

Antifreeze Activity in Temperate Fish from the East Sea, Korea

Hak Jun Kim*

Department of Chemistry, Pukyong National University, Busan 608-739, Korea

Abstract

Antifreeze proteins and glycoproteins [AF(G)Ps] constitute a group of proteins that lower the freezing but not the melting points of aqueous solutions, enabling polar and north-temperate fish to survive in ice-laden environments. However, little is known about antifreeze activity in temperate fish; such work would extend our knowledge on the functions and evolution of AF(G)Ps. In the present study, we screened for antifreeze activity in temperate fish caught off the coast of Jumunjin (37.89°N), Gangneung, Korea. Thermal hysteresis (TH) and the ability to inhibit ice recrystallization (IR) in blood, liver, and muscle samples from nine fish were examined to assess antifreeze activity. As the East Sea off the coast of Jumunjin is ice-free year round, we thought it most unlikely that the fish would express antifreeze proteins. Surprisingly, the blood of *Pleurogrammus azonus* and three types of tissue from *Gymnocanthus herzensteini*, *Zoarces gilli* and *Kareius bicoloratus* exhibited measurable TH values together with the ability to trigger characteristic morphological changes in ice crystals. Blood samples from the three species also evidenced ice recrystallization (IR) inhibition. This implies that AF(G)Ps or other antifreeze-like substances are present in temperate fish even under nonfreezing conditions. These results contribute to our understanding of the functions and origins of antifreeze activity in fish.

Key words: Antifreeze protein, Thermal hysteresis, Ice recrystallization inhibition, Temperate fish, *Gymnocanthus herzensteini*, *Zoarces gilli*, *Kareius bicoloratus*

Introduction

Polar and northern Atlantic fish species survive in ice-laden or subzero environments. Antifreeze proteins and glycoproteins [AF(G)Ps], which are biological antifreezes, seem to be critical in terms of cold adaptation (DeVries and Wohlschlag, 1969; DeVries, 1971). AF(G)Ps, which were first discovered in the serum of Antarctic fish by DeVries and Wohlschlag (1969), are a group of proteins that lower the freezing but not the melting points of aqueous solutions (Jia and Davies, 2002). Solutes in the normal body fluids of temperate fish can depress the freezing point to -0.7°C , but polar and northern temperate fish must tolerate seawater at or below -1.9°C (DeVries, 1971; Davies and Hew, 1990; Fletcher et al., 2001). AF(G)Ps further depress the freezing point by about -1°C , preventing fish fluids from freezing. Such freezing point depression is mediated by an adsorption–inhibition mechanism (Raymond and DeVries,

1977). AF(G)Ps adsorb (or bind) irreversibly to the surfaces of ice crystals in blood and inhibit their further growth, thereby lowering the blood freezing point. The temperature gap thus created, termed thermal hysteresis (TH), is a measure used to quantitatively assess the activity of AF(G)Ps.

Also, AF(G)Ps inhibit ice recrystallization (IR), a process whereby larger ice crystals grow at the expense of smaller crystals (Knight et al., 1984). IR is fatal to overwintering organisms (such as insects and plants) that experience fluctuations in temperature (Raymond and Fritsen, 2001; Raymond and Knight, 2003). IR inhibition likely protects membranes from freezing and helps organisms survive cold conditions (Janech et al., 2006). However, the IR property is not necessarily exploited by fish because they cannot tolerate freezing. In the present work, we regard both TH and IR inhibition as

 © 2015 The Korean Society of Fisheries and Aquatic Science

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received 4 February 2015; Revised 19 March 2015
Accepted 20 March 2015

*Corresponding Author
E-mail: kimhj@pknu.ac.kr

forms of “antifreeze activity.” The unique properties of AF(G)Ps impart many potential applications to such proteins, including their use as cryoprotectants in cell and tissue biobanks, in organ preservation, as food preservatives, as anti-icing agents, and in transgenic technologies seeking to impart cold-resistance (Davies et al., 1989; Hew et al., 1992; Wohrmann, 1996; Barrett, 2001; Ben, 2001; Bouvet and Ben, 2003; Harding et al., 2003; Fuller, 2004).

