Production of a Recombinant Type 1 Antifreeze Protein Analogue by *L. lactis* and Its Applications on Frozen Meat and Frozen Dough

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In this study, a novel recombinant type I antifreeze protein analogue (rAFP) was produced and secreted by *Lactococcus lactis*, a food-grade microorganism of major commercial importance. Antifreeze proteins are potent cryogenic protection agents for the cryopreservation of food and pharmaceutical materials. A food-grade expression and fermentation system (BSE- and antibiotic-free) for the production and secretion of high levels of rAFP was developed. Lyophilized, crude rAFP produced by *L. lactis* was tested in a frozen meat and frozen dough processing model. The frozen meat treated with the antifreeze protein showed less drip loss, less protein loss, and a high score on juiciness by sensory evaluation. Frozen dough treated with the rAFP showed better fermentation capacity than untreated frozen dough. Breads baked from frozen dough treated with rAFP acquired the same consumer acceptance as fresh bread.

INTRODUCTION

Antifreeze proteins (AFPs) are found in polar organisms such as fish, plants, fungi, and insects and are characterized by their ability to cause noncolligative depression of the freezing point (*1*). A more generally applicable name "ice structuring proteins (ISP)" has been proposed, given that all AFPs influence ice crystal growth by controlling the size, morphology, and aggregation of ice crystals but do not prevent freezing (*2*). This feature enables the use of AFPs in cryogenic preservation of cells, tissues, as well as food products (*3, 4*). AFPs may inhibit recrystallization during frozen storage, transport, and thawing, thus preserving food texture by reducing cellular damage and minimizing the loss of nutrients by reducing drip (*4*). AFPs have been applied in ice cream (*5*), frozen meat (*6*), preslaughter lamb (*7*), and frozen dough (*8*). For frozen meat, the large ice crystals formed during frozen process and resulted in drip and loss of nutrition during thawing (*6, 7*). Soaking bovine and ovine muscle in a solution containing 1 mg/mL of AFP (*6*), or injection antifreeze glycoprotein (AFGP) before slaughter of lambs (*7*), reduced drip loss and ice crystal size. For frozen dough, the freezing process weakens the dough structure, decreases the capacity to retain CO_2, and therefore prolongs leavening time of the frozen dough and deteriorates the texture of the product. Concentrated antifreeze protein from carrots (*Daucus carota*) has been shown to improve the leavening fermentation capacity of frozen dough (*8*), resulting in maintenance of loaf volume and improving the softness of the dough during frozen storage (*9*). However, the use of natural AFP is associated with high costs, due to low yield, limitation of

natural resources, and the requirement for extensive purification procedures (*3*). Recombinant production of AFP is not limited by the seasons or natural sources, and it may be an economical, less labor-intensive source of AFPs.

Lactococcus lactis has long been used in food production and has many potential applications, including a delivery vehicle of heterologous protein in foodstuffs and the digestive tract, as well as the production of proteins (*10*). Many heterologous proteins have been produced in *L. lactis*, and industrial-scale food-grade *L. lactis* expression systems have been developed (*11*). We previously designed and synthesized a novel recombinant type I antifreeze protein analogue (rAFP) by overlap extension PCR for functionally expression it in *B. subtilis* (*12*). In this study, we attempt to express rAFP as a secreted protein in the food-grade host *L. lactis*. A food grade, BSE-free, and antibiotic-free culture was investigated to produce the rAFP by *L. lactis*. The activity of rAFP was determined by assaying for inhibition of ice recrystallization by observing the growth of single ice crystals (*12*). Finally, frozen meat and frozen dough were examined as food models to evaluate the potential application of rAFP produced by *L. lactis* in the cryo-food industry.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. The bacterial strains and plasmids used in this study are summarized in **Table 1**. The *Escherichia coli* strains were propagated at 37 °C with agitation in LB broth (Luria−Bertani, Difco) supplemented with 20 μg/mL chloramphenicol (*Cm*) for transformants harboring pNZ8008-derivative plasmids. The *L. lactis* NZ9000 strain was propagated at 30 °C in M17 broth (Difco). For *L. lactis* transformants harboring pNZ8008 derivative-plasmids, 5 μg/mL of *Cm* was supplemented in GM17 (M17 broth supplemented with 0.5% glucose). For food trials, a food-grade beef-free FMB medium (*13*) was used to culture

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Table 1. Strains, Plasmids, and Primers Used in This Study

Strains or plasmids	Relevant characteristics	Source or Reference
Strains		
Escherichia coli JM109	*end*A1 *recA*1 *gry*A96 *thi hsd*R17 ($rK-$, mK^+) *relA*1 *sup*E44 (*lac-pro*AB)[*tra*D36 *pro*AB *lac Z* ΔM15]	Promega Co.
Lactobacillus acidophilus ATCC 4356	Source of P_{SlpA}, SP_{slpA}, and Ter_{slpA}	ATCC
Lactococcus lactis NZ9000	*L. lactis* MG1363 *pepN::nisRK*	Reference *10*
Lactococcus lactis NZ9000 ΔhtrA	*L. lactis* NZ9000 *htrA::ery*^{r}	Reference *29*
Plasmids		
pHY300PLK	Tc^r and Ap^r, a shuttle vector for *E. coli* and *Bacillus subtilis*.	Takara Shuzo Co., Tokyo, Japan
pHYSASM	Tc^r and Ap^r, pHY300PLK derivative. P_{slpA}-SP_{slpA}-MCS (HindIII-KpnI-XmaI-XhoI-SacI-BamHI)	This study
pHYSAST	Tc^r and Ap^r, pHY300PLK derivative. P_{slpA} -SP_{slpA}-T_{slpA}	This study
pVASAFP-1	Tc^r and Ap^r, pHY300PLK derivative. Synthetic expression control sequence-SP_{YaB}-AFP-his$_6$-TerQ.	Unpublished
pHYSASAFP	Tc^r and Ap^r, pHY300PLK derivative. P_{slpA}-SP_{slpA}-AFP-his$_6$-T_{slpA}	This study
pNZ8008	Cm^r, a broad host range vector for *L. lactis* and *E. coli*,	Reference *10*
pNZSASAFP	Cm^r, derivative of pNZ8008. P_{slpA}-SP_{slpA}-AFP-his$_6$-T_{slpA}	This study

