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Characteristics of Antifreeze Protein-1 Induced during Low Temperature Acclimation in the *Protaetia brevitarsis* (Coleoptera; Cetonidae) Larva

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Change of proteins was confirmed during low temperature acclimation of overwintering larva, and some biochemical characteristics of the induced antifreeze protein-1 (AFP-1) were investigated in *Protaetia brevitarsis*. As the freezing point depression by the action of induced AFPs, a considerable thermal hysteresis was observed in the haemolymph and in partially purified proteins. AFP-1 was purified from the cold acclimation larvae by ammonium sulfate precipitation, ion exchange chromatography, gel permeation chromatography, and electroelution. The purified AFP-1 was determined to be a glycoprotein (approximately 320 kDa, pI 5.8) composed of a single type of subunit (80 kDa). The high contents of hydrophilic amino acids (Asp, Glu, Lys, Asn, Gln, Arg, Ser, Thr) were also confirmed, showing similarity with antifreeze proteins from other insects.

Overwintering insects exposed to freezing temperatures are generally classified into two groups: freeze-tolerant and freeze-intolerant (freeze-susceptible). Freeze-tolerant insects avoid extensive supercooling by means of haemolymph ice nucleating proteins (Duman et al., 1985; Lee, 1991). However, not all freeze-tolerant species have haemolymph ice nucleating agents (Miller, 1982; Ring, 1982). Ice nucleating protein has been described in a variety of insects, which functions as ice catalysts in the insect haemolymph (Duman et al., 1984; Lee, 1991). In contrast, freeze-susceptible insects must avoid freezing by seasonally depressing their freezing and supercooling points through proliferation of AFPs (Patterson and Duman, 1978; Duman, 1984). The insect AFPs have not been as extensively investigated as those of the fishes. However, AFPs have been purified from some insect species - *Oncopeltus fasciatus* (Patterson et al., 1981; Zachariassen, 1985), *Tenebrio molitor* (Patterson and Duman, 1982), and *Dendroides canadensis* (Duman, 1984). These proteins enhance low temperature survival by depressing the freezing point, thus producing a difference between the melting and freezing points (Raymond and De Vries, 1977; Duman, 1984; Lee, 1991). Acclimation to different environmental conditions (low temperature, short photoperiod, low relative humidity) induces a significant increase in thermal hysteresis in *Meracantha contracta* (Duman, 1977), *Tenebrio molitor* (Patterson and Duman, 1978), and *Dendroides canadensis* (Horwath and Duman, 1983, 1986; Li et al., 1998).

In this paper, we not only provide information on cold resistance of overwintering larvae in *Proteatia brevitarsis* but also data on the purification and the biochemical properties of AFP-1 induced by cold acclimation.

Materials and Methods

Insects

The overwintering larvae of *P. brevitarsis* were collected from rice straw in the vicinity of Taejon city late October, and then reared on fermented rice straw and soil at 15±1℃, 12L:12D photoperiod, and 75±5% relative humidity.

Cold treatments

For cold acclimation, the insects were isolated from the soil and then transferred to a low temperature incubator maintained at successive temperatures of coldness and warmth (10, 5, 0, 5, 10, 20℃) for 10 d. The insects used in a series of experiments were collected under each of the conditions shown in Table 1.

Collection of haemolymph

Haemolymph was collected into a cold microfuge tube by cutting legs. After addition of a few crystals of phenylthiourea, haemolymph was centrifuged at 10,000 rpm for 20 min to remove cellular debris, and the supernatant was stored at -70℃ until used.

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Table 1. *Temperature acclimation schedule for experiment*

Experimental groups	Conditions
A	10℃/10 days ↓
B	5℃/10 days ↓
C	0℃/10 days ↓
D	5℃/10 days ↓
E	10℃/10 days ↓
F	20℃/10 days

Samples were collected after exposure to each series of acclimation (coldness and wormth) temperature for 10 days.

Protein determination

Protein contents were determined by the method of Lowry et al. (1951) with slight modifications. Bovine serum albumin (BSA) was used as the standard protein.

Measurement of thermal hysteresis

Thermal hysteresis (melting point minus freezing point) activity was measured using a modification of the drop-freezing technique of Vali (1971). The sample (5 mg/ml) to be tested was loaded in 1% paraffin-sealed aluminum foil, and the sample (10 μl) was placed into a temperature-controlled, refrigerated ethylene glycol bath where the seed crystal could be observed.