To date, five structurally diverse types of AF(G)Ps have been isolated from fish of the Antarctic, Arctic, and north Atlantic oceans: termed types I–IV and antifreeze glycoprotein (AFGP) (Davies and Sykes, 1997; Fletcher et al., 2001). We do not further discuss type IV AFP because its function has not been clearly established. Winter flounder and other fish from the east coast (50°N) of Canada express antifreeze proteins, which is important, as sea ice can form off this coast during winter. Nishimiya et al. (2008) extensively surveyed fish from coastal waters off Hokkaido and found that at least 50 species contained AF(G)Ps. This is not surprising; although Hokkaido is at 43.06°N, the coastal sea freezes during winter (Ice Information Center, 2015). Therefore, many fish in this area have evolved AF(G)Ps to facilitate adaptation to cold. Molecular analysis of AF(G)P genes suggested that novel antifreeze functions developed because of selective pressure (e.g., an extremely cold environment); the proteins do not share a common progenitor (Cheng, 1998; Cheng and Chen, 1999; Cheng et al., 2003; Cheng et al., 2006; Graham et al., 2013). Thus, AF(G)Ps constitute a textbook example of convergent evolution, supported by the fact that antifreeze activity (except that of AFGP) is generally absent from temperate and tropical fish. Functional AFGP genes have been described in fish of the sub-order Notothenioidei (Order Perciformes) living in temperate waters off New Zealand; the ancestral gene likely originated in Antarctica (Cheng et al., 2003). Although the AF(G)Ps of polar and north-temperate fish have been well studied, those of temperate fish have not. We considered that further studies on antifreeze activity in fish living in ice-free and temperate oceans might yield insights into the distribution and evolution of AF(G)Ps. Hence, we sought antifreeze activity in temperate fish living off the coast of Jumunjin (37.89°N), Gangneung, Korea.

Materials and Methods

Sample preparation

Nine fish species from the East Sea were purchased January 8, 2010, from the fish market of Jumunjin (37.89°N), Gangneung, Korea (Table 1). Blood, liver, and muscle were flash-frozen in liquid nitrogen on site and brought to the laboratory. Frozen blood was thawed on ice. Fifty μ L amounts of precooled 1 \times phosphate buffered saline (PBS) were added to the same volumes of blood, followed by thorough mixing

using pipettes. These suspensions were frozen at -80°C and then thawed on ice. This freeze–thaw step was repeated three additional times. The suspensions were centrifuged at 13,000 rpm and 4°C for 10 min, and supernatants were collected and kept at -80°C prior to analysis. Frozen liver and muscle samples were thawed on ice and washed with 1 \times PBS to remove residual blood. Two hundred microgram volumes of each tissue sample were weighed, cut into small pieces, and ground in liquid nitrogen in a homogenizer. Each sample was next suspended in 200 μ L precooled 1 \times PBS. The freeze–thaw cycle described above was applied three times, with thorough vortexing between cycles. Each suspension was centrifuged at 13,000 rpm and 4°C for 20 min, and the supernatant was harvested and stored at -80°C prior to use.

Thermal hysteresis (TH) activity

The thermal hysteresis (TH) and ice crystal morphology of each sample were examined using a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand), as described elsewhere (Lee et al., 2010). The osmometer was connected to a cold-well stage mounted on an Olympus CH-2 microscope equipped with a Canon Digital Camera. Briefly, about 1.5 μ L of each sample was loaded into the osmometer, which was then completely filled with oil. The sample well was mounted onto the cold-well stage, rapidly frozen, and held below -20°C. This process created polycrystalline ice. The temperature was next slowly raised until only a single ice crystal remained; this was considered the melting point. The temperature was next lowered again at about 0.05°C/min, and morphological changes in ice crystals were examined. The temperature at which ice crystals began to grow rapidly was considered the freezing point. The TH value was calculated by subtracting the latter value from the melting point. All TH values were measured in triplicate. Ice crystal morphology was observed and recorded during antifreeze activity measurement; the same experimental method was employed.

