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Functional Secretion of a Type 1 Antifreeze Protein Analogue by Optimization of Promoter, Signal Peptide, Prosequence, and Terminator in *Lactococcus lactis*

CHUAN-MEI YEH,* XIN-HUI HUANG, AND CHAN-WHEI SUE

Department of Food Science and Biotechnology, National Chung-Hsing University, Taichung, Taiwan, Republic of China

Lactococcus lactis is a food-grade microorganism of major commercial importance. Antifreeze protein is a potent cryogenic protection agent for the cryopreservation of food and pharmaceutical materials. In this study, extracellular expression of a novel recombinant type I antifreeze protein analogue (rAFP) in *L. lactis* was optimized. An efficient *SlpA* promoter (P_{SlpA}) was fused to various signal peptides (SPs) and propeptide sequences to examine the extracellular expression levels of rAFP. An efficient signal peptide, SP_{sacB}, fused to prosequence AE, enabled higher extracellular rAFP production; use of the *SlpA* terminator (Ter_{SlpA}) was a further improvement. The extracellularly expressed rAFP successfully inhibited ice recrystallization and is thus potentially applicable for cryogenic preservation.

KEYWORDS: Type I antifreeze protein analogue; extracellular production; *Lactococcus lactis*

INTRODUCTION

Genetic manipulation of lactic acid bacteria (LAB) has many potential applications in the food industry. *Lactococcus lactis*, a well-characterized Gram-positive bacterium, has long been used in food production. It is a good candidate for heterologous protein delivery in foodstuffs and in the digestive tract and can be used for protein production in a fermentor (*1*). Many heterologous proteins have already been produced in *L. lactis*, and industrial-scale food-grade *L. lactis* expression systems has been developed (*2*). For industrial gene expression, however, other prokaryotic systems dominate: *Escherichia coli* and *Bacillus subtilis* both offer high yields, ease in genetic handling, long-term experience, and extensive documentation with the U.S. Food and Drug Administration (FDA) (*3*). Nonetheless, *E. coli* has various disadvantages, such as the formation of endotoxins and inclusion bodies; the presence of two membranes, which hampers secretion; and a relatively complicated aerobic fermentation pathway (*4*). *B. subtilis* is superior to *E. coli* by virtue of its GRAS (generally regarded as safe) rating by the FDA, lack of endotoxins and inclusion bodies, and high secretion capacity (*3*). *L. lactis*, on the other hand, has a number of properties that make it an interesting alternative candidate for large-scale gene expression (*1*). It has been used in food production for thousands of years (thus, it is considered to be food-grade), produces no endotoxins or inclusion bodies, has the capacity to secrete proteins, and can be fermented by simple, nonaerated conditions, allowing for direct scale-up from the 1-L scale to the 1000-L scale (*2*).

* Author to whom correspondence should be addressed (telephone 886-4-22840386; fax 886-4-22853165; e-mail cmyeh@nchu.edu.tw).

For industrial production, extracellular expression is favored over cytoplasmic expression. Extracellular expression not only provides a convenient means to obtain large amounts of recombinant proteins in a relatively pure form, without contamination from cellular proteins, but it also greatly facilitates downstream processing and enables the use of continuous cultures for large-scale production (*3*). Although many heterologous proteins have been expressed by *L. lactis*, optimization of host factors affecting heterologous protein production is still a challenge. To optimize the expression of functional target proteins for use in the industrial process, a strong constitutive promoter and an efficient secretion signal are highly recommended (*5*).

In *L. lactis*, as in other Gram-positive bacteria, secreted proteins are synthesized as a precursor containing an N-terminal signal peptide (SP). The signal peptide is recognized by the host secretion machinery and guides the translocation of mature protein across the cytoplasmic membrane. The SP is then cleaved and degraded, and the mature protein is released into the culture supernatant. Most secreted proteins require subsequent folding and maturation steps to acquire their active conformation (*5*). Studies on secretion enhancement have identified several ways to increase production yield: secretion of a reporter nuclease was increased in *L. lactis* by using the USP signal peptide (SP_{USP}) (*6*), and optimization of a propeptide located in the junction between the SP and the mature protein led to an increased secretion efficiency (SE) and yield of heterologous proteins (*7*). There are few data concerning secretion bottlenecks and biotechnological tools for heterologous protein expression in *L. lactis*. For each heterologous protein,

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Table 1. Strains and Plasmids Used in This Study

strain or plasmid	relevant chacteristics	source or ref
strains		
Escherichia coli JM109	*end*A1 *rec*A1 *gry*A96 *thi hsd*R17 (*rK*-, *mK*$^+$) *rel*A1 *sup*E44 (*lac-pro*AB)[*tra*D36 *pro*AB *lac Z* ΔM15], used as DNA manipulation strain	Promega Co.
Lactococcus lactis NZ9000	*L. lactis* MG1363 *pepN::nisRK*	NIZO (The Netherlands)
Lactobacillus acidophilus ATCC 4356	source of P_{SlpA}, $SP_{slpA,}$ and Ter_{slpA}	ATCC
plasmids		
pNZ8008	*Cm*^{r}, a broad host range vector for *L. lactis* and *E. coli*	NIZO (The Netherlands)
pNZter	*Cm*^{r}, a derivative vector of pNZ8008, broad host range vector for *L. lactis* and *E. coli*, P_{nisA}-terminator from pQE30 (*terQ*)	Liu et al. (*23*)
pHYσ^AGUS	Tc^r and Ap^r, *synthetic expression control sequence*(SECS)-*gusA- terQ*	Liu et al. (*23*)
pNZGUS	*Cm*^{r}, pNZ8008 derivative, SECS-*gusA- terQ*	Liu et al. (*23*)
pHYSAG	Tc^r and Ap^r, pHY300PLK derivative, P_{SlpA}-*gusA- terQ*	this study
pNZSAG	*Cm*^{r}, pNZ8008 derivative, P_{SlpA} -*gusA- terQ*	this study
pHYNSR	Tc^r and Ap^r, pHY300PLK derivative, source of P_{NSR}	unpublished
pHYNG	Tc^r and Ap^r, pHY300PLK derivative, P_{NSR}-*gusA- terQ*	this study
pNZNG	*Cm*^{r}, pNZ8008 derivative, P_{NSR}-*gusA-ter*	this study
pUU2ATT	Tc^r and Ap^r, pHY300PLK derivative, source of P_{USP}	unpublished
pHYUG	Tc^r and Ap^r, pHY300PLK derivative, P_{USP}-*gusA- terQ*	this study
pNZUG	*Cm*^{r}, pNZ8008 derivative, P_{USP}-*gusA- terQ*	this study
pNZori177	*Cm*^{r}, pNZ8008 derivative, *rep*A and *rep*C replaced with ori-pACYC177 from pHY300PLK	unpublished
pNZ8008D	*Cm*^{r}, pNZori177 derivative, *Nde*I site within the repA sequence was eliminated by silent mutation	this study
pHYSA1-1ATT	Tc^r and Ap^r, pHY300PLK derivative, P_{SlpA}-SP_{YaB}-AE-*afp*-his t*ag*(his_6)- *terQ*	unpublished
pNZSA1-1ATT	*Cm*^{r}, pNZ8008 derivative, P_{SlpA}-SP_{YaB}-AE-*afp-his*$_6$- *terQ*	this study
pNZDSA1-ale	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}-SP_{YaB}-*afp-his*$_6$- *terQ*	this study
pNZDSA1-usp45	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}-SP_{USP}-*afp-his*$_6$- *terQ*	this study
pNZDSA1-sacB	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}-SP_{SacB}-*afp-his*$_6$- *terQ*	this study
pNZDSA1-slpA	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}-SP_{SlpA}-*afp-his*$_6$- *terQ*	this study
pNZDSA1-sacBA	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}- SP_{SacB} -AE-*afp-his*$_6$- *terQ*	this study
pNZDSA1-sacBL	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}-SP_{YaB}-LEISS-*afp-his*$_6$- *terQ*	this study
pNZDSASm-sacBA14	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}- SP_{SacB} -AE-*afp-his*$_6$- ter_{SlpA14}	this study
pNZDSASm-sacBA2-1	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}- SP_{SacB} -AE-*afp-his*$_6$- $ter_{SlpA12-1}$	this study
pNZDSASm-sacBA2-4	*Cm*^{r}, pNZ8008D derivative, P_{SlpA}- SP_{SacB} -AE-*afp-his*$_6$- $ter_{SlpA12-4}$	this study