Precipitation of haemolymph proteins

Haemolymph proteins of cold acclimated larvae (10, 5, 0, 5, 10, 20℃) were precipitated into 85% ammonium sulfate. The pellet was dialyzed against distilled water and the dialyzed supernatant was then concentrated by freeze-drying in a Max Dry Lyo freeze-dryer.

Purification

Haemolymph of cold acclimated larvae (0℃/10 d) was precipitated into four ammonium sulfate fractions 0~35, 35~50, 50~65, 65~80%, respectively. The pellet was dialyzed against distilled water and the dialyzed supernatant was then concentrated by a freeze-dryer. The lyophilized sample (50~65% ammonium sulfate fraction) containing AFPs was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and chromatographed on a DEAE Sepharose CL-6B column (200×25 mm) using a 0~1.0 M NaCl gradient. Fractions containing AFPs were dialyzed against distilled water to remove the remaining salts and freeze-dried again. Peak D-2 from the DEAE Sepharose CL-6B column was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and chromatographed on a Ultrogel AcA 34 column (1000×15 mm) at a flow rate of 0.15 ml/min. Peaks (U-3) were collected, dialyzed against distilled water, and freeze-dried. Partially purified proteins were electrophoresed on native gel and sliced with a blade. AFP-1 was eluted using a gel eluter (GE 200 SixPac, Hoeffer Co.), dialyzed against distiled water, and freeze-dried.

Native-PAGE

Native polyacrylamide gel electrophoresis was carried out on a 3~10% gradient gel at 4℃. After electrophoresis, gels were fixed, washed overnight in 12% trichloroacetic acid, and stained for 4 h in 0.2% Coomassie brilliant blue R-250.

The molecular weight of native AFP-1 was determined as described by Hedrick and Smith (1968) with some modifications. Standard proteins were thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and albumin (67,000).

Isoelectric focusing (IEF)

Isoelectric focusing was performed as described by O'Farrell (1975) with some modifications. Glass tubes (120×3 mm) were filled to 90 mm with a mixture containing polyacrylamide (5.5%) and ampholyte (2%). After electrofocusing, gels were equilibrated for 30 min in 0.1 M Tris-HCl buffer (pH 6.8) containing 20% glycerine and applied in the second dimension for electrophoresis. The pH gradient of IEF gels was determined by slicing one gel into 5 mm segments and incubating with 1 ml of distilled water for 24 h at room temperature.

Detection of conjugated proteins

Glycoprotein was identified by the method of Caldwell and Pigman (1965). After electrophoresis, the gel was incubated for 1 h at room temperature in 7.5% acetic acid and then for 1 h in 0.2% periodic acid at 4℃. They were subsequently transferred to Schiff's reagent after being rinsed in 15% acetic acid.

For the detection of lipoprotein, the method of Chippendale and Beck (1966) was used. Excess stain was removed with 70% ethanol.

SDS-PAGE

SDS-PAGE was carried out by the method described by Laemmli (1970) with slight modifications. Gel staining was performed in the same manner as in native-PAGE. Molecular weight of AFP-1 was measured by Weber and Osborn (1969) with some modifications. Standard proteins were phosphorylase (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,000).

Amino acid analysis

Protein sample was hydrolyzed for amino acid analysis in 6 N HCl for 16 h at 110℃. Amino acid analysis

Table 2. Melting point, freezing point, and thermal hysteresis of haemolymph (10 μl) in the *Protaetia brevitarsis* larva during successive temperatures of coldness and warmth acclimation

Experimental groups	Freezing point (℃)	Melting point (℃)	Thermal hysteresis (℃)
A	-5.3±0.08	-0.7±0.07	4.6±0.11
B	-5.8±0.07	-0.8±0.08	5.0±0.11
C	-6.5±0.12	-0.6±0.08	5.9±0.13
D	-6.7±0.13	-0.7±0.07	6.0±0.18
E	-6.1±0.11	-0.6±0.06	5.5±0.11
F	-6.2±0.13	-0.6±0.07	5.6±0.13

Each value represents the mean±SD obtained from ten replicate experiments. The abbreviations of experimental groups (A, B, C, D, E, F) are the same as in Table 1.

was performed on the HPLC system (TOSOH 8010 series).

Results

Thermal hysteresis of AFPs

Thermal hysteresis was monitored by measuring the freezing and the melting points. The haemolymph and 50~65% ammonium sulfate-precipitated proteins collected from 0℃ acclimated larvae showed 5.9 and 11.2℃ thermal hysteresis, respectively. In general, the thermal hysteresis of samples of lower temperature acclimation was higher than those of relatively high temperature acclimation (Tables 2, 3).