Ice recrystallization inhibition assay

IR inhibition was measured using the method of Smallwood (Smallwood et al., 1999) with the aid of a Linkam TMHS600 cold stage (Linkam Scientific Instruments, Surrey, UK) mounted on an Olympus BX51 light microscope. Briefly, glycerol was added to each sample to a final concentration of 30% (v/v). Two 1-microliter amounts of each mixture were layered between two coverslips and loaded onto a silver block located within a THMS600 cold stage. The temperature was quickly lowered to -80°C at a rate of 90°C/min, and this temperature was maintained for 10 min; fine ice crystals developed. The temperature was next increased to -6°C, and this temperature was maintained for 60 min, during which time changes in the ice crystals were noted; smaller ice crystals grew into larger crystals.

Results and Discussion

Thermal hysteresis

All fish species studied were caught off the coast of Jumunjin (Table 1), where more than 77 fish species can be found (Yang et al., 2012; Lee et al., 2013; Sohn et al., 2014); all were caught on the morning of the sampling day. The Korea Ocean Observing and Forecasting System reported that the average temperature of surface seawater in the Jumunjin area 1 week before and 1 week after the date of purchase was about 9°C, well above the freezing point of seawater (http://sms.khoa.go.kr/koofs/eng/observation/obs_real_map.asp). Also, sea ice does not form in this area even in winter. Our fish samples comprised three orders, six families, and nine species (Table 1). Four were of the order Scorpaeniformes, one of the order Perciformes, and four of the order Pleuronectiformes. The rightmost column of Table 1 lists fish

species exhibiting antifreeze activity expressed by relevant genes (Graham et al., 2013). As TH and changes in ice crystal morphology are indicative of the presence of antifreeze proteins, the THs of blood, liver, and muscle samples were measured using a nanoliter osmometer, and we simultaneously observed changes in ice crystals. The detection limit of the osmometer was 0.01°C. Intriguingly, of the nine fish species tested, four exhibited discernible blood TH values; these were *P. azonus*, *G. herzensteini*, *Z. gilli*, and *K. bicoloratus* (Table 2). Weak activity was evident in the liver and muscle samples of *G. herzensteini*, *Z. gilli*, and *K. bicoloratus*, but not *P. azonus*, perhaps indicating that the antifreeze protein(s) in *P. azonus* blood are all produced by the liver, whereas the other three species express more than one type of antifreeze protein. As shown in Table 2, the TH values also differed among species. *G. herzensteini* had the highest value of all tested species, and all tissue samples of *K. bicoloratus* exhibited consistent activity. However, the TH

Table 1. List of fish species used in this study and their north-temperate counterparts with functional antifreeze proteins. Habitats of these fishes are described in footnote

Fishes used in this study [‡]			Fishes whose antifreeze proteins are known		
Order	Scorpaeniformes		Order	Scorpaeniformes	
	Family	Hexagrammidae		Family	Liparidae
		<i>Pleurogrammus azonus</i>			<i>Liparis atlanticus</i> [†] (Type I AFP)
		<i>Hexagrammos agrammus</i>			<i>Liparis gibbus</i> [†] (Type I AFP)
	Family	Cottidae		Family	Cottidae
		<i>Gymnocephalus herzensteini</i>			<i>Myoxocephalus octodecemspinosus</i> (Type I AFP)
	Family	Sebastidae			<i>Myoxocephalus scorpius</i> [†] (Type I AFP)
		<i>Sebastes inermis</i>		Family	Scorpaenidae
					<i>Hemitripterus americanus</i> [†] (Type II AFP)
Order	Perciformes		Order	Perciformes	
	Family	Zoarcidae		Family	Zoarcidae
		<i>Zoarces gillii</i> Jordan et stark			<i>Zoarces gillii</i> Jordan et stark (Type III AFP)
				Family	Labridae
					<i>Tautoglabrus adspersus</i> [†] (Type I AFP)
				Family	Nototheniidae
					<i>Dissostichus mawsoni</i> [‡] (AFGP)
Order	Pleuronectiformes		Order	Pleuronectiformes	
	Family	Pleuronectidae		Family	Pleuronectidae
		<i>Pseudopleuronectes herzensteini</i>			<i>Pseudopleuronectes americanus</i> [†] (Type I AFP)
		<i>Kareius bicoloratus</i>			
		<i>Eopsetta grigorjewi</i>			
	Family	Paralichthyidae			
		<i>Paralichthys olivaceus</i>			