the combination of SP and propeptide needs to be optimized for high-level secretion.

Antifreeze proteins (AFPs) cause noncolligative depression of the freezing point and can inhibit ice recrystallization and modify ice morphology (*8*). These properties enable the use of AFPs for cryogenic preservation of cells and tissues or food products (*9*). However, the use of natural AFPs has been limited by the scarcity of natural sources and the requirement for purification procedures. Recombinant production of AFP is a favorable way to overcome this problem (*10*). We previously designed and synthesized a novel recombinant type I antifreeze protein analogue (rAFP) by overlap extension PCR and functionally expressed it in *B. subtilis* (*11*). In this study, we attempted to express the rAFP extracellularly in the food-grade host *L. lactis*. A strong promoter was chosen and fused with various signal peptides and prosequences to determine the optimal secretion signal for extracellularly produced rAFP. The *SlpA* terminator (Ter_{SlpA}) was tested in these constructs as well. The function of rAFP was determined by assaying for inhibition of ice recrystallization and observation of single ice crystals (*11*).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. The bacterial strains and plasmids used in this study are listed in **Table 1**. The *E. coli* strains were propagated at 37 °C with agitation in LB broth (Luria−Bertani, Difco) or supplemented with 20 μg/mL chloramphenicol (*Cm*) for transformants harboring pNZ8008-derivative plasmids or with 25 μg/mL tetracycline (Tc) for transformants harboring pHY300PLK-derivative plasmid. The *L. lactis* NZ9000 strain was propagated at 30 °C in M17 broth (Difco). For transformants harboring pNZ8008-derivative plasmids, 5 μg/mL of *Cm* was supplemented in GM17 (M17 broth supplemented with 0.5% glucose), medium A (*12*), 2GM17B (M17 broth supplemented with 1% glucose and buffered at pH 6.9 with 0.2 M potassium phosphate) (*12*), FMG (5% lactose, 1.5% soy peptone, 1% yeast extract, 1 mM $MgSO_4$, 0.1 mM $MnSO_4$) (*5*), FMB (FMG buffered at pH 7.4 with 0.2 M potassium phosphate)m or FMS (the glucose of FMB was replaced with sucrose), respectively. Cell growth was monitored by measuring the cell density at OD_{600}.

Molecular Techniques. Plasmid DNA was isolated with a Mini-M plasmid DNA extraction system (Viogene, Taipei, Taiwan). Lactic acid bacteria 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. The primers were purchased from Invitrogen Life Technologies (Carlsbad, CA). Ex T*aq* DNA polymerase (Takara, Kyoto, Japan) was used for PCR amplifications. Electrotransformation 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). Electrotransformation of *L. lactis* was performed as described previously (*12*).

Plasmid Construction. In the first part of this study, vectors containing various promoters fused to a β-glucuronidase reporter, *gusA*, were constructed. The pNZ series *L. lactis* expression vectors pNZGUS, pNZSAG, pNZNG, and pNZUG contained, respectively, synthetic control expression sequence (SECS) (*13*), SlpA promoter of S-layer protein of *Lactobacillus acidophilus* (P_{SlpA}) (NCBI accession no. DD459494) (*14*), NSR promoter (P_{NSR}) (NCBI accession no. NC_010901) (*15*), and USP promoter (P_{USP}) (NCBI accession no. M60178 M35374 X53491) (*16*). These vectors were constructed by ligation of *Bgl*II /*Xba*I I-treated pNZter and pHYσ^AGUS, pHYSAG, pHYNGn or pHYUG and electrotransformed into *E. coli* JM109. The desired plasmids were then

Table 2. Primers Used in This Study[a]

Construction	primer	Sequence (5'-3')
pHYSAG	pSA1F	CAATATA**GATCTT**ACAAATAGTATTTTTCGGTCATTTTAACTTGCTA
	pSAR	GAATTA**CATATG**GTCTTTTCCTCCTTGAAATATAAAAAAATGTAATA
pHYUG	pusp45F	CAATATA**GATCT**GTGTTTTGTAATCATAAAGAAATATTAAGGTGGGGTA
	pusp45R	GAATTA**CATATG**TGTTCTTTTTTAATTTTTCCTCCCATTAAGTTC
pHYNG	pnsrF	CAATATA**GATCTT**TGATTATTTAGCCATGACTTGATACC
	pnsrR	GAATTA**CATATG**AAATATGCTCCATCAGTTATTTTAATACTATTAAA
pNZ8008D	repAf	TTATTGGTGCCCTTAAACGCCTGGGGTAATG
	NdeImutr	CTTTGATTCATGAGTCAAATATTCATAAGAACCTTTGATATAATCAAGTATCTCAAC
	NdeImutf	GTTGAGATACTTGATTATATCAAAGGTTCTTATGAATATTTGACTCATGAATCAAAG
	repAr	TAGATA**GTCGAC**TTTCGTCAGGGGGGCTTTTATTTATTC
pNZDSA1-signal peptide	AleSPF2	GTTATA**CATATG**AATAAGAAAATGGGGAAAATTGTT
	AleSPreR	GTTATA**GGATCC**TGCTTGTGCGATCGATGAACT
	Usp45SPF	GTTATA**CATATG**AAAAAAAAGATTATCTCAGCTATTTTAATGT
	Usp45SPR	GTTATA**GGATCC**AGCGTAAACACCTGACAACGG
	SlpASPF	GTTATA**CATATG**AAGAAAAATTTAAGAATCGTTAGCG
	SlpASPR2	GTTATA**GGATCC**AGCGCTAACAGTAGATACAGCAGAA
	SacBSPF	GTTATA**CATATG**AACATCAAAAAGTTTGCAAAACA
	SacBSPreR	GTTATA**GGATCC**CGCAAACGCTTGAGTTGC
	SacBSP2reR	GTTATA**GGATCC**CTCGGCCGCAAACGCTTGAGTTGC
	SacBSP3reR	GTTATA**GGATCC**AGCATCACATGTTGATGAAATTTCTAACGCAAACGCTTGAGTTGCG
pNZDSASm-sacBA	TslpAF	GTTATA**AAGCTT**GTCGTAGCACTAACGCTAACAAAATGA
	TslpAR	GTTATA**CTCGAG**TCTAGAAGAAGATCCTATTAGAACTGTATGTTTAGAAGTGA

