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CLONING AND EXPRESSION OF A NOVEL ANTIFREEZE PROTEIN AFP72 FROM THE BEETLE *Tenebrio molitor*

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A novel antifreeze protein AFP72 cDNA (GenBbank accession No. AY929389) was obtained by RT-PCR from *Tenebrio molitor*. The 216 bp fragment encodes a protein of 72 amino acid residues. Sequence analysis revealed that the cDNA displays a high degree of homology with *T. molitor* antifreeze proteins, ranging up to 90.78%. Recombinant plasmids pMAL-p2X-*afp72* and pMAL-c2X-*afp72* were transferred into *E. coli* TBI to induce a MBP fusion protein by IPTG. The target fusion protein was released from the periplasm and cytoplasm by the cold osmotic shock procedure and sonication respectively. The content of the fusion protein came up to 38.9 and 41.5% of the total dissolved protein, respectively. The fusion protein was purified through an amylose affinity column, and incised by factor Xa. Molecular sieve chromatography was used to achieve a high state of purity of the target protein. The purified target protein displayed a single band in SDS-PAGE. The fusion protein was shown to increase resistance to low temperatures in bacteria. This finding could help in further investigations of the properties and function of antifreeze proteins.

***Keywords:* *Tenebrio molitor*, antifreeze protein, fusion expression, affinity purification, biological activity.**

INTRODUCTION

Antifreeze proteins (AFPs), also known as thermal hysteresis proteins (THPs), are a family of proteins which are able to inhibit growth of ice crystals by binding to the ice's surface by an adsorption–inhibition mechanism [1, 2]. To date, AFPs have been reported in a variety of organisms ranging from fish to bacteria [35]. The sequences of AFPs from two species of insects, the beetle *Tenebrio molitor* and the spruce budworm (Lepidoptera) *Choristoneura fumiferana*, have been elucidated only recently [6, 7]. Insect AFPs tend to have much more specific activities than the fish AFPs, probably reflecting the colder temperatures experienced by insects.

T. molitor antifreeze protein (TmAFP) transcripts are found throughout larval development with peak in the final larval instar. Levels decrease at pupation and decrease again in the adult stage [8]. Larval-stage messenger levels could be experimentally increased by low temperature, desiccation or starvation [9]. Mass spectral analysis of the *T. molitor* hemolymph showed multiple AFP isoforms [10]. Conceptual translation of 17 cDNA clones yielded eight different TmAFP isoforms, which encoded 84, 96, or 120 amino acids, consisting of seven, eight or 10 repeats of the 12 residue sequence Thr-Cys-Thr-X-Ser-X-X-Cys-X-X-Ala-X (where X can be any residue). 1.4 Å resolution crystal structure showed that this repetitive sequence translates into an exceptionally regular β-helix. Not only the 12 amino acid loops are almost identical in the backbone, but also the conserved side chains are positioned in essentially identical orientations, making this AFP perhaps the most regular protein structure yet observed. The first three residues of each amino acid repeat, Thr-Cys-Thr, are stacked and form a flat surface of the β-helix, which is complementary to the surface of ice [11]. Mature TmAFP is a particularly heavily disulfide-bonded protein, having 16 cysteines within less than 9 kDa [12].

The pMAL™-2 vectors provided a method for expressing and purifying a protein produced from a cloned gene or open reading frame. The cloned gene was inserted downstream from the *malE* gene of *Eschericia coli*, which encodes the maltose-binding protein (MBP), and resulted in the expression of an MBP fusion protein. The MBP in these vectors has been engineered for tighter binding to amylose. The method used the strong "tac" promoter and the *malE* translation initiation signals to give high level expression of the cloned sequences, and a one step purification of the fusion protein using MBP's affinity for maltose. The vectors expressed the *malE* gene (with or without its signal sequence relative to pMAL-p2X and pMAL-c2X) fused to the *lacZ*α gene. Restriction sites between *malE* and *lacZ*α were available for inserting the coding sequence of interest. In the vectors, the "-c'' designa-

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tion referred to cytoplasmic expression, i.e. the signal sequence that directed MBP to the periplasmic space was deleted. Vectors that were designated "-p" refer to periplasmic expression, and these contain the wild type *malE* signal sequence. The pMAL-c2X and pMAL-p2X vectors that are included in the system encode the site for factor Xa. Factor Xa cleaves after its four amino acid (IEGR) recognition sequence, so that few or no vector derived residues are attached to the protein of interest. The two types of expression plasmids were transferred into the *E. coli* TBI strains.