[‡]Temperate species, Jumunjin (37.89 °N), East Sea, Korea

[†]North-temperate and polar species, Atlantic coast of North America

[‡]Polar species, Antarctica

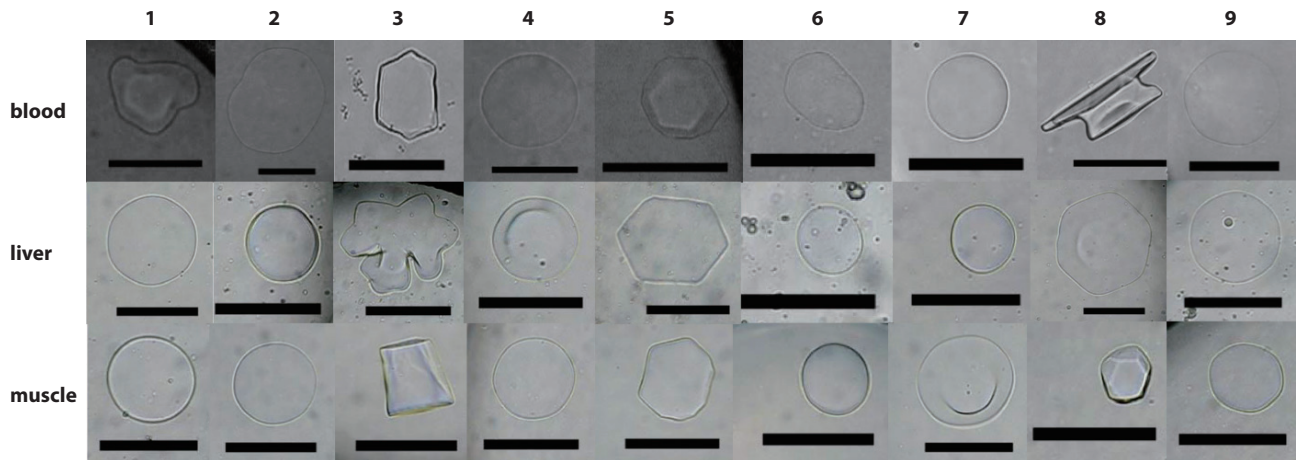


Fig. 1. Ice crystal morphologies of blood, liver, and muscle samples from nine fishes. The hexagonal ice crystal shapes shown in 3, 5, 8 samples indicates existence of antifreeze-like substances. Numbers are used in place of scientific name of fish species: 1, *P. azonus*; 2, *H. agrammus*; 3, *G. herzensteini*; 4, *S. inermis*; 5, *Z. gillii*; 6, *P. oliveceus*; 7, *P. herzensteini*; 8, *K. bicoloratus*; 9, *E. grigorjewi*. The scale bar indicates 100 μ m.

values of the other species were very low (sometimes close to the limit of detection). This may indicate the absence, or minimal presence, of antifreeze proteins in these species. As mentioned above, AF(G)Ps change ice morphologies. When fish AF(G)Ps bind to a specific ice plane, the shape of the ice crystal changes from round to hexagonal bipyramidal or lemon-like (Wathen et al., 2003). As shown in Fig. 1, *P. azonus*, *G. herzensteini*, and *Z. gillii* developed hexagonal ice crystals whereas *K. bicoloratus* blood developed a different form of crystal. Control PBS developed round crystals, indicative of the absence of antifreeze activity (data not shown). Together, the data suggest that four species (*P. azonus*, *G. herzensteini*, *Z. gillii*, and *K. bicoloratus*) appear to exhibit antifreeze activity, indicating that their tissues express antifreeze proteins or antifreeze-like substances. As shown in Table 1, with the exception of *P. azonus*, the three species exhibiting TH are

