 mg, 3810 mg of crude AFP was obtained. In the final step, the adsorbed AFP to Mono Q was eluted as when the KPB was at about 65 mM KPB (Fig. 1). The AFP fractions (7.3 mg) were pooled at this step and dialyzed three times against pure water. The antifreeze activity of the AFP was 0.281°C when the protein concentration was 2 mg/ml. A summary of the purification step of Japanese smelt AFP is shown in Table 1.

Table 1. Yield of Protein During Purification of Japanese Smelt AFP

Step	Protein
Muscle extract (127.4 g)	6307.9 mg
57.5°C for 6 min	3813.9 mg
35–60% ammonium sulfate	868.3 mg
Gel filtration on Superdex 200	171.5 mg
Anion exchange on Super Q	13.3 mg
Anion exchange on Mono Q	7.3 mg

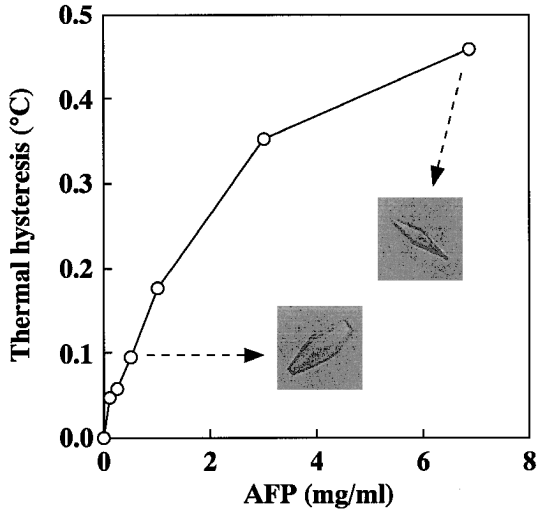

Fig. 2. Effects of Japanese Smelt AFP Concentration on Thermal Hysteresis.

The antifreeze activity of purified Japanese smelt AFP was measured at the concentrations of 0.1, 0.25, 0.5, 1.0, 3.0, and 7.0 mg/ml. Thermal hysteresis was examined and ice crystals were observed as Materials and Methods.

Seventy-five percent of the antifreeze activity disappeared after treatment at 100°C for 60 min, and the optimum pH of the purified AFP was 7.0 (not shown). The effects of the AFP concentration on the antifreeze activity are shown in Fig. 2. The temperature of the antifreeze activity (that is, the concentration dependence between 0 and 7.0 mg/ml of thermal hysteresis) was about 0.45°C. The molecular mass of this AFP was estimated to be 17,000 from the results of SDS-PAGE (Fig. 3). The TOF-mass spectrum and deconvolution analysis showed indicates that the molecular mass of the AFP to be 16,800 (Fig. 4).

This antifreeze activity did not completely disappear after dialysis against 50 mM KPB containing 0.1 M EDTA. The Japanese smelt AFP was stained with ruthenium red before dialysis against 50 mM KPB containing 0.1 M EDTA. After dialysis against same buffer, the AFP was not stained by ruthenium red.

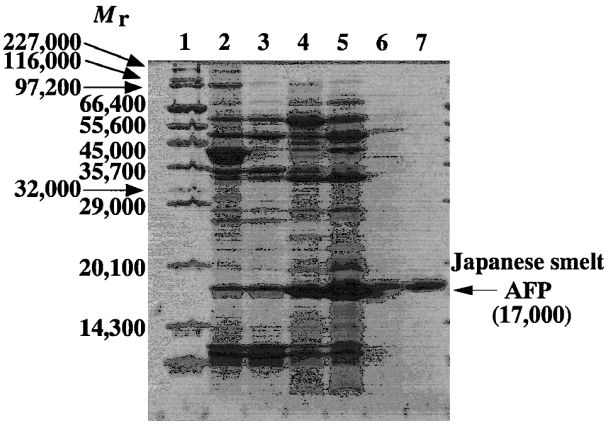

Fig. 3. SDS-PAGE (15%) Analysis of Japanese Smelt AFP.
Lane 2, Muscle extract; lane 3, after heat treatment at 65°C; lane 4, after ammonium sulfate precipitation; lane 5, after gel-filtration with Superdex 200; lane 6, after anion-exchange chromatography with Super Q; lane 7, after anion-exchange chromatography with Mono Q. Lane 1, molecular weight markers.