[a] The restriction sites are bolded; boxed sequences are *Nde*I elimination site.

electrotransformed into *L. lactis* NZ9000. The intermediate cloning plasmid pHYσ^AGUS, in which the reporter gene *gusA* is driven by the SECS, was constructed previously (*13*). Other intermediate cloning plasmids, pHYSAG, pHYNG, and pHYUG, were constructed by ligation of *Nde*I/*Bgl*II-treated pHYσ^AGUS and PCR products containing P_{SlpA}, P_{NSR}, and P_{USP} sequences and electrotransformed into *E. coli* JM109. PCR reactions were performed with Ex T*aq* DNA polymerase; templates were the *Lb. acidophilus* chromosome for P_{SlpA1} promoter, plasmid pHYNSR for P_{NSR}, and plasmid pUU2ATT for P_{USP}. The PCR reaction conditions were 94 °C (5 min), 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (20 s), and 72 °C (5 min) for P_{SlpA}; 94 °C (5 min), 94 °C (5 min), 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (27 s), and 72 °C (5 min) for P_{NSR}; and 94 °C (5 min), 35 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (12 s), and 72 °C (5 min) for P_{USP}.

For examining extracellular expression, plasmids containing various signal peptides and propeptides were constructed. Plasmid pNZSA1-1ATT was made by ligation of *Bgl*II/*Xba*I-treated pNZ8008 and pHYSA1-1ATT (unpublished), which created a $P_{SlpA}-SP_{YaB}-$AE-afp fusion fragment with extra amino acids AE in the junction of $SP_{YaB}-$rAFP.

To facilitate the cloning procedure, plasmid pNZ8008D was constructed, wherein the *Nde*I site in the repA sequence was ablated by silent mutation. Overlap extension PCR (OEPCR) was performed by using two sets of primers (repAf, NdeImutr) and (NdeImutf, repAr) to amplify overlap PCR products, then using repAf and repAr primers to amplify the *Nde*I site-mutated *repA* gene. The PCR reaction conditions were 94 °C (2 min), 35 cycles of 94 °C (30 s), 60 °C (30 s), 72 °C (90 s), and 72 °C (7 min) for repAf, NdeImutr amplification; 94 °C (5 min), 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (40 s), and 72 °C (7 min) for NdeImutf, repAr amplification; and 94 °C (2 min), 35 cycles of 94 °C (30 s), 58 °C (30 s), 72 °C (2 min), and 72 °C (7 min) for repAf and repAr amplification.

The other rAFP expression vectors pNZDSA1-ale, pNZDSA1-usp45, pNZDSA1-sacB, and pNZDSA1-slpA were constructed next. Plasmid pNZDSA1-ale was made by ligation of *Bgl*II/*Xba*I-treated pNZ8008D and pNZSA1-1ATT; it contains the rAFP gene, *afp*, fused downstream of the signal peptide of subtilisin YaB DNA sequence (SP_{YaB}) (*13*) and driven from the P_{SlpA} promoter. The extra amino acids AE in the junction of SP_{YaB}-rAFP (from plasmid pNZSA1-1ATT) were eliminated. Various signal peptides were inserted into pNZDSA1-ale by ligation of *Nde*I/*Bam*HI-treated pNZDSA1-ale and PCR products containing signal peptides of USP45 (SP_{usp}) (*16*), *B. subtilis* levansucrase (SP_{sacB}) (*17*), and *Lb. acidophilus* S-layer protein (SP_{SlpA}) (*14*) to create pNZDSA1-usp45, pNZDSA1-sacB, and pNZDSA1-slpA, respectively. The PCR reaction conditions were 94 °C (2 min), 35 cycles of 94 °C (30 s), 56 °C (30 s), 72 °C (20 s), and 72 °C (7 min) for AleSPF2, AleSPreR to amplify SP_{YaB}; Usp45SPF, Usp45SPR to amplify SP_{USP}; SacBSPF, SacBSPreR to amplify SP_{SacB}; and SlpASPF, SlpASPR2 to amplify SP_{SlpA}.

To enhance extracellular expression, extra prosequences, AE and LEISSTCDA, were introduced into the junction of SP and rAFP in pNZDSA1-sacB to create pNZDSA1-sacBA and pNZDSA1-sacBL, respectively. The codons of prosequences were designed according to the codon preference of *B. subtilis* and were amplified by PCR, digested with *Nde*I/*Bam*HI, and then ligated into pNZDSA1-sacB. The PCR reaction conditions for SacBSPF, SacBSP2reR to amplify *SPsac-A* and SacBSP2reR, SacBSP3reR to amplify *SPsac-LEIS* were the same as for SacBSPF, SacBSPreR.

Terminator replacement vectors were also constructed. The PCR-amplified terminator sequence (Ter_{SlpA}) of *Lb. acidophilus* S-layer protein (*14*) was digested with *Hin*dIII/*Xho*I and ligated into pNZDSA1-sacBA to create terminator replacement vectors pNZDSASm-sacBA14, pNZDSASm-sacBA2-1, and pNZDSASm-sacBA2-4. The PCR reaction conditions were 94 °C (2 min), 35 cycles of 94 °C (30 s), 57.2 °C (30 s), 72 °C (10 s), and 72 °C (7 min) for TslpAF, TslpAR to amplify the SlpA terminator.

All constructs were confirmed by diagnostic digest and DNA sequencing (Tri-I Biotech Inc., Taipei, Taiwan). The flow sheet of the constructions can be seen in the Supporting Information. Primers are listed in **Table 2**.

Measurement of β-Glucuronidase (GusA) Activity. β-Glucuronidase activity was monitored by plate assay using X-Gluc as substrate (*18*). Plates of *L. lactis* transformants cultured for 48 h were incubated at 4 °C for 2 h to develop the blue color. The specific activity assay was performed with PNPG (*p*-nitrophenyl-β-D-glucose) as substrate. Cell pellets were treated with 0.5 mg/mL lysozyme before acetone/toluene (9:1) extraction. The units of β-glucuronidase were expressed in terms of the OD_{600} of cultures.