Primers	Sequence (5'-3')	Construction
pSAF	GATATA<u>GAATTC</u>AGA<u>TCT</u>TACAAATAGTATTTTTCGGTCATTTTAACT EcoRI BglII TGCTATT	pHYSASM
pSAR	GATATA <u>AAGCTT</u> <u>GGTACC</u> <u>CCCGGG</u> <u>CTCGAG</u> <u>GAGCTC</u> <u>GGATCC</u> HindIII KpnI XmaI XhoI SacI BamHI TGAACTTGCGTTAATAGT AGTAGCAGCGC	
pSTF	GATATA<u>CCCGGG</u>TCGTAGCACTAACGCTAACAAAATGAAAA XmaI	pHYSAST
pSCTR	GATATA<u>AAGCTT</u>CAGAAGATCCTATTAGAACTGTATGTTTAGAAGTGAAA HindIII	
P300F	TTATAA<u>CCCGGG</u>AATCATTGTCATTAGTTGGCTGGTTAC XmaI	pHYSASAFP
P300R	TTATAA<u>TCTAGA</u>GCTCCTGAAAATCTCGCCAAGC XbaI	

transformants under antibiotic-free conditions. Cell growth was monitored by measuring the cell density at 600 nm.

Molecular Techniques. Plasmid DNA was isolated with a Mini-M plasmid DNA extraction system (Viogene, Taipei, Taiwan). *Lactobacillus acidophilus* genomic DNA was isolated with a Blood and Tissue genomic DNA extraction system (Viogene). PCR products were purified with a PCR-M cleanup system (Viogene). DNA fragments were recovered from gels with a Gel-M gel extraction system (Viogene). Plasmids, genomic DNA, and PCR products were analyzed by agarose gel electrophoresis. Primers were purchased from Invitrogen Life Technologies (Carlsbad, CA). Ex Taq DNA polymerase (Takara, Kyoto, Japan) was used for PCR amplifications. Electro-transformation of *E. coli* was carried out at a field strength of 12.5 kV/cm, capacitance of 25 μF, and resistance of 200 Ω using a Gene Pulser II electroporation apparatus (Bio-Rad, Hercules, CA). Electro-transformation of *L. lactis* was performed as described previously (*13*).

Plasmid Construction. Because of the limitation of restriction sites, serial constructions were prepared in pHY300PLK derivative plasmids, followed by the assembly of an expression vector for *L. lactis*. Plasmid pHYSASM was constructed first and contained DNA sequences of the promoter and signal peptide of surface layer protein SlpA (P_{slpA}-SP_{slpA}) from *L. acidophilus* ATCC4356 (NCBI accession no. DD459494) (*13*) fused to multiple cloning sites (MCS; *Hin*dIII-*Kpn*I-*Xma*I-*Xho*I-*Sac*I-*Bam*HI). The DNA fragment containing P_{slpA}-SP_{slpA}-MCS was PCR amplified using genome of *L. acidophilus* ATCC4356 as template and primer pair pSAF/pSAR, in which the MCS was appended to the pSAR primer (**Table 2**). The PCR fragment and vector pHY300PLK was restriction digested by *Eco*RI/*Hin*dIII, ligated to obtain pHYSASM. The T_{slpA} was PCR amplified using primer pair pSTF/pSCTR, restriction digested with *Xma*I/*Hin*dIII, ligated to *Xma*I/*Hin*dIII-digested pHYSASM to obtain pHYSAST (P_{slpA}-SP_{slpA}-MCS-T_{slpA}) in which the SlpA terminator (T_{slpA}) was added. The rAFP gene containing six histidine tag afp-his$_6$ was PCR amplified from pVASAFP-1 (unpublished) using the

Table 2. Consumer Preference Analysis of Frozen Meats Treated with CIS or ACIS

treatments	tenderness	juiciness	flavor	overall
ACIS	5.29^a	5.41^a	5.20^a	5.33^a
CIS	5.12^a	4.96^b	5.18^a	4.98^a
control	4.71^b	4.45^c	4.73^b	4.45^b

^{a} The fillets were soaked in cryo-immersion solution [CIS, dissolve 0.9% NaCl and 15% food grade alcohol in distilled water] or antifreeze cryo-immersion solution (ACIS, 0.048 g/mL CAFP dissolved in CIS) at −12 °C and stored at −20 °C for 30 days. Untreated frozen fillets were used as controls. ^{b} Differences among samples were determined by Duncan's multiple range tests at $p < 0.05$. The same letter indicates no significant difference among samples. ^{c} All data are mean scores (1 = extremely dislike, 4 = neither like nor dislike, 7 = extremely like) of 53 consumers.

P300F/p300R primer pair. The PCR product and pHYSAST were restriction digested with *Bam*HI/*Xho*I, ligated to obtain pHYSASAFP (P_{slpA-1}-SP_{slpA}-afp-his$_6$-T_{slpA}). Finally, the secretion vector pNZSASAFP for *L. lactis* was constructed by *Bgl*II/*Hin*dIII restriction digestion of both pHYSASAFP and pNZ8008, ligation of the insert into pNZ8008. All the constructed plasmids were electro-transformed into *E. coli* JM109 to manipulate the plasmids and confirmed by diagnostic digest and DNA sequencing (Tri-I Biotech Inc., Taipei, Taiwan). Primer sequences are listed in **Table 2**. The following PCR cycling conditions were employed: 94 °C (5 min), 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (20 s) (pSAF/pSAR) or 72 °C (27 s) (pSTF/pSCTR, P300F/p300R), and 72 °C (5 min).