Some protocols were reported in which a soluble fused protein with antifreeze activity was expressed at a lower temperature [1316]. Recently a new *T. molitor* antifreeze protein cDNA fragment of 339 bp encoding a protein of 112 amino acid residues was reported [12]. Today another novel antifreeze protein cDNA fragment *afp72* was cloned, expressed and purified in *E. coli,* as a base for studying the structure and function of antifreeze proteins.

EXPERIMENTAL

Materials and reagents. The *E. coli* JM109 strain came from our laboratory, the *E. coli* TBI and the plasmid pMAL-p2X, pMAL-c2X were obtained from "New England Biolab" (Beijing), the plasmid pUCm-T vectors were purchased from "Sangon" (Shanghai) company. Spin Column RNA Purification Kit, Spin Column DNA Gel Extraction Kit, Taq DNA polymerase, restriction endonucleases (EcoRI, SalI), and T4 DNA ligase were also purchased from "Sangon" (Shanghai) company. GeneRuler 1 kb DNA ladder, Revert Aid TM M-MuLV Reverse Transcriptase were obtained from "MBI". 100 bp DNA ladder, protein marker, amylose affinity column, factor Xa, Sephacryl-S 100 HR came from "New England Biolab".

Total RNA preparation. *T. molitor* were kept in the dark at 22 ± 1°C, and provided with wheat bran and moist paper, as previously described [8]. Large, near-last stage larvae were incubated at 4°C for four weeks. Total RNA was extracted from larva using the Spin Column RNA Purification Kit.

RT-PCR. PCR primers were designed according to AFP cDNA sequences reported in GenBank (GenBank Accession No. AF159116, AF159115, AF160494): P1(sense) 5' TCGAATTCCAAT GCACTGGTGGT-GCTGATTGTA 3' and P2(antisense) 5' GTGTC-GACTTAATGTCCGGGA CATCCTGTTG 3'. According to the information on the expression vector, 5' primer and 3' primer were introduced into the EcoRI and SalI sites (underlined letters) respectively. According to the codon bias in *E. coli*, a GGT (shadowed letters) replaced the original GGG. The complementary sequence of the stop codon TAA (TTA) is bolded. First strand cDNA synthesis was performed by using M-MuLV reverse transcriptase and the Oligo $(dT)_{36}$(A/G/C) according to the manufacture's instructions: a mixture of 10 μL total RNA and 3 μL Oligo was incubated at 70°C for 5 min, followed by a chill on ice. 4 μL 5 × buffer, 10 mM dNTPs were added into the mixture after chilling, and a 5 min incubation at 37°C was applied. An additional 60 min incubation at 42°C was performed after adding 1 μL M-MuLV reverse transcriptase. The reaction was stopped by heating at 70°C for 10 min and chilled on ice. In the following PCR amplification 100 μL of the reaction mixture contained: 5 μL of 10 μM 5' primer and 5 μL of 10 μM 3' primer, 20 μL cDNA template, 8 μL 10 × PCR buffer, 4.5 μL of 25 mM $MgCl_2$, 57 μL ddH_2O, 0.5 μL of 5 U/μL Taq DNA polymerase. The PCR reaction was started by a preliminary denaturation step at 94°C for 10 min, followed by 30 amplification cycles consisting of 2 min at 94°C, 60 s at 59°C and 60 s at 72°C, and a final extension at 72°C for 10 min. 10 μL of RT-PCR products were examined by electrophoresis on a 1.5% agarose gel.

Construction of the prokaryotic expression plasmid. DNA fragments were recovered according to the instructions from the Spin Column DNA Gel Extraction Kit. The target DNA fragment was ligated into the pUCm-T vector for 2 h at 16°C. Competent *E. coli* JM109 cells were transformed with the above ligation product, then grown on LB-agarose plates containing 60 μg/mL ampicillin, 40 μg/mL X-gal and 4 μg/mL IPTG. White colonies were selected and grown in a 3 mL LB culture. Plasmid DNA was isolated, digested by EcoRI and SalI, and amplified by PCR for sequencing ("Sangon"). The recombinant plasmid pUCm-T-*afp72* and the expression plasmids pMAL-p2X, pMAL-c2X were digested by EcoRI and SalI respectively. The recovered DNA fragments were ligated overnight at 16°C. Competent *E. coli* TBI cells were then transformed with the ligation products, positive clones were randomly picked by growing on LB-agar plates containing 60 μg/mL ampicillin and further identified by PCR and double restriction enzyme digestion. Preparation of component cells, transformation of ligation products, isolation of plasmid DNA and identification of recombinant plasmids were done according to Sambrook et al. [17].