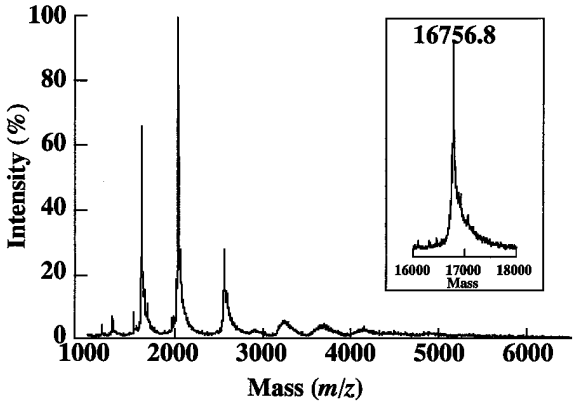

Fig. 4. TOF-Mass Spectrum Analysis of Japanese Smelt AFP.

N-terminal and DNA sequence of Japanese smelt AFP

The N-terminal sequence of Japanese smelt AFP, underlined in Fig. 5, was 83.0% identical to that from herring and 54.0% to smelt AFP. The results were consistent with the Japanese smelt AFP being of a Type II AFP.

The DNA fragment containing the Japanese smelt AFP gene was amplified as cDNA by the PCR method using the two primers described in Materials and Methods. By 10% agarose electrophoresis, only one amplified fragment was found. The 0.5-kbp PCR product was ligated into the overhanging U residue of pDrive vector. The gene sequence and deduced amino acid sequence are shown in Figs. 5 and Fig. 6. The gene encoding Japanese smelt AFP was 444 bp long and had 91.0 and 62.0% identity with herring and smelt genes for Type II AFPs. Our AFP had a signal peptide with 11 amino acids residues. The number of residues coded for by the Japanese smelt AFP gene was identical with that coded for by the

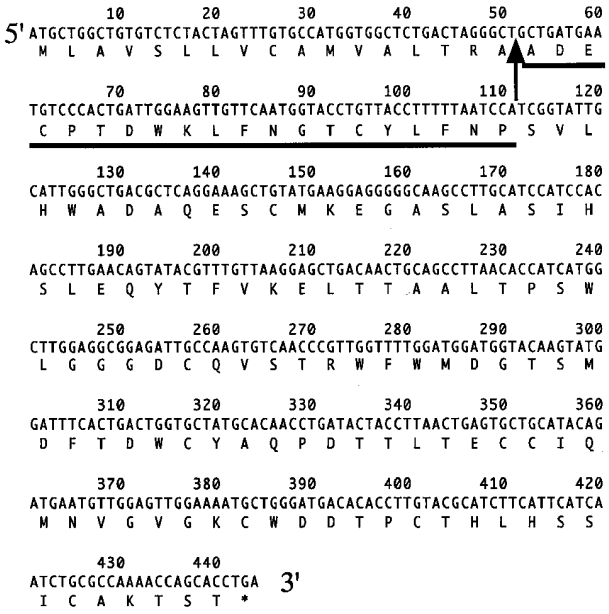

Fig. 5. DNA Sequence of Japanese Smelt AFP Gene and the Deduced Amino Acid Sequence.
The underline indicates the N-terminal sequence of Japanese smelt AFP. The amino acid sequence upstream from the arrow is the signal peptide.

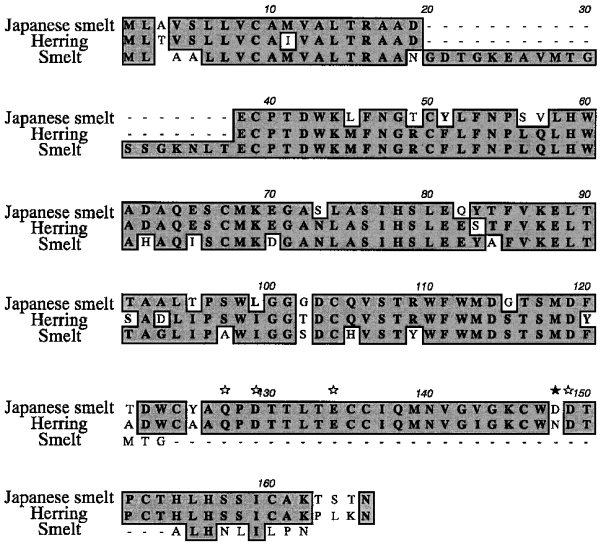

Fig. 6. Amino Acid Sequence Alignment of Japanese Smelt AFP with Herring and Smelt Type II AFPs.
Sequence alignment of Japanese smelt AFP and herring and smelt AFPs was done with the Mac Vector. Identical amino acid residues in two or all three species are shown in gray boxes. Open stars, calcium-binding residues of herring AFP. Closed stars, difference of calcium-binding amino acid residues between Japanese smelt and herring AFP.

herring AFP gene. The amino acid compositions of various AFPs are shown in Table 2. The Cys, Leu, Phe, Ser, His, Ala, Trp, and Met proportions of this Japanese smelt AFP and the herring AFP were identical. The amino acid sequence of Japanese smelt AFP was similar to that of herring AFP, but dissimi-