Expression, Purification, Tricine-SDS-PAGE Analysis and N-terminal Amino Acids Analysis of Recombinant Antifreeze Protein Analogue. Single colony of *L. lactis* NZ9000 (pNZSASAFP) or *L. lactis* NZ9000htrA (pNZSASAFP) was inoculated in GM17 or food-grade BSE-free FMB medium (with or without antibiotic) in a flask and cultured at 30 °C. At time intervals of 2 to 48 h, the cell densities were

measured and the rAFPs in supernants were analyzed. The cultures were centrifuged at 14000g for 10 min at 4 °C and the supernatant proteins were analyzed by Tricine-SDS-PAGE (14). Total secreted proteins from 2 to 48 h culture in GM17 or FMB medium were precipitated as described previously (15) and suspended in 1/10 volume of TSE buffer (10 mM Tris-HCl, 10 mM EDTA, 300 mM NaCl, pH8.0). An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, boiled for 5 min, and loaded on an SDS-PAGE gel. The purification of rAFP was preceded by adjusting the supernatants to pH 8.0 using a buffer containing 500 mM NaH_2PO_4, 1.5 M NaCl, 100 mM imidazole, followed by native Ni-NTA affinity chromatography according to the QIAexpressionist instructions (4th edition, Qiagen). Purified rAFP was quantified using a DC Protein Assay kit (Bio-Rad Laboratories Inc., USA). The yield of secreted rAFP was estimated by comparing the density of rAFP bands to the standard purified recombinant AFP protein band of fixed concentration on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co., Ltd., Taipei, Taiwan) as described previously (13). To prepare samples for N-terminal amino acid analysis, proteins were electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories Ltd., USA) using an electro-blotter (model VEP-2, Owl Scientific, Inc., NH) as described previously (16). The partial amino N-terminal sequence was analyzed by automated Edman degradation using the Procise Protein Sequencing System (Max Planck Institute for Molecular Genetics, Berlin, Germany).

Ice Recrystallization Inhibition Activity and Single Ice Crystal Photomicroscopy. Recrystallization inhibition activity was assayed using a variation of the splat assay (17). A 1 μL droplet of control PBS buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) or rAFP in PBS buffer (24−144 μg/μL) was spread between two coverslips. The coverslips were placed in the cooling chamber of a Linkam BCS196 cold stage (Linkam Scientific Instruments Ltd., Tadworth, UK) attached to an Olympus BX41 microscope equipped with a CCD camera (Roper Scientific, USA). The sample was flash-cooled to −40 at 90 °C/min, held for 1 min, warmed at 90 °C/min to −7 °C, and held for 60 min and photographed. Ice recrystallization inhibition activity was measured by recording changes in the size and number ice crystals.

The ability of AFP to bind to single ice crystals and modify their growth behavior was also assayed with a Linkam BCS196 cold stage attached to an Olympus BX41 microscope. A 2 μL droplet of PBS buffer or AFP in PBS buffer on a coverslip was placed in the cooling chamber and crash-cooled to −40 at 90 °C/min rate, held for 1 min, and warmed at 90 °C/min to just above the melting point (∼−1.5 °C). The temperature was maintained until a single crystal formed and was photographed.

Preparation of Crude Recombinant Antifreeze Protein Powder (CAFP). *L. lactis* transformants (pNZSASAFP) were cultured under food grade FMB medium. A 24 h culture was centrifuged at 14000g for 10 min at 4 °C to remove the bacteria, and the supernatant was lyophilized (Freezon 4.5, Labconco Corporation, Kansas City, MO) to achieve a safe, crude rAFP powder (CAFP). The crude powder from the supernatant of a nontransformed *L. lactis* culture was designated as CPS.

Frozen Meat Preparation, Cryo-storage and Quality Evaluation. Fresh pork fillet was purchased from a local market, the fat removed, and the pork fillet fixed to equivalent slices (10 ± 0.5 g). The cryo-immersion solution [CIS, dissolve 0.9% NaCl and 15% food grade alcohol (95% stock) in distilled water] and antifreeze cryo-immersion solution (ACIS, 0.045 g/mL CAFP dissolved in CIS) were stirred in beakers maintained at −12 °C in a −20 °C chamber. The pork fillets were immersed in CIS or ACIS for varying time intervals, and the surface moisture from the fillets was removed using paper towels. The fillets were then sealed in plastic zip lock bags and stored at −20 °C. Pork fillets that were not immersed in the solutions were sealed and stored at −20 °C to serve as controls. The drip loss and protein loss of frozen fillets were analyzed after 15 and 30 days of storage. In a second experiment, frozen fillets were stored for 15 days and then transferred to room temperature for 1 h. The fillets were then returned to −20 °C until day 30 and analyzed for drip and protein loss. Drip loss was assayed by wrapping the frozen meat in a nylon string and hanging the meat vertically in a scale tube, followed by centrifugation at 40g for 90 min. The volume of the liquid collected in the tube represented the drip loss. The protein concentration of the collected liquid was quantified by a DC Protein Assay kit (Bio-Rad Laboratories Inc., USA).

Frozen Dough Preparation, Thawing and Baking, and Evaluation of Dough and Bread Quality. The dough was made by mixing 100 g of flour (already mixed with yeast) and 60 mL of water (contained sugar and CAFP) thoroughly. The dough was allowed to rest for 10 min prior to further experimentation. Varying amounts of yeast (0.25−1.5%) and CAFP (0−0.06 g/mL) were examined to determine the optimal composition of the dough. The final dough (20 g) was placed in a plastic scale tube, molded to a constant volume, capped, and stored at −20 °C for 7 days. The dough was then subjected to thawing (4 °C, 12 h) and proofing (fermented at 37 °C for 1 h), and the dough volume was measured. Control dough was not frozen and was subjected to proofing directly.

For bread quality evaluation, the dough was molded and then stored at −20 °C. At day 7, 14, 21, and 28, the frozen dough was thawed (4 °C, 12 h), fermented at 37 °C for 1 h, and baked at 195 °C for 12 min. The baked bread was transferred to room temperature for 10 min, and the bread volume was measured by a salt displacement method. The density of bread (*D*) was measured as ratio of mass (*M*) to volume (*V*) ($D = M/V$) (18).