Isolation and purification of AFP

Electrophoretic analysis of haemolymph proteins revealed two induced proteins (AFP-1 and AFP-2), and three bands (D-1, D-2, and D-3) decreased or disappeared in larvae of coldness acclimation (0℃/10 d) and warm temperature (5℃/10 d). Their induction correlated to a decreasing or disappearing trend of some protein bands (D-1, D-2, and D-3) (Fig. 1).

AFPs were obtained in the 50~65% ammonium sulfate fraction of relatively high purity (Fig. 2), providing a simple way for isolating these proteins. AFP-1 was further purified by ion exchange chromatography, gel permeation chromatography, and electroelution (Fig. 3). The purity of the AFP-1 after each purification step was assayed by native-PAGE (Figs. 4, that

Table 3. Melting point, freezing point, and thermal hysteresis measured from haemolymph proteins (50 μg/10 μl) precipitated in 50~65% ammonium sulfate in the *Protaetia brevitarsis* larvae during successive temperatures of coldness and wormth acclimation

Experimental groups	Freezing point (℃)	Melting point (℃)	Thermal hysteresis (℃)
A	-11.0±0.14	-0.7±0.07	10.3±0.18
B	-11.0±0.13	-0.6±0.08	10.5±0.16
C	-11.8±0.11	-0.6±0.07	11.2±0.13
D	-11.7±0.14	-0.7±0.07	11.0±0.16
E	-11.4±0.12	-0.7±0.07	10.7±0.09
F	-11.4±0.10	-0.8±0.09	10.6±0.16

Each value represents the mean±SD obtained from ten replicate experiments. The abbreviations of experimental groups (A, B, C, D, E, F) are the same as in Table 1.

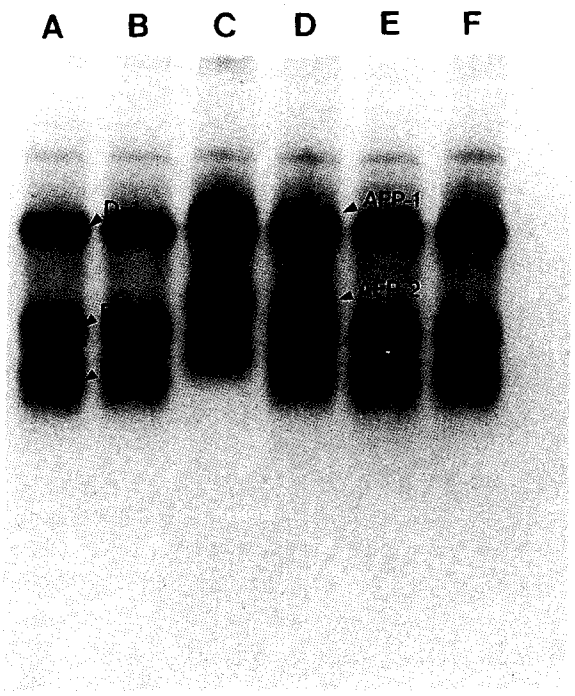

Fig. 1. Native-PAGE (3~10% gradient gel) of haemolymph proteins precipitated in 0~80% ammonium sulfate in the *Protaetia brevitarsis* larvae during successive temperatures of coldness and wormth acclimation. The abbreviations of experimental groups (A, B, C, D, E, F) are same as in Table 1.

5A). Electrophoresis of eluted AFP-1 on gradient polyacrylamide gel electrophoresis showed