Protein expression. Recombinant expression plasmids pMAL-p2X-*afp72* and pMAL-c2X-*afp72* were transformed into *E. coli* TBI strains. A single colony of the freshly transformed cells was inoculated into 3 mL of LB culture containing 100 μg/mL ampicillin. Cultures were grown at 37°C with shaking (175–200 rpm) to $OD_{600} \approx 0.6$, and then sub-cultured into 300 mL of rich LB culture (with glucose & amp) to generate the cell biomass for induction. When cell density reached $OD_{600} \approx 0.6$, the temperature was then lowered to 25°C, and overexpression was induced by addition of IPTG to a final concentration of 0.5 mM. Cultures were further shaken for 8 h at 25°C and harvested by centrifugation (10 min, 6000 g, 4°C). Cell pellets were flash-frozen and stored at –20°C. The cell pellets of

expression products contain internal MBP tag proteins.

Fusion protein MBP-AFP72 affinity purification and analysis. The fusion protein MBP-AFP72 was extracted from the bacteria pMAL-p2X-*afp72*/TBI strains by following the cold osmotic shock procedure. Cell pellets harvested by centrifugation in Tris/sucrose were suspended in 20 μL 0.5 M EDTA (1 mM final concentration) and incubated for 510 min at room temperature with shaking or stirring. After a 10 min centrifugation step at 8000 g at 4°C, the cell pellets were resuspended in 10 mL of ice-cold $MgSO_4$ of various concentrations. Shaked or stired for 10 min in an ice-water bath. Centrifuge as above. The supernatant containing MBP-AFP72 was used as the cold osmotic shock fluid. With regard to the recombination bacteria pMAL-c2X-*afp72*/TBI strains, cells pellets harvested by centrifugation were lysed by sonication (with pulses of 15 s or less) in 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA. Cell debris was removed by centrifugation at 9000 g at 4°C for 40 min. The supernatant (crude extract) containing MBP-AFP72 was decanted and saved on ice.

2 mL of the amylose pre-packed column were incubated at 4°C, and the amylose resin was washed with 10 × column volumes of ddH_2O. The amylose pre-packed column was balanced with 18 mL of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). The supernatant (crude extract) was then added into the amylose pre-packed column, at a controlled flow velocity of 0.51 mL/min. Finally the column was washed with 24 mL of column buffer and the fusion protein was eluted with 6 mL of the elution buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose). Six to eight fractions of 1 mL each were collected. The fusion protein was eluted within the first four fractions, and detected by UV absorbance at 280 nm and SDS-PAGE (stained by Coomassie brilliant blue G-250). The protein bands were then quantified extensively using Bandscan 4.3 software.

Digestion of the fusion protein with factor Xa protease. The N-terminal extension of MBP-AFP72 was removed by factor Xa protease. 1 μL of factor Xa and 50 μg MBP-AFP72 were added and incubated at 23°C in 50 μL of a buffer (pH 8.0) containing 20 mM Tris-HCl, 100 mM NaCl, and 2 mM $CaCl_2$. Samples were mixed 1 : 1 with SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 0.1% bromphenol blue, 2% SDS, and 10% glycerol) and immediately applied onto a SDS-PAGE gel.

Molecular sieve chromatography purification and quantitative analysis. Molecular sieve chromatography was used to separate MBP from AFP72. Approximately 6 mL of Sephacryl-S 100 HR was washed in 20 mL of 150 mM Tris-HCl, 2 mM NaCl, pH 7.4 a few times, letting the resin settle and pouring off the supernatant between washes. The resin was poured into a 1 × 10 cm column to give a bed volume of 5 mL (67 cm bed height). The column was washed with 15 mL of the same buffer. The cleaved fusion protein was loaded onto the column and 2.5 mL fractions of the column flow-through were collected. The column was further washed with 35 column volumes of the same buffer, and 2.5 mL fractions were collected. The column eluate was monitored at 280 and 260 nm, and the fractions containing high protein concentrations were collected and analyzed by SDS-PAGE and silver staining.

After the fraction containing high protein concentration was desalted and condensed by ultrafiltration (3000 D interception molecular weight (Microcon)), the target protein was quantified by the Bradford assay. For the sake of testing the folding degree, the DTNB method [15] was employed to test dissotiated-SH quantitavely.