Sensory Evaluation. A panel consisted of 53 members aged from 18 to 30 was selected randomly from local staff members. Frozen meats were boiled for 5 min, and tenderness, juiciness, and flavor were evaluated. Bread made from frozen dough or fresh dough was evaluated for volume, shape, crust color, crumb color, grain, texture, odor, and overall acceptance. Visual and tactile comparisons were conducted under constant illumination from an identical source for each experiment. A hedonic scale of 1−7 (1 = extremely dislike, 2 = dislike, 3 = slightly dislike, 4 = neither like nor dislike, 5 = slightly like, 6 = like, 7 = extremely like) was used to quantify the consumer preference. Water was provided for rinsing purposes. Statistical analyses of variance (ANOVA) were analyzed with SPSS (Windows 10.0).

RESULTS AND DISCUSSION

Expression and Secretion of the Functional rAFP by *L. lactis*. For industrial-scale production of recombinant proteins for
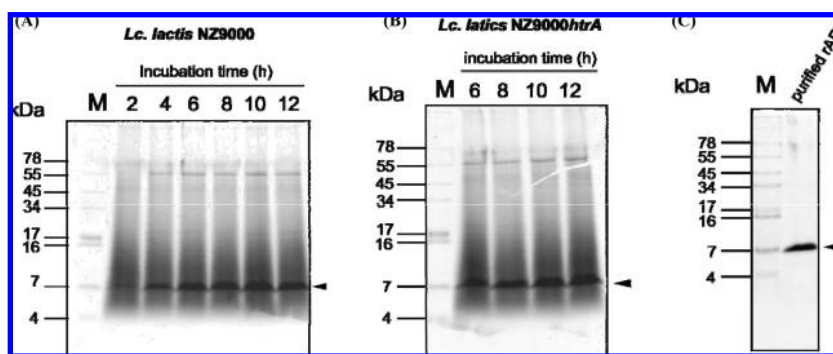

Figure 1. SDS-PAGE analysis of recombinant type 1 antifreeze protein analogue secreted by *Lactococcus lactis* NZ9000 (A), *Lactococcus lactis* NZ9000htrA (B), and the purified rAFP (C). The cell-free supernatants of GM17-cultured transformants were concentrated 10-fold by TCA precipitation and analyzed by Tricine-SDS-PAGE. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, boiled for 5 min, and loaded into the lane of an SDS-PAGE gel.
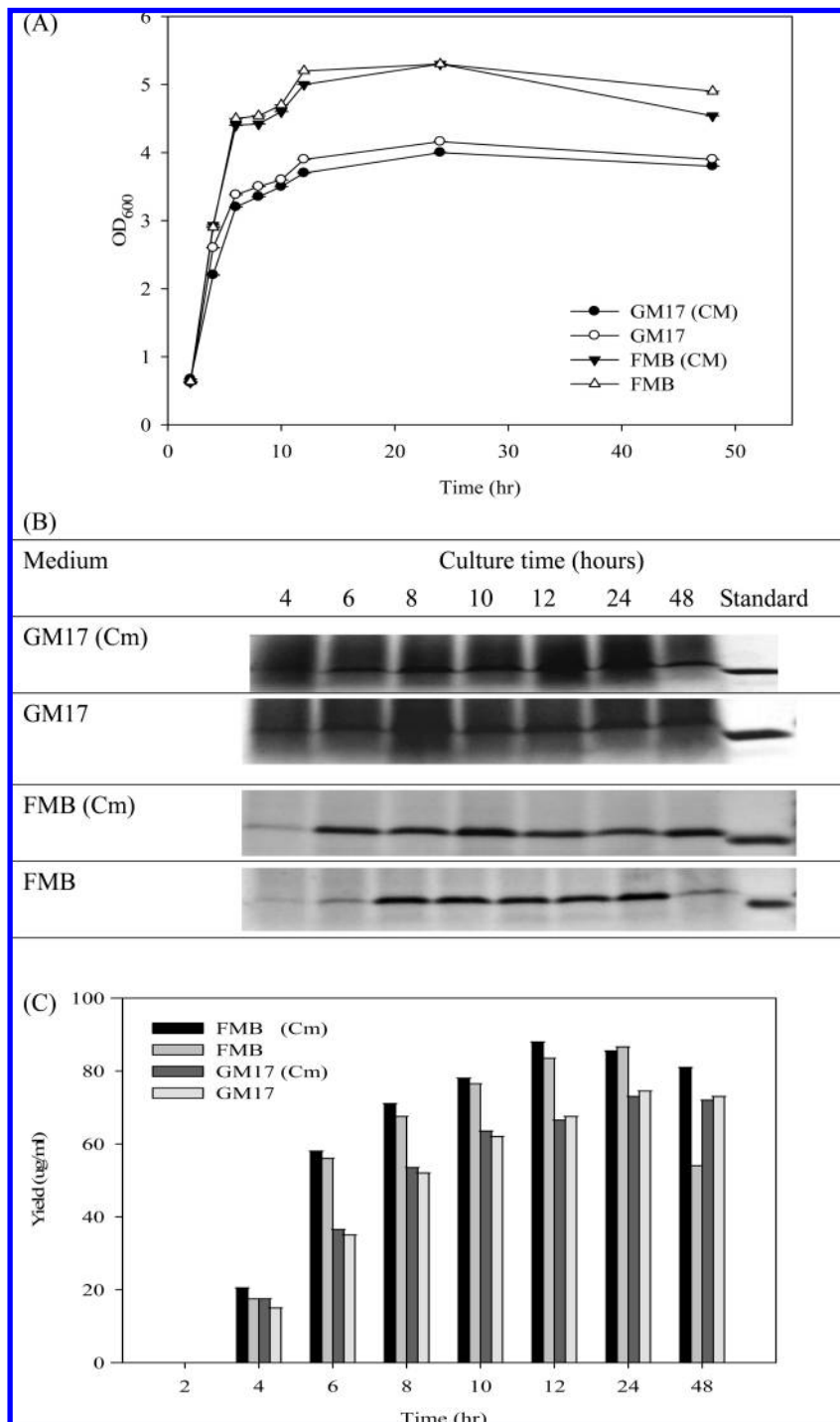
Figure 2. The growth (A), SDS-PAGE analysis of recombinant type I antifreeze protein expression (B), and protein yields (C) of *L. lactis* NZ9000 (pNZSASAFP) cultured in various media. The cell-free supernatants of various medium cultured transformants were concentrated 10-fold by TCA precipitation and analyzed by Tricine-SDS-PAGE. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, boiled for 5 min, and then loaded into the lane of an SDS-PAGE gel. The yield of extracellular rAFP was estimated by comparing the density of rAFP bands to the standard purified recombinant AFP protein (5.4 kDa) band of fixed concentration on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co., Ltd., Taipei, Taiwan). The cell densities (OD_{600}) at each time interval represent the growth of the culture.