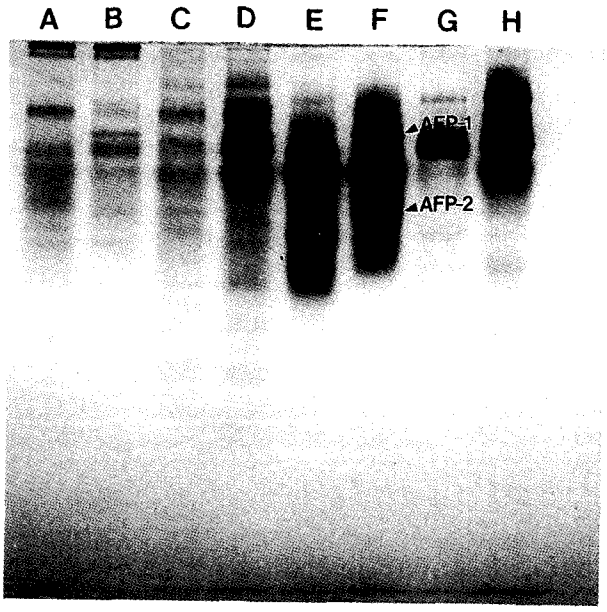

Fig. 2. Native-PAGE (3~10% gradient gel) of haemolymph protein in *Protaetia brevitarsis* larvae acclimated to 10℃ for 10 days (control) and to 0℃ for 10 days (cold acclimation). A and B, 0~35%; C and D, 35~50%; E and F, 50~65%; G and H, 65~80% saturated (control; A, C, E, G, cold acclimated; B, D, F, H); AFP, antifreeze protein.

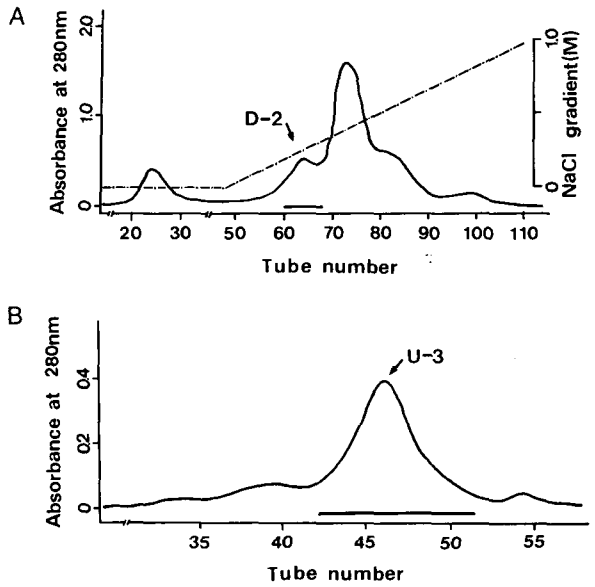

Fig. 3. Isolation of antifreeze protein-1 (AFP-1) from *Protaetia brevitarsis*. A, Ion exchange chromatography of precipitated haemolymph protein in 50~65% ammonium sulfate in *Protaetia brevitarsis* larvae acclimated to low temperature (0℃/10 days). The proteins were loaded on a DEAE Sepharose CL-6B column (200×25 mm), and then eluted at a flow rate of 0.3 ml/min with a linear gradient (0~1.0 M sodium chloride) of 50 mM Tris-HCl buffer (pH 7.5). B, Gel permeation chromatography of D-b fraction collected from ion exchange chromatography. The fraction (D-2) containing AFP-1 was applied to an Ultrogel AcA 34 column (1000×15 mm), and then eluted at a flow rate of 0.15 ml/min with Tris-HCl buffer (pH 7.5) containing 100 mM NaCl.