Determination of the biological activity of the recombinant protein AFP72. The method of measuring the biological activity of AFP72 involved analysis of enhancement of cold resistance of different *E. coli* strains treated with purified and refolded AFP72. *E. coli* TBI strains containing pMAL-c2X cells were grown overnight at 37°C (220 rpm) in LB media containing 100 μg/mL ampicillin and then subcultured (1 : 100) until the cell density reached $OD_{600} \approx 0.6$. The cells were treated with a 10^6 fold gradient dilution with sterilized water. Diluted cells were transferred to 1.5 mL Eppendorff tubes and AFP72 was added to a final concentration of 10, 30, 50, 70, and 90 μg/mL and negative control (10, 30, 50, 70, and 90 μg/mL BSA and sterilized buffer without the AFP72 protein). The total volumes were adjusted to 1.5 mL with water. Samples were incubated at 0°C for 24 h (the cells were not frozen during the treatment) and then spread on LB plates containing 100 μg/mL ampicillin at the beginning and the end of cold treatment. Plates were incubated for 12 h at 37°C and colonies were counted. Experiments were performed in triplicate, and the bacterial survival account during cold treatment as related to AFP72 treatment, was regarded as antifreeze activity. To investigate the longer-term improvements of bacterial cold resistance by antifreeze protein expression, additional cold treatments for 24, 48, 72, 96, 120 h and 144 h were performed and analyzed as described above.

RESULTS

Cloning of afp72 cDNA and nucleotide sequence alignments

The target cDNA fragment, coding for AFP72, was reverse transcribed from total RNA using RT-PCR, and then cloned into the vector pUCm-T. Following the sequencing of the insect, the cDNA was registered in GenBank under the name *afp72* (Accession No. AY929389). The total length of the cDNA is 216 bp. Multiple nucleotide sequences were aligned with DNAMAN 2.9 software (fig. 1).

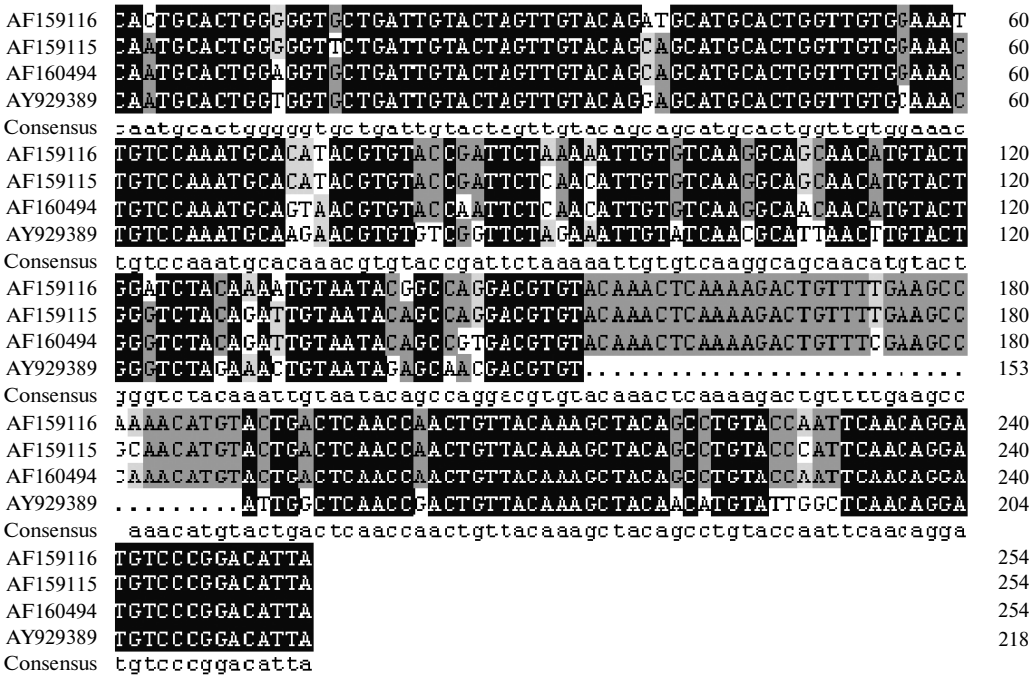

Fig. 1. Alignment of the sequence of *afp72* (AY929389) and three antifreeze protein genes from *Tenebrio molitor*. Black shaded areas indicate 100% homology, deep gray 75%, and light grey 50% homology.