application in the food industry, a food-grade expression system is highly recommended. *L. lactis* has long been used in the food industry and is a good candidate for heterologous protein production (*10*). An industrial-scale food-grade *L. lactis* expression system has been developed (*11*). We previously designed and synthesized a novel recombinant type I antifreeze protein analogue (rAFP) by overlap extension PCR and functionally expressed in *B. subtilis* (*12*). In the present study, a new construct

was designed for rAFP expression in *L. lactis* consisting of a strong promoter from *Lactobacillus acidophilus* S-layer protein (P_{slpA}) (*19*, *20*), a signal peptide of S-layer protein (SP_{slpA}) with predicted cleavage site (*19*), and terminator of S-layer protein (T_{slpA}). **Figure 1A** shows that the rAFP was secreted into the medium; the expression levels were calculated to be 6.8, 35, 45, 54, 94, and 94 mg/L at culture time 2, 4, 6, 8, 10, and 12 h, respectively.
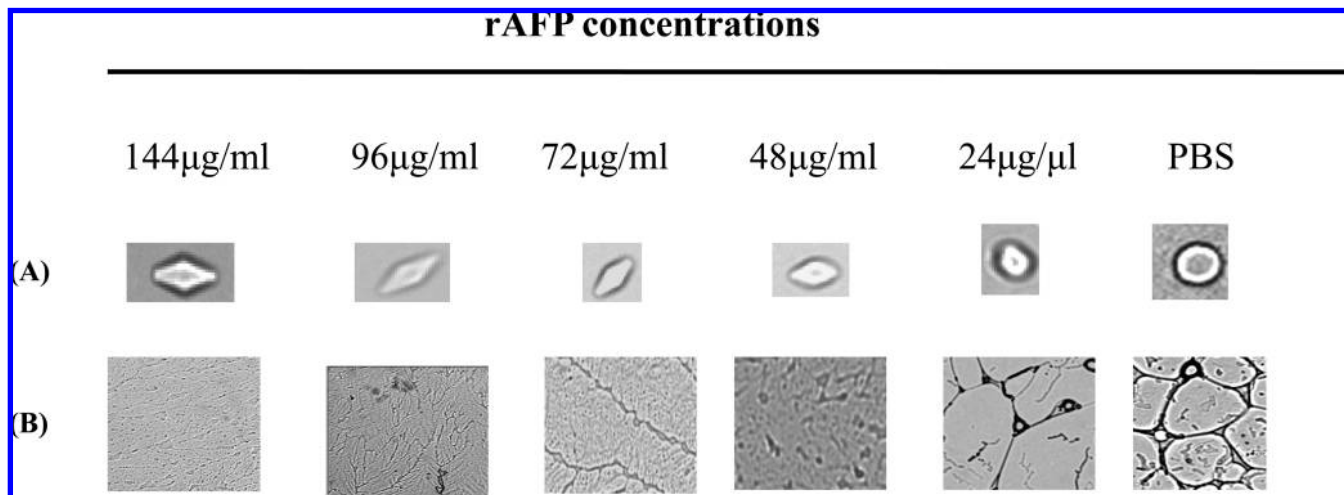
Figure 3. The ice crystal morphology (A) and ice recrystallization inhibition activity (B) of recombinant type I antifreeze protein analogues purified from *L. lactis* NZ9000 (pNZSASAFP). The supernatants of 24 h cultures were collected, adjusted to pH 8.0, and rAFPs were purified by native Ni-NTA affinity chromatography. A 1 μL droplet of PBS buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0) or rAFP in PBS buffer (0$-$144 μg/μL) was spread between two coverslips and placed in the cooling chamber of a Linkam BCS196 cold stage attached to a Olympus BX41 microscope mounted with CCD camera (Roper Scientific, USA). For ice morphology, the sample was flash-cooled to -40 °C, held for 1 min, and the temperature was then elevated until a single ice crystal formed. This temperature was maintained and crystal growth and the morphology observed. For ice recrystallization inhibition activity, the sample was flashed cooled to -40 °C and held for 1 min. The temperature was elevated to -7 °C and held for 60 min to observe the ice crystal size. The rates of temperature shifts were all 90 °C/min. The shaded areas represent the space between aggregated ice crystals, and the spots are small air droplets.

In a previous study, the rAFP was secreted by a *B. subtilis* strain, which possessed a truncated cell wall protease (*12*). To examine whether the cell wall protease affect the expression of rAFP in *L. lactis*, a cell wall protease deficient *L. lactis* strain, NZ9000-*HtrA,* was used to examine whether the cell wall protease affects the production levels of rAFP. The expression levels of *L. lactis* NZ9000-*HtrA* (pNZSASAFP) (**Figure 1B**) were calculated to be 42, 78, 83, and 82 mg/L at culture times of 6, 8, 10, and 12 h, respectively, values which were not significantly different from *L. lactis* NZ9000 (pNZSASAFP). HtrA is the sole extracellular protease that degrades abnormal exported proteins and is responsible for the pro-peptide processing and maturation of native secreted proteins. Others have suggested it was responsible for the housekeeping of exported proteins in *L. lactis* (*21*). Heterologous proteins expressed previously by *L. lactis* NZ9000-*HtrA* exhibited higher secretion levels (*13, 21*). Compared to these other proteins, the relatively small rAFP protein may escape degradation by *HtrA*, contributing to the negligible differences in protein secretion levels between strains.