in a family that contains polar or north-temperate species that express antifreeze proteins (Graham et al., 2013). Thus, AF(G)Ps or antifreeze-like substances are present in temperate fish even under nonfreezing conditions. Although all fish exhibiting TH in the present study were coldwater species, our findings are surprising because the fish never encounter a subzero environment. The sea temperature is well above freezing all year round. Family members from Hokkaido and the east coast of Canada have antifreeze proteins because they are seasonally exposed to subzero conditions (Nishimiyama et al., 2008; Graham et al., 2013). Just as the nototheniids of New Zealand evolved from an Antarctic ancestor, the fish that we studied may have had an Arctic ancestor, because the last glaciation in the Korean peninsula terminated around 0.15 My ago. It remains unclear why TH activity is required by fish living in temperate waters.

Table 2. Thermal hysteresis of nine fishes used in this study

Order	Species	Thermal hysteresis ($^{\circ}$ C)		
		Blood	Liver	Muscle
Scorpaeniformes	<i>Pleurogrammus azonus</i>	0.06 \pm 0.01	0.03 \pm 0.01	0.02 \pm 0.01
	<i>Hexagrammos agrammus</i>	0.03 \pm 0.01	0.02 \pm 0.01	0.01 \pm 0.01
	<i>Gymnocanthus herzensteini</i>	0.1 \pm 0.01	0.06 \pm 0.01	0.04 \pm 0.01
	<i>Sebastes inermis</i>	0.02 \pm 0.01	0.03 \pm 0.01	0.01 \pm 0.01
Perciformes	<i>Zoarces gillii</i> Jordan et starks	0.07 \pm 0.01	0.05 \pm 0.01	0.04 \pm 0.01
Pleuronectiformes	<i>Paralichthys oliveceus</i>	0.02 \pm 0.01	0.01 \pm 0.01	0.01 \pm 0.01
	<i>Pseudopleuronectes herzensteini</i>	0.02 \pm 0.01	0.01 \pm 0.01	0.01 \pm 0.01
	<i>Kareius bicoloratus</i>	0.03 \pm 0.01	0.04 \pm 0.01	0.03 \pm 0.01
	<i>Eopsetta grigorjewi</i>	0.01 \pm 0.01	0.01 \pm 0.01	0.01 \pm 0.01