Expression, Purification, Tricine-SDS-PAGE Analysis, and N-Terminal Amino Acids Analysis of Recombinant Antifreeze Protein Analogue. Single colonies of *L. lactis* transformed with various rAFP expression vectors were cultured, and the cell density was measured at various time intervals. The cultures were centrifuged at 14000*g* for 10 min at 4 °C, and the supernatant proteins were analyzed by Tricine-SDS-PAGE (*19*). The supernatant proteins of *L. lactis* transformants were precipitated as described (*12*) and resuspended to achieve a 10-fold concentrate. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, and the mixture was boiled for 5 min and then loaded in each lane of an SDS-PAGE gel. The purification of rAFP was preceded by adjusting the supernatants to pH 8.0 using adjusting buffer (500 mM NaH_2PO_4, 1.5 M NaCl, 100 mM imidazole) before native Ni-NTA affinity chromatography purification and preceded

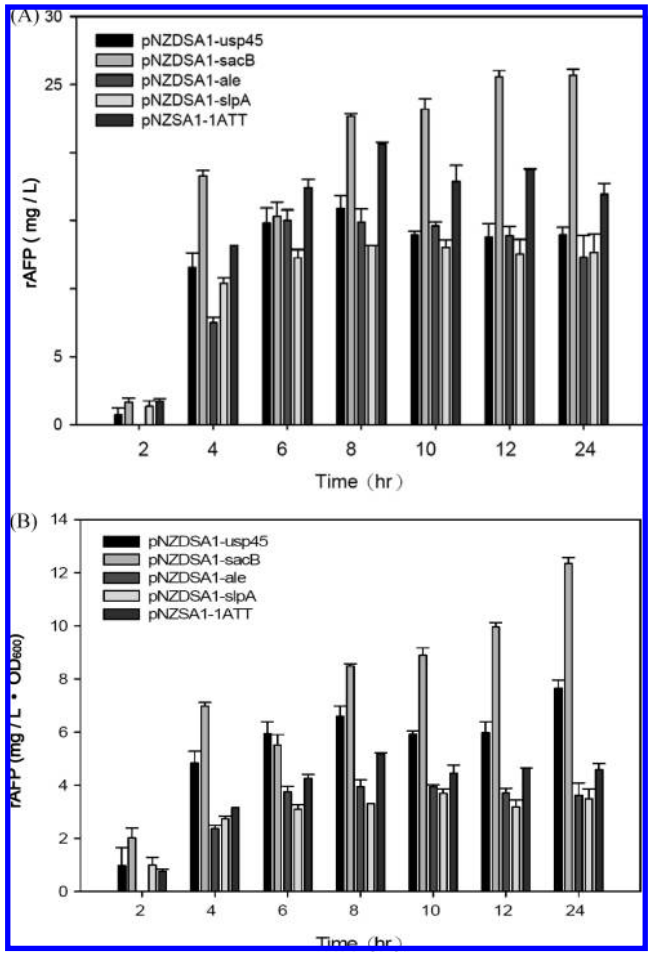

Figure 1. Productivity (**A**) and yield (**B**) of extracellular rAFPs expressed by *L. lactis* NZ9000 (pNZDSA1-sacB), NZ9000 (pNZDSA1-usp45), NZ9000 (pNZDSA1-ale), NZ9000 (pNZDSA1-slpA), and NZ9000 (pNZDSA1-1ATT). The GM 17 cultured transformants were centrifuged at 14000*g* for 10 min at 4 °C, and the supernatant proteins were precipitated by TCA, washed with cold acetone resolved to 10-fold concentrations, and analyzed by Tricine-SDS-PAGE. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, and the mixture was boiled for 5 min and then loaded in each 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 band of fixed concentraion on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co., Ltd., Taipei, Taiwan). The milligrams of rAFP per liter of supernatant represents the productivity; the productivity per cell mass (OD_{600}) represents the yield.

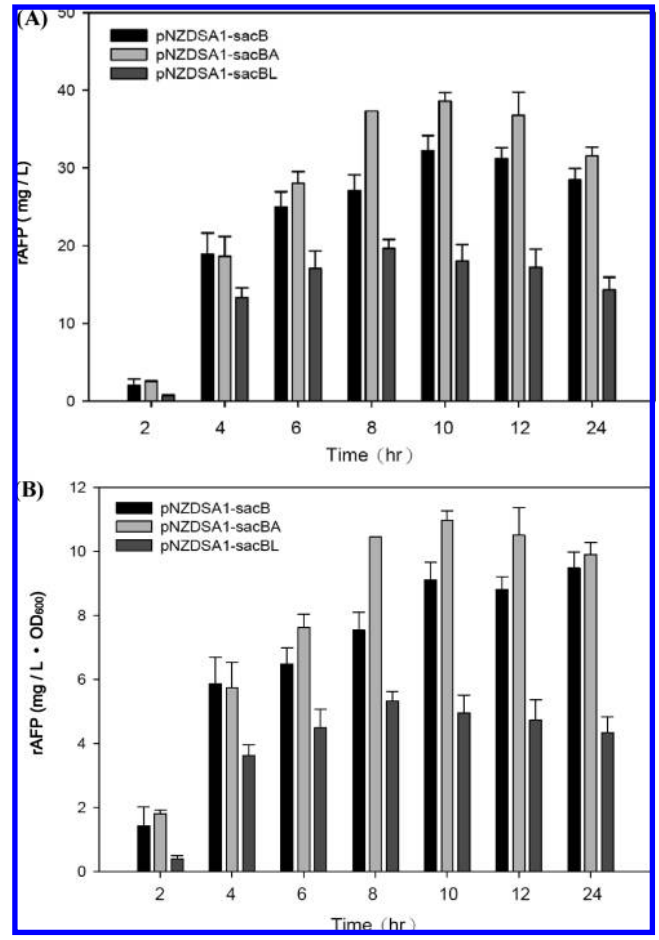

Figure 2. Productivity (**A**) and yield (**B**) of extracellular rAFPs expressed by *L. lactis* NZ9000 (pNZDSA1-sacB), NZ9000 (pNZDSA1-sacBA), and NZ9000 (pNZDSA1-sacBL). The GM 17 cultured transformants were centrifuged at 14000*g* for 10 min at 4 °C, and the supernatant proteins were precipitated by TCA, washed with cold acetone resolved to 10-fold concentrations, and analyzed by Tricine-SDS-PAGE. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, and the mixture was boiled for 5 min and then loaded in each 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 band of fixed concentraion on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co., Ltd.). The milligrams of rAFP per liter of supernatant represents the productivity; the productivity per cell mass (OD_{600}) represents the yield.

according to the QIAexpressionist instructions (4th ed., Qiagen). Purified rAFP was quantified by a DC Protein Assay kit (Bio-Rad Laboratories Inc. USA). The yield of extracellular rAFP was estimated by comparing the density of rAFP bands to the standard purified recombinant AFP protein band of fixed concentraion on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co., Ltd., Taipei, Taiwan). N-Terminal amino acid analysis was performed by electroblotting of the SDS-PAGE gel onto poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad Laboratories Ltd.) using an electroblotter (model VEP-2, Owl Scientific, Inc.) as described previously (*20*). The partial amino N-terminal sequences were analyzed by automatic 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 according to a variation of the splat assay (*21*). A 1-μL droplet of control PBS buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) or AFP in PBS buffer (400 μg/μL) was spread between two coverslips and placed in the cooling chamber of a Linkam BCS196 cold stage (Linkam Scientific Instruments Ltd., Tadworth, U.K.) attached to an Olympus BX41 microscope mounted with a CCD camera (Roper Scientific, USA). The sample was crash-cooled to −40 at 90 °C min^{-1}, held for 1 min, and warmed at 90 °C min^{-1} to −7 °C, held for 60 min, and photographed. The observed changes in size and number of crystals represent the ice recrystallization inhibition activity.