AF159116, AF160494 and AF159115 are GenBank sequences coding for 84 aa AFP genes from *T. molitor*. The alignment showed that *afp72* shares a high degree homology with other AFP genes in *T. molitor*, 90.78% at the nucleotide level, and the different nucleotides are in the high variational regions of the series genes. According to the high homology of the series genes, the *afp72* sequence may come from mutation and deletion of the other AFP genes

Analysis of the deduced amino acid sequence

The amino acid sequence deduced from the cDNA of AFP72, which encodes a putative 72 aa mature protein and has a theoretical molecular mass of 7.9 kDa, was aligned to the amino acid sequences of three other AFPs.

1

HCTGGADCTSCTDACTGCGNCPNA-
HTCTDSKNCVKAATCTGSTKCNTARTCTNS
KDCFEAK TCTDSTNCYKATACTNSTGCPGH

2

QCTGGAGCTSCTAACTGCGNCPNAVTCTN-
SQHCVKATCTTGSTDCNTAVTCTNSK
DCFEAQ TCTDSTNCYKATACTNSTGCPGH

3 QCTGGS
DCTSCTAACTGCGNCPNAHTCTDSQHC-
VKAATCTGSTDCNTARTCTNSKDCFEAA
TCTDSTNCYKATACTHSTGCPGH

4 QCTGGADCTSCTGACTGCANCPNARTCV-
GSRNCINALTCTGSRNCNRAT

TCIGSTDCYKATTCIGSTGCPGH

The tandem repeats of a 12 amino acid sequence (TCTxSxxCxxAx) are underlined alternately and the highly conserved amino acids are shadowed. Sequences 1, 2, 3 and 4 are deduced amino acid sequences of AF159116, AF160494, AF159115 and AY929389 (AFP72). These antifreeze proteins are all comprised of tandem repeats of a 12 amino acid sequence (TCTxSxxCxxAx), the amino acid sequence of the AFP72 protein lacks one of the 12 aa repeat sequences as compared with the other 84 aa AFPs.

Expression and analysis of the MBP-AFP72 fusion protein

Stable cell lines that could express MBP-AFP72 on a larger scale for purification were then established. In our studies, optimal induction conditions were found to be $OD_{600} \approx 0.6$ (the concentration of *E. coli* before induction); 0.5 mM IPTG (the final concentration); (the induction temperature) 25°C; 8 h (the time of induction) in rich LB culture yielding visible bands upon SDS-PAGE analysis (*2* in fig. 2 and *1* in fig. 3). Based on staining with Coomassie blue, MBP-AFP72 in pMAL-p2X-*afp72*/TBI strains was the major secreted protein in the cell periplasm, and in pMAL-c2X-*afp72*/TBI strains MBP-AFP72 was expressed in the cell cytoplasm.

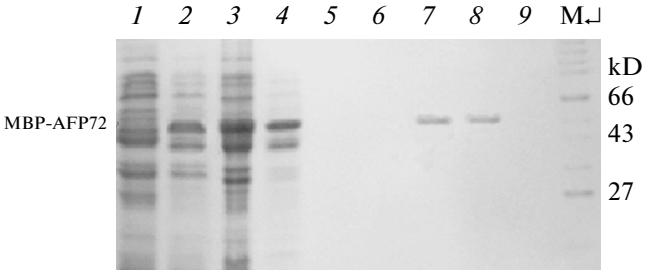

Fig. 2. SDS-PAGE analysis of affinity chromatography of fusion protein released from the periplasm (stained with Coomassie blue). *1* – Total protein from uninduced bacteria; *2* – total protein from induced bacteria; *3* – the precipitate after treatment with $MgSO_4$; *4* – the supernatant after treatment with $MgSO_4$; *5* – eluted protein from the first tube; *6* – eluted protein from the second tube; *7* – eluted protein from the third tube; *8* – eluted protein from the fourth tube; *9* – eluted protein from the fifth tube; M – molecular weight markers.

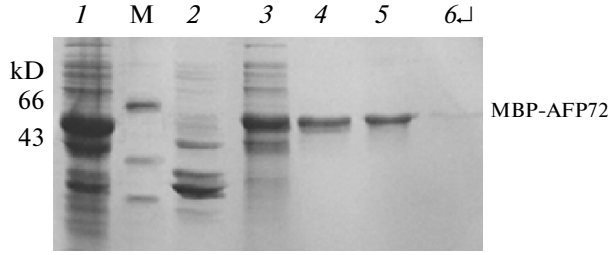

Fig. 3. SDS-PAGE analysis of affinity chromatography of fusion protein released from the cytoplasm (stained with Coomassie blue). *1* – Total protein from induced bacteria; M – molecular weight markers; *2* – total protein from uninduced bacteria; *3* – the supernatant after sonication; *4* – eluted protein from the third tube; *5* – eluted protein from the fourth tube; *6* – eluted protein from the fifth tube.