Table 2. Amino Acid Compositions (mol%) of Various Antifreeze Proteins

Amino acid residue	AFP (Japanese smelt)	Type II[13] (herring)	Type II[13] (smelt)	AFGP[23] (polar cod)	Type I[5] (winter flounder)	Type III[6] (ocean pout)
Cys	7.4	7.4	3.5	0.0	0.0	0.0
Leu	9.5	9.5	9.7	0.0	5.8	3.4
Phe	3.4	3.4	5.3	0.0	0.0	1.7
Ser	8.1	8.1	7.9	0.0	3.3	3.9
His	2.7	2.7	4.4	0.0	0.0	0.0
Ala	8.8	8.8	7.9	50.0	62.8	10.4
Met	4.1	4.1	5.3	0.0	0.0	8.2
Trp	5.3	5.3	4.4	0.0	0.0	0.0
Asp/Asn	8.8	10.1	10.5	0.0	11.5	6.3
Glu/Gln	7.4	8.1	6.1	0.0	1.6	12.1
Gly	5.4	4.1	8.8	0.0	0.0	7.9
Arg	1.4	2.0	0.9	0.0	1.6	0.0
Thr	11.5	8.8	7.9	33.3	9.9	9.1
Pro	3.4	4.1	3.5	16.7	0.0	10.4
Tyr	2.0	0.7	1.8	0.0	0.0	1.5
Val	5.4	4.1	2.6	0.0	0.0	11.5
Ile	2.0	4.7	4.4	0.0	0.0	7.2
Lys	3.4	4.1	4.4	0.0	3.3	6.4

lar to that of smelt AFP (the two fishes belong to the same species). The portion of Thr residues in our AFP was higher than in the two other Type II AFPs.

Discussion

Antifreeze proteins depress the freezing temperature of water by their binding to planes of ice crystals, thereby inhibiting crystal growth and ice recrystallization.[20] Wierzbicki *et al.* have identified the ice-binding mechanism of Type II fish AFP, including there being a surface that is a specific ice-binding site.[21] Type II AFPs are found in the sea raven (*Hemitrypterus americanus*),[11] smelt (*Osmerus mordax*),[13] and Atlantic herring (*Clupea harengus*).[13] The characteristic feature of this class is that they are Cys rich molecules (up to 9.1 mol%); Ala is the most common amino acid in them (up to 14.4 mol %). Smelt Type II AFP differs from the other two just named in its small amount of glucosamine (~3%). In spite of being the same species as smelt, the AFP from Japanese smelt had no sugars in its structure from the result of TLC after hydrolysis (not shown). Type II AFPs resemble the carbohydrate-recognition domain of calcium-dependent C-type lectins; their three-dimensional folds are similar. However, such AFPs have no sugar binding activity, although they have a galactose-binding motif.

On the basis of their calcium requirements, Type II AFPs can be divided into calcium-dependent (herring and smelt)[13] and calcium-independent (sea raven)[11] kinds. The AFPs of herring and smelt are unique among all known AFPs in their absolute requirement for a cofactor, Ca^{2+}, for antifreeze activity. These two Ca^{2+} dependent AFPs can be studied for the identification of the role of metal ions in the ice-binding.[11,13] The replacement of Ca^{2+} in calcium-dependent Type II AFP with another divalent metal ion not only reduces activity but also gives ice crystals of different shape. Ca^{2+} ions seem be directly involved in ice-binding and may stabilize the protein conformation. These results suggested that the antifreeze activity of Japanese smelt AFP was not be affected by Ca^{2+} ions. The ruthenium red staining indicated that Japanese smelt AFP had at least one Ca^{2+}-binding site. Although the sequence of Japanese smelt AFP was 83.0% identical with that of herring AFP (83.0%), its antifreeze activity did not disappeared completely with the removal of Ca^{2+} ions. The smelt and herring AFPs have conserved residues that form a Ca^{2+}-binding site corresponding to Ca^{2+}-binding site 2 of the galactose-binding C-type lectins.[18,22] By amino acid replacement of herring AFP Ca^{2+}-binding motif, from herring AFP with the sequence Gln-Pro-Asp to a mutant type of herring AFP with the sequence Glu-Pro-Asn, a mutant AFP had normal Ca^{2+}-binding, folding, and stability, but had no antifreeze activity.[21] The 130th amino acid residue, Asp of Japanese smelt AFP, is different from Ca^{2+}-binding amino acids of the herring AFP. The Asp seems to play a role in the stabilization of Japanese smelt AFP structure and it seems to be important to activate its antifreeze activity. However, the role of the Asp on antifreeze activity remains unknown.

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