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^{-1}, held for 1 min, warmed at 90 °C min^{-1} to just above the melting point (~ −1.5 °C), and held until a single crystal formed and was photographed.

RESULTS AND DISCUSSION

Expression Levels of Reporter β-Glucuronidase by Promoters P_{USP}, P_{NSR}, and P_{SlpA1} and Synthetic Expression Control Sequence (SECS) in *L. lactis*. Although many

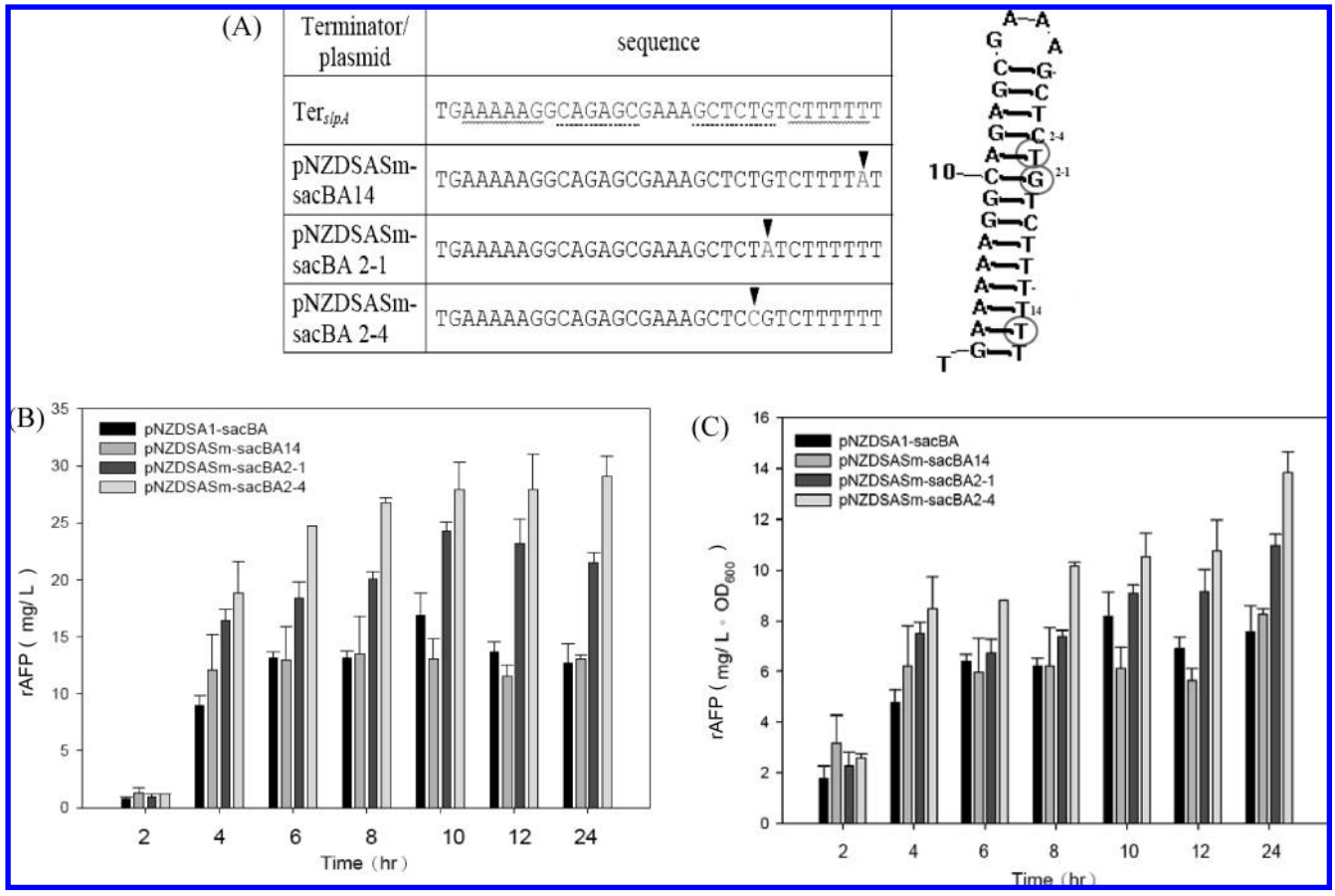

Figure 3. Sequences and predicted secondary structures of site-mutated *slpA* transcription terminator (**A**), productivity (**B**), and yield (**C**) of extracellular rAFP expressed by *L. lactis* NZ9000 (pNZDSA1-sacB), NZ9000 (pNZDSASm-sacBA14), NZ9000 (pNZDSASm-sacBA2-1), and NZ9000 (pNZDSASm-sacBA2-4). The GM 17 cultured transformants were centrifuged at 14000*g* for 10 min at 4 °C, and the supernatant proteins were precipitated by TCA, washed with cold acetone resolved to 10-fold concentrations, and analyzed by Tricine-SDS-PAGE. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, and the mixture was boiled for 5 min and then loaded in each 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 band of fixed concentraion on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co.). In (**A**), the sequences to form stem loop structure of Ter$_{SlpA}$ are underlined; the mutated bases are indicated and circled. In (**B**), the milligrams of rAFP per liter of supernatant represents the productivity, and in (**C**) the productivity per cell mass (OD$_{600}$) represents the yield.

heterologous proteins have been expressed by *L. lactis*, the expression and secretion levels have been lower than in *B. subtilis* (*5, 12*). Optimization of host factors affecting heterologous protein production in *L. lactis* is still a challenge (*4*). To optimize the expression of functional target proteins ready for use in the industrial process, a strong constitutive promoter is preferable to an inducible system (*22*). In this study, several promoters were evaluated for driving rAFP expression. A homologous chromosome Usp45 promoter (P_{USP}) was reported to have higher transcription efficiency than promoters derived from plasmid and bacteriophage 4SK11G (*16*). We had previously synthesized a synthetic expression control sequence (SECS), which was extremely efficient in *B. subtilis* (*13*); the SECS showed a consensus sequence identical to those of the predicted strong promoters of *L. lactis* (*22*). NSR promoter (P_{NSR}) showed higher transcription efficiency than the SECS in *Lactobacillus paracasei* (*23*). The promoter of *Lb. acidophilus* S-layer protein (P_{SlpA}) was reported to be strong in *L. lactis* (*14, 22*). These promoters were assayed with a β-glucuronidase reporter in *L. lactis*. The specific activities of β-glucuronidase in 48-h cultures of pNZSAG, pNZNG, pNZUG, and NZGUS were 2150, 36.5, 25.4, and 16.2 units/OD$_{600}$, respectively. Promoter P_{slpA} was the most efficient. There are no distinct differences among the consensus sequences of these promoters; other factors may contribute to the higher expression of the P_{SlpA} promoter. A previous study showed that the 5′-untranslated leader sequence (UTLS) of the *slpA* gene produced a 5′ stem−loop structure, which stabilized the mRNA (*22*); this UTLS was included in our construct.