Purification of the MBP-AFP72

The target fusion protein from the pMAL-p2X-*afp72*/TBI and pMAL-c2X-*afp72*/TBI strains was released from the periplasm and cytoplasm through a cold osmotic shock procedure and sonication, respectively. For the pMAL-p2X-*afp72*/TBI strain, 5 mM $MgSO_4$ led to the highest yield of protein. The initial step in the purification was the capture of the MBP-tagged proteins using an amylose affinity column. Most recombinant proteins that bound to the amylose affinity column were recovered with a very good yield. And a band of ~52 kDa could be detected by running a small sample of induced cells on an SDS-PAGE gel, this band was absent in the total protein from uninduced bacteria. Flow-through fractions were reapplied to the column to capture any remaining fusion protein. Almost all of the expressed recombinant proteins were present in the supernatants. The protein concentration was the highest in the third tube on the amylose affinity column (*7*, *8* in fig. 2 and *4*, *5* in fig. 3), which is the same as detected by UV absorbance at 280 nm. Analysis with Bandscan 4.3 software showed the contents of the target protein in the total protein were 38.9 and 41.9%, indicating that a single step of affinity chromatography results in highly purified material.

Purification of the target protein and quantitive analysis

We focused our efforts on the MBP-AFP72 fusion construct for large-scale production of AFP72. The construct was designed with a specific recognition site for cleavage by the factor Xa protease between the MBP and AFP72 portions of the fusion protein. In our protocol, to obtain the target protein AFP72, the fusion proteins MBP-AFP72 cleaved with factor Xa were purified by molecular sieve chromatography. This method purifies the target protein away from MBP and the protease, but also provides an additional purification step for removing trace contaminants. Two distinct peaks were present, and analysis showed that the first peak contained the MBP protein and the second peak, the protein of interest (AFP72). Fractions from the peak corresponding to the AFP72 fragments were concentrated and further analyzed by SDS-PAGE (fig. 4). The target protein purified by molecular sieve chromatography appears as a single band.

The concentrated fractions of the target protein AFP72 contained 0.35 mg/mL of protein. Dissociated-SH content was tested quantitatively, and results showed that no dissociated-SH was present throughout the standard curve of the DTNB method. These results implied that the recombinant protein folded correctly.

Determination of the biological activity of the AFP72 protein

All samples displayed greater antifreeze activity than the negative control. To eliminate the possibility that the enhanced cold resistance of bacteria was created or increased by the osmotic shock caused by protein addition, we used BSA (10, 30, 50, 70, 90 mg/mL) under the same conditions as a control. The results showed that the curve of the BSA control was almost identical to that of the buffer control (fig. 5). This suggested that the enhanced cold resistance of the bacteria was due to the antifreeze activity of the AFP72 protein, and not to the osmotic effect. Numbers of surviving bacteria increased obviously after a 24 h treatment at 0°C compared to the BSA control and the buffer control. Numbers of surviving bacteria increased when they were treated at 0°C for 96 h or longer compared to 72 h, suggesting that bacteria have been propagated owing to protection by the AFP72 protein. But as the biological activity showed almost no distinction between different concentrations of the antifreeze protein, we conclude that cold resistance caused by

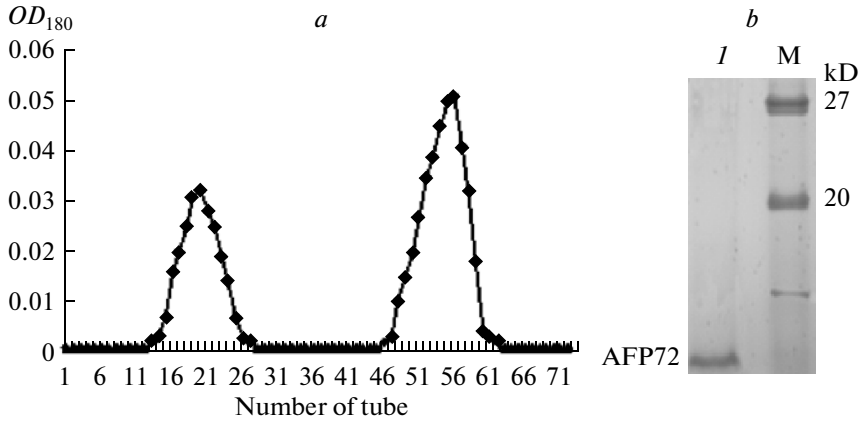

Fig. 4. Map of molecular sieve chromatography and SDS-PAGE analysis of purification of the target protein (stained with silver). *a* – Map of molecular sieve chromatography of the target protein purification; *b* – SDS-PAGE analysis of the target protein purification. *1* – The target protein; *2* – molecular weight markers.