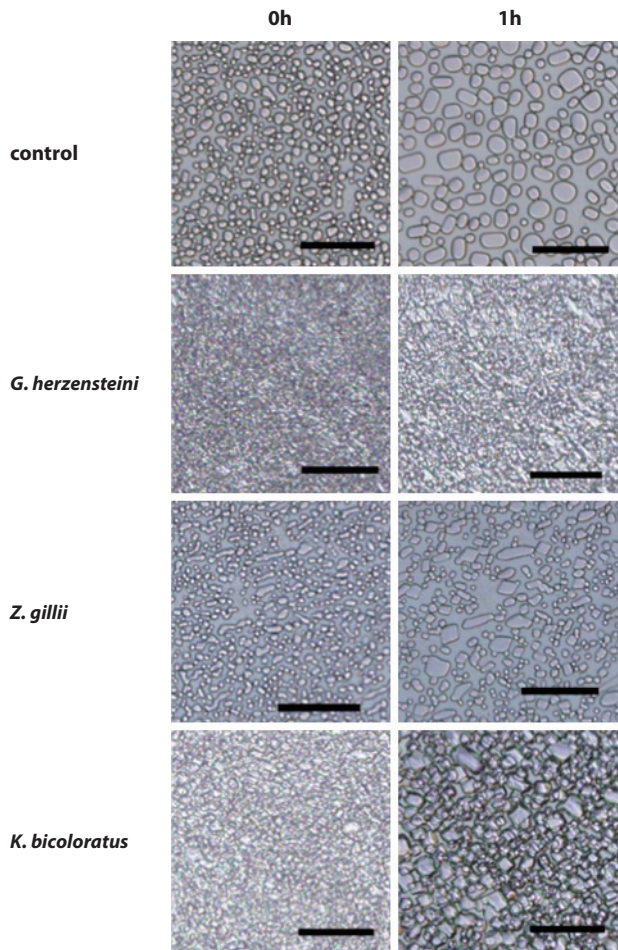


Fig. 2. Ice recrystallization inhibition activity by blood samples from *G. herzensteini*, *Z. gillii*, and *K. bicoloratus*. PBS was used as control. After rapid freezing, the thin disc of sample solution was maintained at -80°C for 1 h and the temperature was raised to at -6°C to provoke ice recrystallization and maintained for at least 1 h. Ice crystal images were collected every 5 min for 60 min. The control underwent distinct ice recrystallization, however three samples showed lesser recrystallization. The scale bar indicates 100 μm .

Ice recrystallization inhibition

IR inhibition by *G. herzensteini*, *Z. gillii*, and *K. bicoloratus* was investigated. Blood samples (at -80°C) of these three species are shown in the left panel of Fig. 2. Rapid freezing created polycrystalline ice (left panel). Microcrystals in such suspensions grow into a small number of larger crystals when the temperature is raised to -6°C and this temperature is maintained for 1 h. Crystals thus formed are shown in the right panel. Compared with the PBS control, all samples displayed IR inhibition to some extent; *G. herzensteini* exhibited the highest such activity. As the high salt level (8 mg/mL NaCl) of PBS compromises IR, the activity that we noted was significant. To the best of our knowledge, this is the first report on antifreeze activity in temperate fish other than no-

totheniids. Further efforts should be made to isolate AF(G)Ps from fish sera, and genetic analysis would shed light on AF(G)P expression in fish from the East Sea, in turn yielding information on the function, regulation, and origin of AF(G)Ps.

Acknowledgments

The author appreciates Dr. Woong Sic Jung for conducting some of experiments. This work is supported by Creative Research Grant (2013) of Pukyong National University.

References

- Barrett J. 2001. Thermal hysteresis proteins. *Int J Biochem Cell Biol* 33, 105-117.
- Ben RN. 2001. Antifreeze glycoproteins--preventing the growth of ice. *Chembiochem* 2, 161-166.
- Bouvet V and Ben RN. 2003. Antifreeze glycoproteins: Structure, conformation, and biological applications. *Cell Biochem Biophys* 39, 133-144.
- Cheng CH. 1998. Evolution of the diverse antifreeze proteins. *Curr Opin Genet Dev* 8, 715-720.
- Cheng CH and Chen L. 1999. Evolution of an antifreeze glycoprotein. *Nature* 401, 443-444.
- Cheng CH, Chen L, Near TJ and Jin Y. 2003. Functional antifreeze glycoprotein genes in temperate-water new zealand nototheniid fish infer an antarctic evolutionary origin. *Mol Biol Evol* 20, 1897-1908.
- Cheng CH, Cziko PA and Evans CW. 2006. Nonhepatic origin of nototheniid antifreeze reveals pancreatic synthesis as common mechanism in polar fish freezing avoidance. *Proc Natl Acad Sci USA* 103, 10491-10496.
- Davies PL, Fletcher GL and Hew CL. 1989. Fish antifreeze protein genes and their use in transgenic studies. *Oxf Surv Eukaryot Genes* 6, 85-109.
- Davies PL and Hew CL. 1990. Biochemistry of fish antifreeze proteins. *Faseb J* 4, 2460-2468.
- Davies PL and Sykes BD. 1997. Antifreeze proteins. *Curr Opin Struct Biol* 7, 828-834.
- DeVries AL. 1971. Glycoproteins as biological antifreeze agents in antarctic fishes. *Science*, 172 1152-1155.
- DeVries AL and Wohlschlag DE. 1969. Freezing resistance in some antarctic fishes. *Science* 163, 1073-1075.
- Fletcher GL, Hew CL and Davies PL. 2001. Antifreeze proteins of teleost fishes. *Annu Rev Physiol* 63, 359-390.
- Fuller BJ. 2004. Cryoprotectants: The essential antifreezes to protect life in the frozen state. *Cryo Letters* 25, 375-388.
- Graham LA, Hobbs RS, Fletcher GL and Davies PL. 2013. Helical antifreeze proteins have independently evolved in fishes on four occasions. *PLoS One* 8, e81285.
- Harding MM, Anderberg PI and Haymet AD. 2003. 'Antifreeze' glyco-