Extracellular Expression of rAFP Using the SlpA Promoter and Various Signal Peptides. To date, secretion bottlenecks and biotechnological tools for heterologous expression have been characterized in dominant prokaryotic systems for industrial-scale production, such as *E. coli* and *B. subtilis* (*3, 4*). For *L. lactis*, although gene expression can now be tightly controlled, secretion efficiency (SE, the proportion of secreted mature protein vs that of the intracellular precursor) remains low, and few data are available (*23*). Previous reports used lactococcal signal peptides (*6*) and synthetic propeptides (*7*) to increase heterologous protein secretion rates in *L. lactis*. However, the peptides used for wild-type heterologous proteins might not function as efficiently in mutated heterologous proteins (*5*). Therefore, it is necessary to examine the secretion signals for individual heterologous proteins. In this study, homologous SP_{USP}, which was reported to be efficient in *L. lactis*, and heterologous SP_{sacB}, SP_{YaB}, SP_{SlpA}, which were shown to drive heterologous protein secretion in *L. lactis* on our unpublished data, were analyzed for extracellular expression of

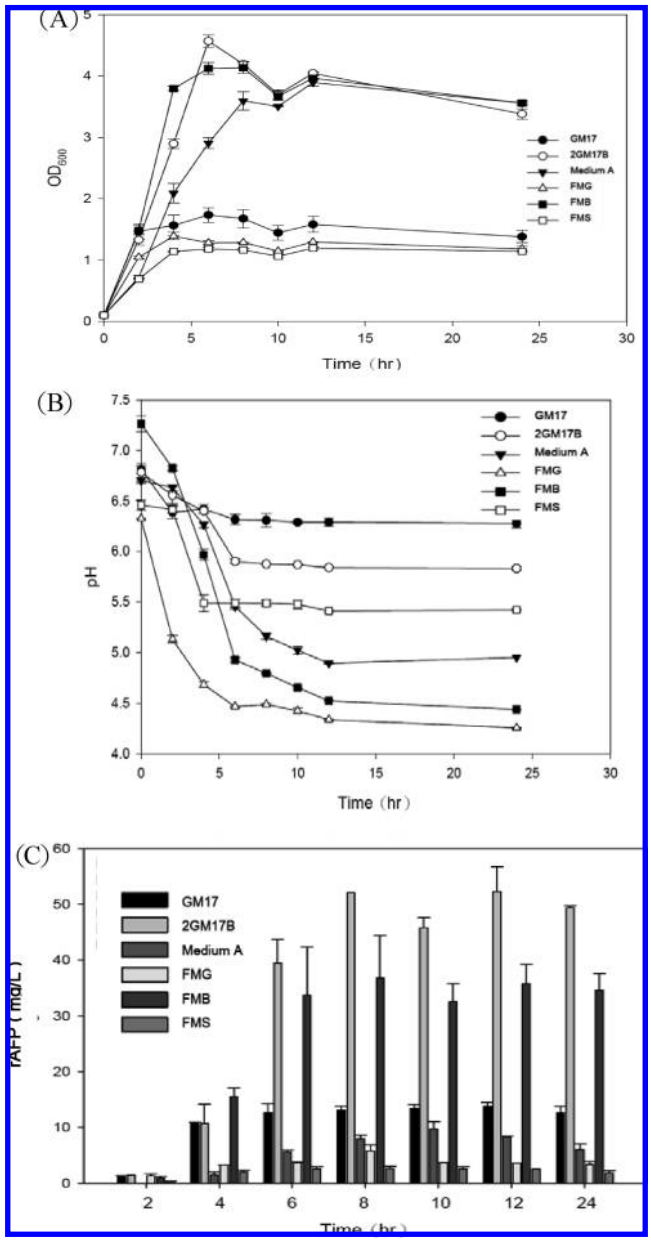

Figure 4. Cell growth (**A**), medium pH (**B**), and productivity of extracellular rAFPs (**C**) of *L. lactis* NZ9000 (pNZDSASm-sacBA2-4) cultured in various media. The transformants were cultured under various media, cell growth was monitored by measuring the cell density at OD_{600}, and the pH of each medium was measured. The transformants cultured under various media were centrifuged at 14000*g* for 10 min at 4 °C, and the supernatant proteins were precipitated by TCA, washed with cold acetone resolved to 10-fold concentrations, and analyzed by Tricine-SDS-PAGE. An aliquot (16 μL) of concentrate was mixed with 4 μL of loading buffer, and the mixture was boiled for 5 min and then loaded in each 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 band of fixed concentration on the SDS-PAGE gel using the Gel-Pro Analyzer version 3.0 (Total-Integra Technology Co., Ltd.). The milligrams of rAFP per liter of supernatant represents the productivity.

rAFP. rAFP secreted by SP_{sacB} achieved 25 mg/L, higher than extracellular rAFPs expressed by other SPs (**Figure 1A**). To choose a more efficient SP, we compared the amount of secreted rAFPs per cell mass. **Figure 1B** shows that the yield of 12 mg/L·OD_{600} by *L. lactis* NZ9000 (pNZDSA1-sacB) was 1.6-, 3.0-, 3.2-, and 2.4-fold higher than NZ9000 (pNZDSA1-usp45), NZ9000 (pNZDSA1-ale), NZ9000 (pNZDSA1-slpA), and NZ9000 (pNZDSA1-1ATT), respectively. The *L. lactis* transformant harboring pNZDSA1-1ATT exhibited slightly higher extracellular rAFP expression than pNZDSA1-ale. The difference between pNZSA1-1ATT and pNZDSA1-ale was that pNZSA1-1ATT included two extra amino acids, AE, in the junction of SP_{YaB}−rAFP. As indicated in previous papers, the prosequence might affect the extracellular expression of heterologous proteins (*5, 7*). Therefore, we engineered the prosequence to examine its effect on extracellular rAFP production.