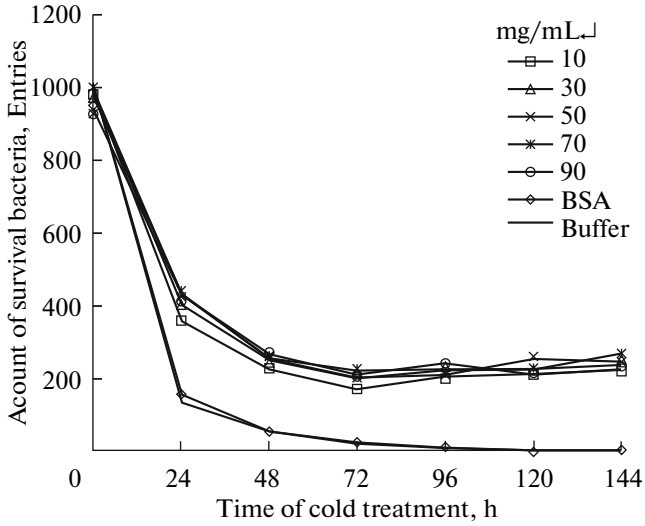

Fig. 5. The effect of AFP72 on enhancing the cold resistance of bacteria.

the AFP72 protein is concentration independent. Our research revealed that the biological activity of the AFP72 protein obtained from pMAL-c2X-*afp72*/TBI and pMAL-p2X-*afp72*/TBI was nearly the same (data not shown).

DISCUSSION

Antifreeze proteins are of great interest because of their potential applications in various fields such as food industry [18], cryo-preservation of cells [19], organs [20, 21], tissues [22], and cryosurgery [23]. Insect AFPs display considerably higher antifreeze activities than fish and plant AFPs, even at lower concentrations, what makes them more suitable for industrial applications [21]. Because of their unique structure and biological activities, AFPs have been in the focus of many scientific investigations. Both scientific studies and practical applications of insect AFPs would be facilitated by improved methods of their production.

The beetle *T. molitor* produces several isoforms of antifreeze proteins, which are comprised of seven or eight or ten tandem repeats of a 12 aa sequence [6, 10]. Genomic clones were isolated from three different libraries [9]. To obtain the coding region, we used primers designed from highly conserved regions of other AFP genes of *T. molitor*. In order to express target proteins efficiently in *E. coli*, a GGG was substituted for a GGT in the 5' primer according to the codon bias of *E. coli*. Several bands could be amplified by RT-PCR, and the shortest DNA fragment was selected and sequenced. It was shown that the full length of the nucleotide sequence was 216 bp, which has never been reported in GenBank. Homology analysis indicates a

90.78% identity between *afp72* and other 84aa AFPs genes, and a deletion of 36 bp. Apart from this deletion, the other differences in the nucleotide sequences of *afp72* and other AFP genes are located in the highly divergent regions of this series of genes, what may come from a loss or mutation during the process of evolution. In addition, AFP72 is composed of six repeats, and is shorter than the other AFPs, but the number of amino acid residues is also a multiple of the 12 aa repeat unit. More over, AFP72 has many cysteines, which is also characteristic of other AFPs. To conclude, *afp72* is a shorter, novel AFP gene. The research on the differences in structure and biological activity between AFP72 and the other AFPs is underway.