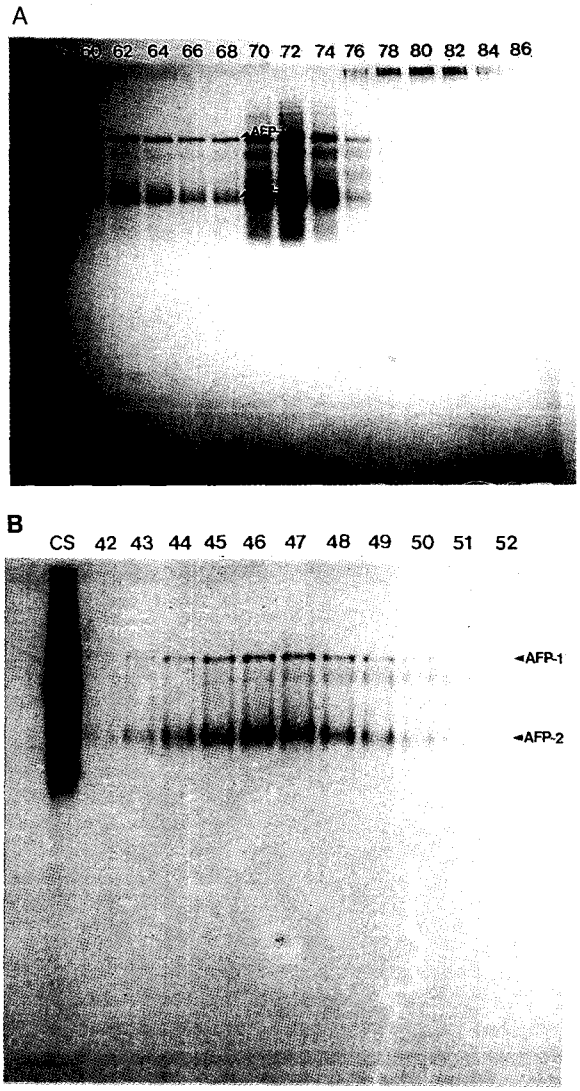

Fig. 4. Native-PAGE of fractions from Fig. 3. A, Native-PAGE (3~10% gradient gel) of *Protaetia brevitarsis* haemolymph proteins collected by ion exchange chromatography. The numbers represent the collected fractions. B, Native-PAGE (3~10% gradient gel) of *Protaetia brevitarsis* haemolymph proteins (U-3) collected by gel permeation chromatography. The numbers represent the collected fractions. CS, 0℃/10 days; AFP-1, antifreeze protein-1.

that this purified protein consisted of a single band when stained with Coomassie brilliant blue R-250 (Fig. 5A). Consequently, it was assumed that the eluted protein was pure.

Properties of AFP-1

The AFP-1 was found to have a native molecular weight of 320 kDa by electrophoretic analysis. Purified AFP-1 was isoelectrically focused on a 5.5% polyacrylamide gel, and the isoelectric point of AFP-1 was determined to be approximately pH 5.8 (Fig. 6). Native electrophoresis gels of the purified protein were detected with Schiff's reagent. The AFP-1 was found to be a glycoprotein (Fig. 5B), and the protein was composed of a single type subunit having a molecular weight of 80 kDa when determined with SDS-PAGE (Fig. 7). The amino acid composition of AFP-1 is shown in Table 4. As seen from this table, the high contents of lysine and histidine are notable. While relatively high contents of hydrophilic amino acids (Asp, Glu, Lys, Asn, Gln, Arg, Ser, Thr) were detected, proline, alanine and cysteine were not detected.

Discussion

The acclimation to different environmental conditions (low temperature, short photoperiod, low relative humidity) induces a significant increase in thermal hysteresis in *Meracantha contracta* (Duman, 1977), *Tenebrio molitor* (Patterson and Duman, 1978), and *Dendroides canadensis* (Horwath and Duman, 1983; Horwath and Duman, 1986). Insects adapt to the cold by producing AFPs (thermal hysteresis proteins) and cryoprotectants (polyhydroxy alchol) to prevent freeze damage (Miller and Smith, 1975; Storey and Storey, 1988; Lee, 1989, 1991). These proteins enhance low temperature survival by producing thermal hysteresis (melting point minus freezing point) (Raymond and De Vries, 1977; Duman, 1984; Lee, 1991). In this work with *P. brevitarsis*, the acclimation to low temperature induced AFPs and increased thermal hysteresis. The haemolymph and the partially purified proteins containing AFPs demonstrated a hyperbolic relationship between the induced AFPs concentration and thermal hysteresis

Table 4. Amino acid composition of antifreeze protein-1 (AFP-1) purified from *Protaetia brevitarsis* larvae acclimated at low temperature (0℃/10 days)

Amino acids	Antifreeze protein-1 (mole %)
Aspartic acid	3.2
Threonine	2.9
Serine	3.3
Glutarmic acid	3.2
Proline	-
Glycine	4.7
Alanine	-
Cysteine	-
Valine	2.8
Methionine	10.8
Isoleucine	6.7
Leucine	3.8
Tyrosine	3.0
Phenylalanine	3.0
Histidine	15.5
Lysine	30.4
Arginine	6.8
Total	100.1

activity (Tables 2, 3 and Figs. 1, 2). These data demonstrate that the induction of AFPs is responsible for the cold acclimation, and that will enhance insect survival at low winter temperatures.