Prosequence AE Improves the Extracellular Expression of rAFP. Prosequences are N terminal to the mature protein moiety and may greatly affect the translocation efficiency across the cytoplasmic membrane (*5*). Some propeptides function as chaperones in the cytoplasm and guide the folding of the active protein extracellularly (*24*). Some propeptides play a positive role in SE (*24*). In *E. coli*, the charge balance between the N termini of the SP and of the mature moiety may be critical for SE (*25*). In *L. lactis*, the propeptide of reporter nuclease (Nuc), sequence LEISSTCDA, has been shown to promote secretion. Insertion of negatively charged residues in the N terminus of the mature moiety affected the SE (*5, 7*). Here, we examine the effects of changing the propeptide on the secretion of rAFP. The rAFP expressed by pNZDSA1-sacBA in which extra AE were introduced into the junction of SP and rAFP achieved higher secretion yield than pNZDSA1-sacB; the mg/L productivity and mg/L·OD_{600} yield were 1.19- and 1.16-fold higher (**Figure 2**). By contrast, pNZDSA1-sacBL, in which extra LEISSCTDA was introduced into the junction of SP and rAFP, exhibited lower secretion of rAFP among three transfromants; the mg/L productivity and mg/L·OD_{600} yield were 0.53- and 0.45-fold lower than in NZ9000 (pNZDSA1-sacBA).

In pNZDSA1-sacBA, the N terminus of rAFP (AEGSDTASD) contains three acidic amino acids; in pNZDSA1-sacB, the N terminus (GSDTASD) contains two acidic amino acids and lacks a common negatively charged residue at position 2 of the SP (*26*). For efficient translocation, the positive-inside rule posits that the charge at the N terminus of a precursor protein should be superior to the charge surrounding the cleavage site (including the N terminus of the mature protein) (*26*). In this study, the N terminus (AEGSDTASD) of rAFP produced by pNZDSA1-sacBA enhanced the secretion of rAFP, probably due to the distribution and charges of the acidic amino acids, which could enhance the SE and increase extracellular production. The extra LEISSTCDA prosequence has been reported to enhance the extracellular expression of heterologous proteins (*5*−*7*). Surprisingly, in this study, the LEISSTCDA prosequence lowered the amount of secreted rAFP. A previous study found that negatively charged and neutral propeptides enhanced Nuc secretion in *L. lactis* and exhibited a dual effect of improving SE and increasing protein yield (*27*). Unexpectedly, in pNZDSA1-sacBL, the extra LEISSTCDA in front of the rAFP N terminus GSDTASD actually lowered the secretion of rAFP. Further investigation is needed to determine whether the extra negative charges or other side chains in GSDTASD downstream of LEISSTCDA affect the SE and extracellular production. However, our result indicates that LEISSTCDA may not enhance the SE of all heterologous proteins and that the N-terminal sequence of the heterologous protein itself may influence the SE.

Enhancement of Extracellular rAFP Expression by Terminator Replacement. Previous studies have shown that the terminator not only reduces plasmid destabilization resulting from transcriptional read-through but also increases the half-

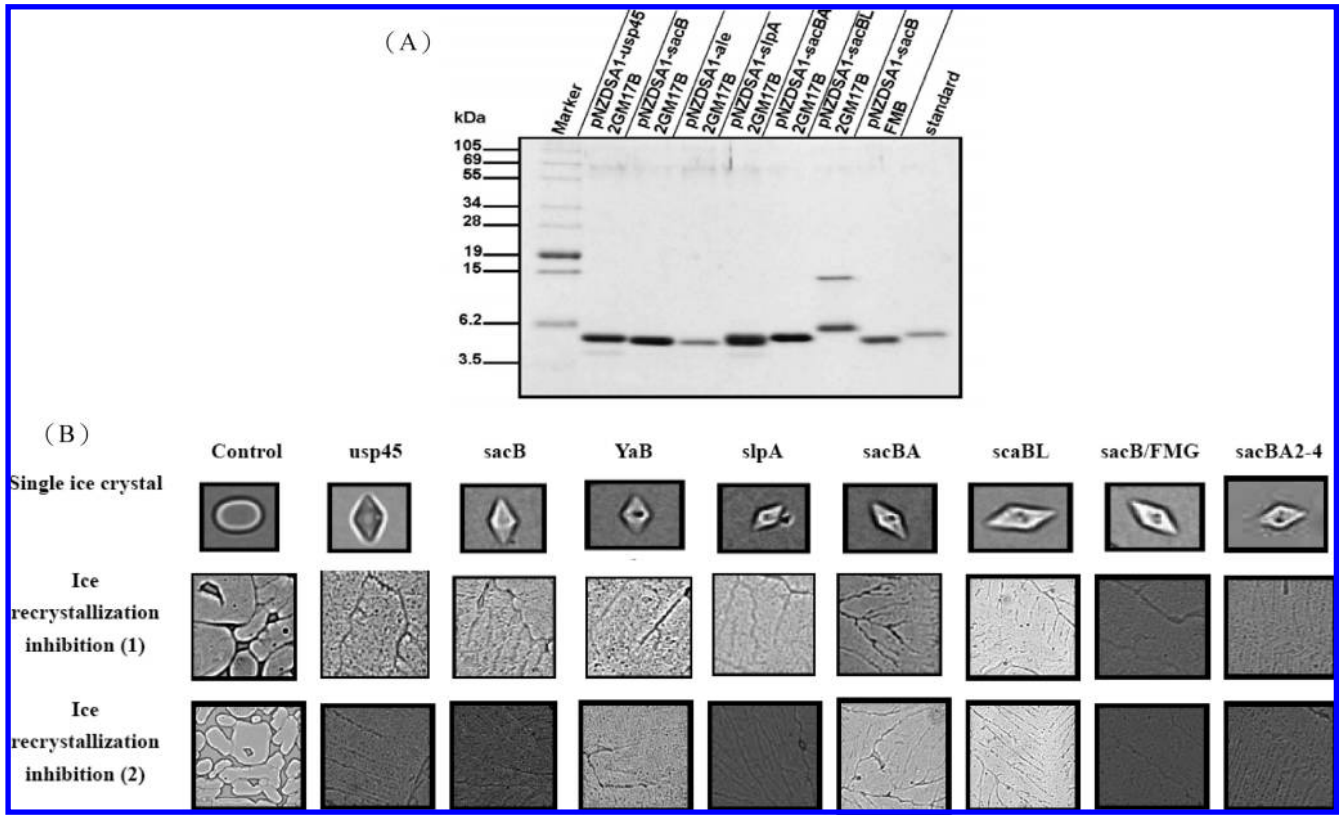

Figure 5. Tricine-SDS-PAGE analysis of purified rAFPs (**A**) and ice morphology and ice recrystallization inhibition activity (**B**) of rAFPs. For (**A**), the supernatants of 8 h of culture were collected, adjusted to pH 8.0, then rAFPs were purified by native Ni-NTA affinity chromatography. A 1 μL droplet of PBS buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) or rAFP in PBS buffer (400 μg μL^{-1}) was spread between two coverslips and placed in the cooling chamber of a Linkam BCS196 cold stage attached to an Olympus BX41 microscope mounted with a CCD camera (Roper Scientific). For ice morphology, the sample was crashed cooled to −40 °C and held for 1 min, and the temperature was then elevated until a single ice crystal was formed and the temperature was held to let the ice crystal grow; the ice crystal morphology was observed. For ice recrystallization inhibition activity, the sample was crashed cooled to −40 °C and held for 1 min, then the temperature elevated to −7 °C and held for 60 min to observe the ice crystal size. All rates of temperature shift were 90 °C min^{-1}. Shaded areas are the space between aggregated ice crystal; spots a\re the small air droplets.