The rAFP was purified by Ni-NTA chromatography (**Figure 1C**) and the N-terminal sequence was determined to be ATTINASS. The cleavage of signal peptide did not occur at the site predicted in a previous report (*18*). The secreted rAFP had an apparent molecular weight of 6.5 kDa, slightly larger than our previous expressions (5.4 kDa) (*12, 13*). The activity of rAFP was confirmed by assaying the ability of rAFP to inhibit ice recrystallization and single ice crystal formation (data not shown). Many chimeric proteins containing the antifreeze domain have exhibited antifreeze activities (*22*). In this study, the extra amino acids at the N-terminal and 6xHis tag at C-terminus did not affect the antifreeze activity.

Expression of rAFP in Food Grade Culture. For commercial application of heterologous proteins produced by *L. lactis*, food-grade fermentation and expression systems are strongly recommended. For food-grade expression, the product must be guaranteed antibiotic-free and beef-free. The GM17 medium contained bovine spongiform encephalomyelitis (BSE) and considered not beef-free. We previously modified a plant-based, beef-free FMB medium, which achieved high protein expression in *L. lactis* (*13*). In this study, antibiotic-free GM17 and FMB were used and the expressions were analyzed. **Figure 2** show that the FMB achieved better expression levels than GM17 with the highest production level of 86 mg/L at 24 h, after which rAFP dropped, possibly due to plasmid instability. Therefore, the 24 h culture of *L. lactis* NZ9000 (pNZSASAFP) was used to produce rAFP.

The Activity of rAFP Produced by Food Grade *L. lactis* System. Activity of the rAFP purified from the food grade FMB medium cultured *L. lactis* NZ9000 (pNZSASAFP) was analyzed. The ability of rAFP to inhibit ice recrystallization was demonstrated by observing crystal size and shape in a frozen film of solution (**Figure 3B**). The ability of rAFP to cause characteristic changes in ice morphology was also observed, while control PBS buffer did not exhibit ice crystal modification ability (**Figure 3A**). At 48 μg/mL concentration of rAFP, the shape of single ice crystal begins to alter, becoming sharper. The ice crystal for the 48 μg/mL sample was smaller than both the control and the 24 μg/mL rAFP sample. As designated by Miller (*22*), the minimal inhibition concentration of the rAFP was about 48 μg/mL. At concentrations greater than 72 μg/mL, all ice crystals were relatively small. The shaded area was the space between aggregated ice crystals and less in 144 ug/mL rAFP sample than 96 ug/mL rAFP sample. All the ice crystals were relatively smaller as the rAFP concentrations increase. (**Figure 3A**).

Treatment of Frozen Meat with Food grade rAFP. There is great promise in the application of AFP in foods that are frozen only for preservation. AFP may inhibit recrystallization during storage, transport, and thawing, thus preserving food texture by reducing cellular damage and minimizing the loss of nutrients by reducing drip (*23*). In chilled and frozen meat, large ice crystals may form intracellularly, resulting in drip and loss of nutrition during thawing (*6, 7, 24*). Many AFPs localize extracellularly in freeze-tolerant organisms, suggesting that the AFP can be added to food by physical means such as mixing, injection, soaking, or vacuum infiltration (*24*). Soaking bovine and ovine muscle in a solution containing 1 mg/mL of AFP (*6*), or injection antifreeze
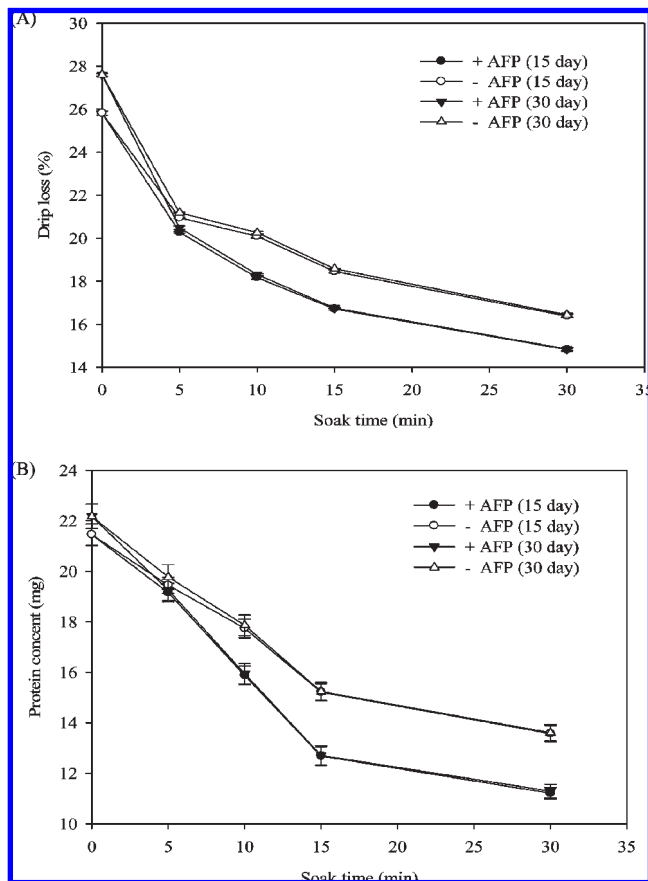
Figure 4. Effect of various immersion times and preservative agents on thawing drip loss (%) (A) and protein loss (B) of frozen meat. The fat-removed fresh pork fillets were cut into equivalent slices, and the fillets were immersed in CIS or ACIS at −12 °C for varying lengths of time. The surface moisture of the fillets was removed using paper towels, and the pork was sealed in plastic zip lock bags and stored at −20 °C. The drip loss and protein loss of frozen fillets were analyzed after 15 and 30 days of storage. Drip loss was assayed by wrapping the frozen meat in a nylon string and hung vertically in a scale tube, centrifuged at 40g for 90 min. The volume of the liquid collected represented the drip loss. The protein concentration of the drip was quantified by a DC Protein Assay kit (Bio-Rad Laboratories Inc., USA). Pork fillets that were not immersed in preservative were sealed and stored at −20 °C as control experiments.