- proteins from polar fish. *Eur J Biochem* 270, 1381-1392.
- Hew CL, Davies PL and Fletcher G. 1992. Antifreeze protein gene transfer in atlantic salmon. *Mol Mar Biol Biotechnol* 1, 309-317.
- Ice information center J. 2015. Sea ice statistics. Retrieved from http://www1.kaiho.mlit.go.jp/KANI/drift_ice/statistics/statistics_e.html
- Janech M, Krell A, Mock T, Kang J-S and Raymond J. 2006. Ice-binding proteins from sea ice diatoms (bacillariophyceae). *J Phycol* 42, 410-416.
- Jia Z and Davies PL. 2002. Antifreeze proteins: An unusual receptor-ligand interaction. *Trends Biochem Sci* 27, 101-106.
- Knight CA, DeVries AL and Oolman LD. 1984. Fish antifreeze protein and the freezing and recrystallization of ice. *Nature* 308, 295-296.
- Lee JK, Park KS, Park S, Park H, Song YH, Kang SH and Kim HJ. 2010. An extracellular ice-binding glycoprotein from an arctic psychrophilic yeast. *Cryobiology* 60, 222-228.
- Lee SJ, Jung Y-S, Kweon S-M and Kim J-K. 2013. First record of the eyeshade sculpin *nautichthys pribilovius* (hemitripterae: Pisces) from the east sea, korea. *Fish Aquat Sci* 16, 207-209.
- Nishimiya Y, Yasuhiro M, Hirano Y, Kondo H, Miura A and Tsuda S. 2008. Mass preparation and technological development of an antifreeze protein. *Synthesiology - English Edition* 1, 7-14.
- Raymond JA and DeVries AL. 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *Proc Natl Acad Sci USA* 74, 2589-2593.
- Raymond JA and Fritsen CH. 2001. Semipurification and ice recrystallization inhibition activity of ice-active substances associated with antarctic photosynthetic organisms. *Cryobiology* 43, 63-70.
- Raymond JA and Knight CA. 2003. Ice binding, recrystallization inhibition, and cryoprotective properties of ice-active substances associated with antarctic sea ice diatoms. *Cryobiology* 46, 174-181.
- Smallwood M, Worrall D, Byass L, Elias L, Ashford D, Doucet CJ, Holt C, Telford J, Lillford P and Bowles DJ. 1999. Isolation and characterization of a novel antifreeze protein from carrot (*daucus carota*). *Biochem J* 340, 385-391.
- Sohn MH, Yoon BS, Park J-H and Choi YM. 2014. Species composition and distribution of trammel net catches in the coastal waters of gangwon province, Korea. *Korean J Fish Aquat Sci* 47, 945-959.
- Wathen B, Kuiper M, Walker V and Jia Z. 2003. A new model for simulating 3-d crystal growth and its application to the study of antifreeze proteins. *J Am Chem Soc* 125, 729-737.
- Wohrmann A. 1996. Antifreeze glycoproteins in fishes: Structure, mode of action and possible applications. *Tierarztl Prax* 24, 1-9.
- Yang JH, Yoon SC, Lee SI, Chag HK, Kim JB, Choi YM and Park JH. 2012. Age and growth of blackfin flounder *glyptocephalus stelleri* in the east sea, Korea. *Fish Aquat Sci* 15, 169-176.