life of the mRNA (*28*). Adding a strong terminator improved the expression levels of heterologous proteins (*13*). In this study, the terminator of *slpA* (Ter_{slpA}) was cloned from *Lb. acidophilus* (*14*). After five cloning attempts, no clones of correct Ter_{slpA} sequence were obtained. However, clones with improved rAFP extracellular expression were picked, and the Ter sequences were analyzed. All clones were mutated at the terminator (**Figure 3A**). Among them, *L. lactis* NZ9000 (pNZDSASm-sacBA2-4) expressed extracellular rAFP at up to 28 mg/L and yielded 14 mg/L·OD_{600}. These *slpA* terminators, although mutated, improved the extracellular rAFP productivity.

Extracellular Expression of rAFP in Various Media. To enhance the productivity of rAFP, a variety of media were tested. For acid-tolerant fermentative bacteria such as *L. lactis*, culturing under pH-controlled conditions lessens acidification, improves energy utilization, and leads to higher biomass production (*29*). In our previous study, pH-controlled and properly glucose-supplemented media (2GM17B) enhanced the production of heterologous protein in *L. lactis* (*12*). The GM17 and 2GM17B media were assayed for rAFP expression. Medium A enhanced heterologous protein production by increasing cell mass in *B. subtilis* (*12*) and was tested in this study as well. For products applicable in human and animal pharmaceuticals, it is important that the product be guaranteed bovine spongiform encephalomyelitis (BSE) agent-free. A plant-based, BSE-free medium for *L. lactis* was previously reported (*30*). In this study, BSE-free FMG (*12*), modified FMB, and FMS were assayed for rAFP expression. Our results show that buffered media 2GM17B, FMB, and medium A increase cell mass (**Figure 4A**); 2GM17B and FMB increase extracellular rAFP production to 3.47- and 2.47-fold higher than GM17 media, respectively (**Figure 4C**). The 2GM17B medium, although buffered, had a final pH that was slightly lower than that of GM17 (**Figure 4B**), probably due to the influence of cell mass (**Figure 4A**); however, highest rAFP productivity was achieved (**Figure 4C**). The safer FMB medium achieved higher cell mass (**Figure 4A**) and higher rAFP productivity (**Figure 4C**) than FMB and FMS, but not buffered after 5 h of culture, the time point at which cell mass started to increase (**Figure 4B**). Medium FMS did not achieve high cell mass or rAFP productivity. Medium A, which achieved a high cell mass, was not a proper medium to produce rAFP. The 2GM17B and the safer FMB media are candidates for the industrial production of rAFP.

Purification, N-Terminal Sequence, and Functional Analysis of rAFPs. The rAFPs produced by *L. lactis* NZ9000 (pNZDSA1-usp45), NZ9000 (pNZDSA1-sacB), NZ9000 (pNZDSA1-ale), NZ9000 (pNZDSA1-slpA), NZ9000 (pNZDSA1-sacBA), and NZ9000 (pNZDSA1-sacBL) at 8 h of culture in 2GM17B were purified by Ni-NTA chromatography (**Figure 5A**). The N-terminal sequences were confirmed as GSDTA for *L. lactis* NZ9000 (pNZDSA1-usp45), NZ9000 (pNZDSA1-sacB), NZ9000 (pNZDSA1-ale), and NZ9000 (pNZDSA1-slpA); AEGSD was confirmed for NZ9000 (pNZDSA1-sacBA); and LEISS was confirmed for NZ9000 (pNZDSA1-sacBL), indicating correct cleavage of signal peptides. An unknown band of correct cleaved LEISS N terminus appeared at about 14 kDa

in purified rAFP from *L. lactis* NZ9000 (pNZDSA1-sacBL) cultured in 2GM17B. The rAFP produced by NZ9000 *L. lactis* (pNZDSA1-sacB) cultured in FMB did not achieve higher expression level of rAFP, and its N-terminal sequences were analyzed as mixtures of GSDTA and SDTAS. How the FMB medium influences the cleavage of signal peptide remains to be determined.

The rAFP function was assessed by several assays. The ability of rAFP to inhibit recrystallization was demonstrated by observing crystal size and shape in a frozen film of solution. The PBS solution containing rAFPs produced smaller ice crystals than the control PBS buffer after recrystallization occurred (**Figure 5B**). The ability of rAFP to bind to ice and cause characteristic changes in morphology was also observed (**Figure 5B**). This results from the specific binding of rAFP to the hexagonal prism faces, inhibiting the deposition of water at the surfaces and forcing ice growth toward the less-preferred *c*-axis of the crystals. The single ice crystal grown in PBS buffer containing rAFP showed the elongated hexagonal crystal form, whereas flat circular disks were observed for PBS buffer (**Figure 5B**). The presence of the six-histidine tag and variable N-terminal peptide did not affect ice recrystallization inhibition by rAFP. The six-histidine tag facilitated purification by one-step chromatography. These functional rAFPs should therefore serve as potent cryoprotective food ingredients.

The highest rAFP productivity among the constructions was *L. lactis* NZ9000 (pNZDSASm-sacBA2-4) culturing under 2GM17B, which achieved 54.5 mg/L extracellular rAFP. The rAFP purified from *L. lactis* NZ9000 (pNZDSASm-sacBA2-4) of 8 h of 2GM17B culture exhibited a single band rAFP with AEGSD at the N terminus, indicating correct signal peptide cleavage as *L. lactis* NZ9000 (pNZDSASm-sacBA); the function of this rAFP was confirmed by the single ice crystal observation and ice recrystallization inhibition (**Figure 5B**). The transformant *L. lactis* NZ9000 (pNZDSASm-sacBA2-4) can be seen as a good candidate for high-level industrial production or rAFP.

In summary, a strong promoter P_{SlpA1} was chosen and fused with more efficient SP_{sacB} and AE prosequence to increase the extracellular expression of rAFP in *L. lactis*. Terminator $Ter_{SlpA2-4}$ and use of 2GM17B media provided further enhancements. A modified BSE-free FMB medium is a safer production tool for rAFP. The expressed rAFPs were functional and potentially applicable for cryogenic preservation. This study provided not only tools for high-level extracellular expression but also a safer medium for heterologous protein production in food-grade *L. lactis*.

Supporting Information Available: Flow sheet of the DNA constructions. This material is available free of charge via the Internet at http://pubs.acs.org.

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