Generally, AFPs of fish have been found to be glycoprotein (3~15 kDa) which consist of the same tripeptide repeating unit (alanylalanylthreonyl) (De Vries, 1971; Raymond and Devries, 1977; Wen and Laursen, 1992). However, AFP of *Dendroides canadensis* has been 70 kDa (Wu and Duman, 1991), and 8.7 kDa proteins consisting of seven 12- or 13-mer repeating units were identified recently (Li et al., 1998). In the present work, the native molecular weight of AFP-1 was determined to be approximately 320 kDa. It was also determined to be composed of a single type subunit with a molecular weight of approximately 80 kDa (Fig. 7). The isoelectric point of AFP-1 was determined to be approximately 5.8 (Fig. 6). The AFP-1

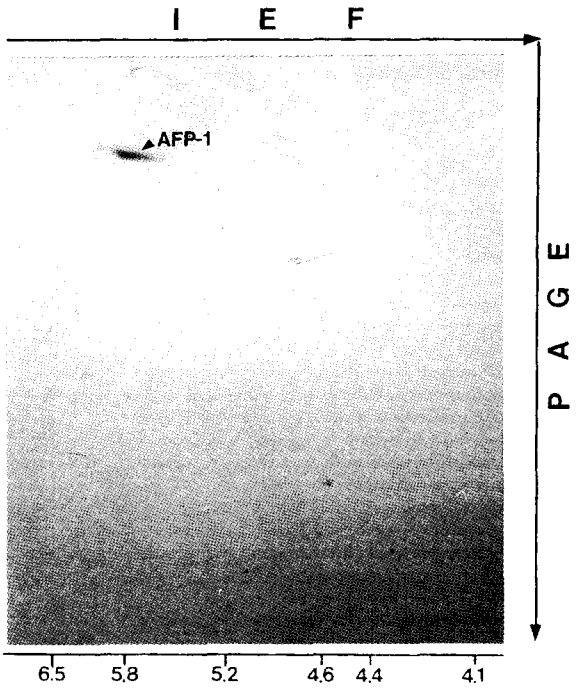

Fig. 6. Two-dimensional gel electrophoresis of the purified AFP-1 in *Protaetia brevitarsis*.

of *P. brevitarsis* was also found to be a glycoprotein (Fig. 5B). The amino acid composition of AFPs (TC-1 and TC-2) shows high contents of hydrophilic amino acids and cysteine as in *Tenebrio molitor* (Pattern and Duman, 1982). In the present work with *P. brevitarsis*, relatively high contents of hydrophilic amino acids (Asp, Glu, Lys, Asn, Gln, Arg, Ser, Thr) were also confirmed showing similarity with antifreeze proteins from other insects. In spite of the considerable structural variation, these antifreezes inhibit ice crystal

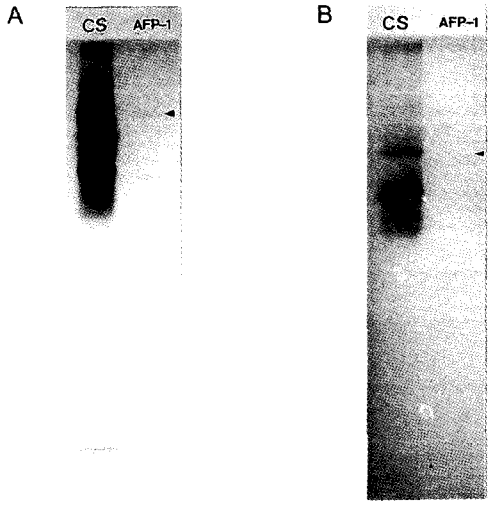

Fig. 5. Native-PAGE (3~10% gradient gel) of purified AFP-1 in *Protaetia brevitarsis*. A, The gel was stained with Coomassie blue R-250. B, The gel was stained with periodic acid Schiff's reagent. CS, 0℃/10 days; AFP-1, purified antifreeze protein-1.

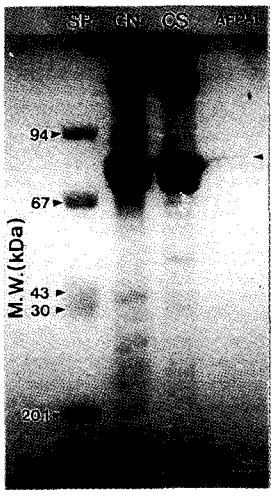

Fig. 7. SDS-PAGE (10% gel) of purified AFP-1 in *Protaetia brevitarsis*. SP, standard marker protein; CN, control (10℃/10 days); CS, cold acclimation (0℃/10 days).

growth by a noncolligative mechanism whereby the freezing point is depressed below the melting point, thus producing a so called thermal hysteresis (De Vries, 1986; Yang et al., 1988; Chapsky and Rubinsky, 1997).

In future studies, we will investigate both the biochemical properties of AFP-2, and the relationship between the AFPs and the disappearance of proteins (band D-1, D-2, and D-3).

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