Both the scientific studies and the practical applications of insect AFPs would be facilitated by better methods for their production. Attempts at expressing secreted eukaryotic proteins in bacteria frequently fail or result in formation of inclusion bodies. The high cysteine content of antifreeze proteins makes their production especially challenging. Several recombinant systems have been tried previously for the production of various isoforms of AFP. None of these systems yielded high levels of active AFPs. We demonstrated a procedure for the preparation of fully folded AFP either fused to carrier proteins or cleaved and isolated from the carrier. In our research, the pMAL™ protein fusion and purification system was employed to express and purify the antifreeze protein AFP72, which includes two vectors, pMAL-c2X and pMAL-p2X. pMAL-p2X encoded the recombinant AFP72 tagged at its N-terminal with MBP with its signal sequence. The signal peptide on pre-MBP directs fusion proteins to the periplasm. For fusion proteins that can be successfully exported, this allows folding and disulfide bond formation to take place in the periplasm of *E. coli*, as well as allowing purification of the protein from the periplasm. As for pMAL-c2X, the recombinant AFP72 is tagged with an MBP without its signal sequence, which leads to the cytoplasmic expression of the fusion protein. This was done to facilitate the expression and purification of these difficult target proteins. Expression was carried out in *E. coli* strain TBI, the recombination strain pMAL-p2X-*afp72*/TBI was developed to enhance disulfide bond formation in the cell periplasm, and the recombination strain pMAL-c2X-*afp72*/TBI was designed for advanced expression efficiency.

Different inducing conditions influence the final expression of target protein, which is very important for determining the optimal expression conditions. The inducing temperature and initial concentration (value of OD_{600}) of expression bacteria have distinct influence on the expression of the target protein; in addition, inducing time and final IPTG concentrations were also important. Therefore, we optimized these conditions with a designed orthogonal experimental protocol (data not shown) and obtained the best expression conditions, which are the following: bacteria initial concentration value of $OD_{600} \approx 0.6$; the final concentration of IPTG 0.5 mM; the induction temperature 25°C; the time of induction 8 h. Low temperature (25°C) of incubation promotes AFP folding in its wild-type disulfide bonding pattern, which is necessary for AFP to exert its antifreeze activity. Our purification procedure yields high quality AFP with a high antifreeze activity. However, even at the same temperature, the two expression vectors showed different levels of fused target proteins and different percentages of fused proteins in the total protein level. The expression efficiency of pMAL-c2X-AFP72/TBI was higher than that of the pMAL-p2X-AFP72/TBI expression system.

We have used two methods to obtain the fusion protein according to the characteristics of the vectors pMAL-c2X and pMAL-p2X. The fusion protein AFP72 was released from pMAL-c2X-*afp72*/TBI by the ultrasonic wave method. Both supernatants and pellets of the sonicated cell lysates were screened by SDS-PAGE for the presence of the recombinant protein MBP-AFP72. Almost all of the expressed recombinant proteins were present in the supernatant. The yield of the recombinant protein in the crude extracts was evaluated to be 41.9% of the total cellular protein, indicating that the expression system was highly efficient. As to the secreted fusion expressing plasmids pMAL-p2X-*afp72*, the fusion protein could be released from the periplasmic space and was dissolved by using $MgSO_4$ (0.05 mM). Analysis showed that the content of the target protein was 38.9% of the total cellular protein. The single-step purification of the recombinant protein by the amylose pre-packed column will make this expression system attractive for future structure/function studies.

We used cold resistance of bacteria to determine the activity of AFP72 expressed in this study. Although this system does not enable quantitative measurements of activity, we demonstrated qualitatively that the recombinant proteins enhance the cold resistance of bacteria. At the same time, reaction with DTNB demonstrated that there were no free thiol groups within the protein, suggesting that the vast majority of the purified protein was correctly folded and fully active.

T. molitor antifreeze proteins consist of seven, eight or 10 repeats of the 12 residue sequence Thr-Cys-Thr-X-Ser-X-X-Cys-X-X-Ala-X (where X can be any residue). The conservation of the Thr-Cys-Thr motif and the number of β-helixes plays an important role in maintaining the biological activity of these antifreeze proteins. Compared with the known TmAFPs, in the recombinant protein AFP72, which codes only 72 amino acids, i.e. three Thr-Cys-Thr-X-Ser-X-X-Cys-X-X-Ala-X domains, two threonine residues have been replaced by other amino acids. Through the analysis in this research, these replacements have some affect on the activity of the protein AFP72, the biological activity of the protein AFP72 was lower than

that of the protein AFP84 and AFP120 (data not shown). These results were consistent with previous studies [24, 25], replacement of the Thr residue, and especially the second Thr residue, in the Thr-Cys-Thr motif can cause reduced biological activity. Within a certain range, the higher is the number of β-helixes the higher is the biological activity.

It is a highly promising route for expressing other recombinant target proteins that are poorly expressed in bacteria, including cysteine-rich proteins. This finding could lead to further investigation of the properties and function of antifreeze proteins.

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