Figure 5. Effect of rAFP pretreatment and frozen storage on thaw drip loss (%) (A) and protein loss (B) of frozen meat. Fresh pork fillet was fixed and cut into equivalent slices, and the fillets were immersed in CIS or ACIS at −12 °C for time intervals. The surface moisture of fillets was removed using paper towels, sealed in plastic zip lock bags, and stored at −20 °C. At day 15, some frozen fillet samples were transferred to room temperature for 1 h and then returned to −20 °C. At day 30, the drip loss and protein loss were analyzed. Drip loss was assayed by wrapping the frozen meat in a nylon string, hanging vertically in a scale tube, and centrifuging at 40g for 90 min. The volume of the liquid collected in the tube represented the drip loss. The protein concentration of the collected liquid was quantified by a DC protein assay kit (Bio-Rad Laboratories Inc., USA). Fresh pork fillet and frozen nonimmersion pork fillets were compared as controls.

glycoprotein (AFGP) before slaughter of lambs (*7*), reduced drip loss and ice crystal size.

In this study, we developed a cryo-immersion solution (CIS) consisting of food grade NaCl plus food grade alcohol and an antifreeze cryo-immersion solution (ACIS) consisting of CAFP dissolved in CIS. Both CIS and ACIS enabled the soaking of meat under conditions (−12 to −20 °C) that avoided microbial contamination or enzymatic degradation to enhance the quality of frozen meat. The salt in ACIS may enhance antifreeze activity of AFP (*25*). **Figure 4A** shows that the drip loss of the frozen meats treated with ACIS were lower than CIS. The protein loss of frozen meats treated with CIS and ACIS were lower than control frozen meat (**Figure 4B**). As expected, the ACIS showed less drip loss and protein loss than CIS; soaking for 30 min decreases the drip loss by approximately 50%.

During the cold chain transport of frozen food, the quality of frozen meat can be damaged by freeze−thaw cycles. The thawing time is usually limited to 30 min. **Figure 5** shows the effect of CIS and ACIS on frozen meat after a freeze−thaw cycle. The drip loss of the frozen meats treated with ACIS was lower than CIS, and
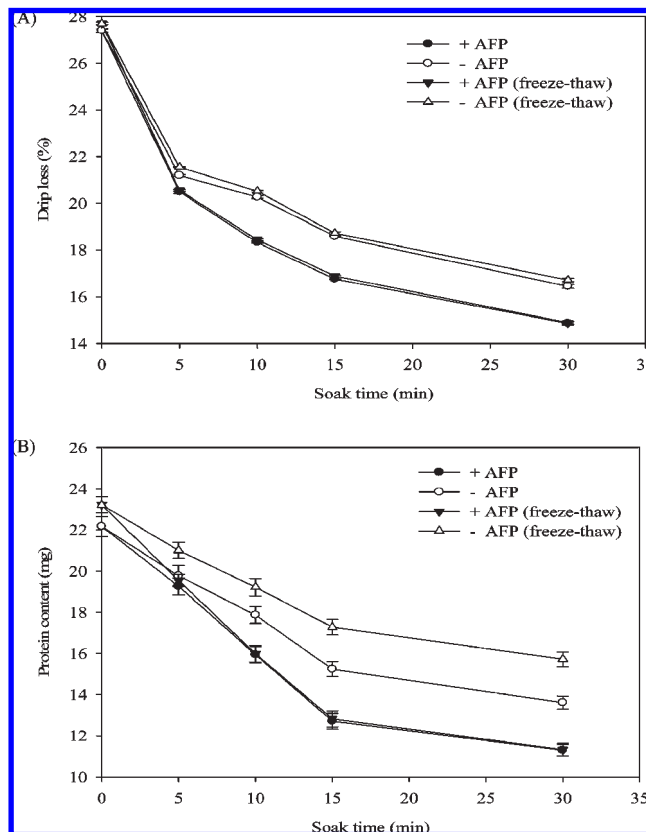
thawing did not increase the drip loss for both CIS and ACIS treated frozen meat (**Figure 5A**). The protein loss of frozen meats treated with CIS and ACIS were lower than that of control frozen meat. The effect of ACIS on the protein loss was significant. CIS-treated frozen meat showed higher protein loss after thawing once, while ACIS-treated frozen meat did not lose significantly more protein after one freeze−thaw cycle than ACIS-treated frozen meat that was not thawed and refrozen (**Figure 5B**). Protein loss in frozen meat is a main problem of meat processing industry. The rAFP provides solution to this problem.

Consumer sensory evaluation of the frozen meat is summarized in **Table 2**. A panel consisting of 53 members ranging in age from 18 to 30 were selected randomly from local staff members. The tenderness, juiciness, flavor, and total acceptance of boiled (5 min), previously frozen meats from various experimental groups (CIS, ACIS or untreated) were analyzed. All parameters of CIS and ACIS-treated frozen meats were significantly better than untreated frozen meat. The juiciness of ACIS-treated frozen meats was significantly better than other frozen meat. These results suggest that application of the antifreeze protein improved the quality of frozen meat. Compared to the previous reports
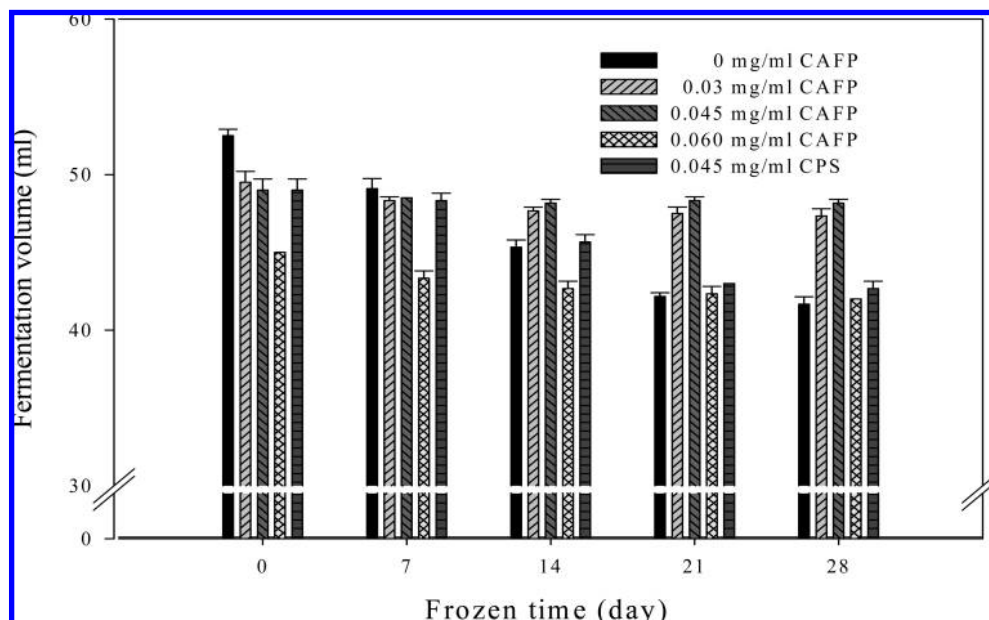
Figure 6. Effect of recombinant freeze-dried *L. lactis* supernatant on the dough volume after leavening. Yeast was mixed with flour, and sugar (±CAFP) was dissolved fully in distilled water. The two portions were mixed thoroughly to form dough. The dough was stored at −20 °C for varying intervals. Proofing (fermentation at 37 °C for 1 h) of frozen dough was conducted after thawing (4 °C, 12 h), and the dough volumes were measured. Control dough was not frozen but instead fermented directly.

Table 3. Consumer Preference Analysis of Breads Made from Fresh or Frozen Doughs

breads	volume	shape	crust color	crumb color	grain	texture	odor	overall acceptance
frozen dough	4.55[b]	4.23[b]	4.53[a]	5.45[b]	4.41[c]	4.38[b]	4.32[b]	4.57[b]
CPS	4.79[b]	4.53[a]	4.55[a]	5.55[a,b]	4.51[b,c]	4.87[a]	3.87[c]	4.58[b]
CAFP	4.89[b]	4.55[a]	4.57[a]	5.79[a]	4.81[a,b]	4.96[a]	3.98[c]	4.91[a]
fresh dough	4.91[a]	4.62[a]	4.62[a]	5.77[a]	4.91[a]	5.00[a]	4.85[a]	5.08[a]

[a] Fresh dough, frozen dough, CPS (crude powder from the supernatant of a untransformed *L. lactis* culture) treated frozen dough, or CAFP (crude rAFP powder of the supernatant of transformed *L. lactis* culture) was thawed (4 °C, 12 h), incubated at 37 °C for 1 h, and baked at 195 °C oven for 12 min. The baked bread was transferred to room temperature for 10 min before the consumer test. [b] Differences among samples were determined by Duncan's multiple range tests at $p < 0.05$. The same letter indicates no significant difference among samples. [c] All data are mean scores (1 = extremely dislike, 4 = neither like nor dislike, 7 = extremely like) of 53 consumers.

which used AFP from natural resources (*6, 7*), the recombinant AFP analogue was not only economically easy to obtain but also provided an efficient way to apply in frozen meat or other forms of refrigerated processed meats.

Quality of Bread Made with Food grade rAFP Treated Frozen Dough. Frozen storage has the potential to solve the problem of short shelf life of conventional bread dough although several problems need to be considered (*8*). For example, frozen storage of dough typically results in loss of bread quality, reflected by a reduced volume, longer leveling time, and an decrease in the proportion of gas content (*26*). The problem of freeze-weakening the dough structure and decreasing of the CO_2 retention capacity can be mitigated by using strong wheat flour or freeze-tolerant yeasts (*27*). Another problem is that freezing dough prolongs leavening time of the dough and deteriorates the texture of the product. Several additives can minimize this problem; however, the effect of each additive on the quality of bread made from treated dough should be carefully evaluated (*9*). AFPs have also been used to improve the quality of frozen dough. Expression of a recombinant antifreeze peptide (GS-5) in industrial baker's yeast (*Saccharomyces cerevisiae*) enhanced the gassing rate and total gas production in frozen sweet dough (*27*). Concentrated antifreeze protein from carrots (*Daucus carota*) has been shown to improve the leavening of frozen dough (*8*), resulting in maintenance of loaf volume and improving the softness of the dough during frozen storage (*9*). In this study, the effect of CAFP on frozen dough was

examined. **Figure 6** shows that the dough treated with CAFP retained its leavening capacity. The specific densities of loaves were 0.241, 0.252, and 0.248 for fresh control dough, CPS, or CAFP treated dough, respectively, and 0.276, 0.262, and 0.248 for frozen dough, CPS, or CAFP treated frozen dough, respectively. The specific density of loaf made from CAFP treated frozen dough was the same as unfrozen dough and similar to bread made from fresh dough. The results of the consumer test are summarized in **Table 3**. The volume, shape, crust color, crumb color, grain, texture, odor, and overall acceptance were evaluated. The bread made from frozen dough treated with CAFP showed no significant difference in scores of all parameters. Breads made from frozen dough treated with CAFP or CPS received lower scores for odor, although this may be attributed to components of the lyophilized culture medium. However, the overall acceptance of bread made from frozen dough treated with CAFP was not significantly different from bread made with fresh dough. CAFP may therefore be considered a beneficial additive for frozen dough processing. From results, the food grade production of recombinant AFP analogue can be considered as genetically modified food production which has been used in many food enzymes, additives production and therefore is much acceptable than the genetically modified baker's yeast (*27*). The rAFP also provides more viable applications than the concentrated antifreeze protein from carrots (*8*).

Taken together, our results show that safety and high level production of a novel recombinant type I antifreeze protein

analogue (rAFP) can be produced safely and expressed at high levels by *L. lactis*. Lyophilized, crude rAFP was shown to improve the quality of frozen meat and frozen dough. The rAFP also has potential applications in the cryo-preservation of other foods or pharmaceutical materials.

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