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Optimization of the pilot-scale production of an ice-binding protein by fed-batch culture of *Pichia pastoris*

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Abstract Ice-binding proteins (IBPs) can bind to the ice crystal and inhibit its growth. Because this property of IBPs can increase the freeze–thaw survival of cells, IBPs have attracted the attention from industries for their potential use in biotechnological applications. However, their use was largely hampered by the lack of the large-scale recombinant production system. In this study, the codon-optimized IBP from *Leucosporidium* sp. (LeIBP) was constructed and subjected to high-level expression in methylotrophic *Pichia pastoris* system. In a laboratory-scale fermentation (7 L), the optimal induction temperature and pH were determined to be 25 °C and 6.0, respectively. Further, employing glycerol fed-batch phase prior to methanol induction phase enhanced the production of recombinant LelBP (rLeIBP) by ~100 mg/l. The total amount of secreted proteins at these conditions (25 °C, pH6.0, and glycerol fed-batch phase) was ~443 mg/l, 60 % of which was rLeIBP, yielding ~272 mg/l. In the pilot-scale fermentation (700 L) under the same conditions, the yield of rLeIBP was 300 mg/l. To our best knowledge, this result reports the highest production yield of the recombinant IBP. More importantly, the rLeIBP secreted into culture media was stable and active for 6 days of fermentation. The thermal hysteresis (TH) activity of rLeIBP was about 0.42 °C, which is almost the same to those reported previously. The availability of large quantities of rLeIBP may accelerate further application studies.

Keywords Ice-binding protein · Fed-batch culture · LeIBP · *Leucosporidium* sp. · *Pichia pastoris*

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Introduction

Ice-binding proteins (IBPs) can interact directly with ice. This interaction can give rise to two properties of IBPs. One is thermal hysteresis (TH), the difference between the melting and freezing points. TH occurs when IBPs bind to the ice crystal and inhibition of its growth. The other is ice recrystallization inhibition (RI). Ice recrystallization is the process of forming larger grains of ice on the expense of smaller ones. Since this phenomenon can cause damage of cell membranes, RI property can increase the freeze–thaw survival of cells (Knight et al. 1984; Knight and Duman 1986; Raymond and Fritsen 2001; Raymond and Knight 2003). These properties seem to be favorable for cold-adaptation strategies of many polar organisms (D'Amico et al. 2006; Davies et al. 2002; Graether and Sykes 2004). To date, IBPs have been isolated and characterized from a wide variety of psychrophilic organisms, including fish (Antson et al. 2001; Chen and Jia 1999; Kwan et al. 2005; Liu et al. 2007; Nishimiya et al. 2008; Patel and Graether 2010; Siemer and McDermott 2008), plants (Atici and Nalbantoglu 2003; Middleton et al. 2009), bacteria (Garnham et al. 2008; Kawahara 2002; Muryoi et al. 2004), fungi (Lee et al. 2010; Xiao et al. 2010), and insects (Doucet et

al. 2000; Leinala et al. 2002a, b; Mok et al. 2010). IBPs include any protein that binds to ice such as antifreeze proteins (AFPs), ice recrystallization inhibition proteins, and ice nucleation proteins and so on.

The intriguing properties of IBPs have attracted attention from academia and industries because these proteins have broader potential applications, including cryopreservation, food preservation, transgenic technology, and cryosurgery (Barrett 2001; Ben 2001; Bouvet and Ben 2003; Davies et al. 1989; Fuller 2004; Harding et al. 2003; Hew et al. 1992; Wohrmann 1996). However, production of IBPs from their natural sources, for example, from fish, is not only hard to achieve due to the limited sources but also cost ineffective. Heterologous protein expression offers one of the best ways to produce large quantities of IBPs. To date, many IBPs have been recombinantly expressed in a variety of expression systems (Table 1). However, as listed in Table 1, none of recombinant expression systems seemed to work effectively for the cost-effective production of IBPs. The production yield varied from 16 to 132 mg/l, which is by far insufficient to scale up the system for large-scale production. The main problem in this failure was due to the low expression level and folding issues of IBPs (see references in Table 1). Hence, establishing a high-yield expression system of recombinant IBP may provide a key to the development of its applications.

Very recently, we have isolated an ~25 kDa extracellular IBP (LeIBP) from the Arctic yeast *Leucosporidium* sp. AY30 (Lee et al. 2010). The amino acid sequence alignment showed that this IBP does not share significant similarity in primary structure to other known AFPs, but it has similar three-dimensional structures to hyperactive AFPs. Despite of the structural resemblance to hyperactive AFPs, LeIBP is moderately active. Interestingly, native LeIBP formed a homodimer via the C-terminal swapping (Lee et al. 2012a; Park et al. 2012). However, the C-terminal deleted monomeric LeIBP was also functional, meaning that the active ice-binding site was not formed by dimerization. The native and recombinant LeIBPs have the TH activities of 0.87 °C at 15 mg/ml of native LeIBP and 0.42–0.43 °C 10.8 mg/ml of recombinant LeIBP (rLeIBP). The TH difference of native and recombinant LeIBPs was, as described elsewhere, probably due to the unknown TH enhancer in the native LeIBP solution, not due to the structural instability or aggregation of rLeIBP. They also had RI activity down to 0.001 mg/ml (Park et al. 2012). Like other IBPs, the depression of the freezing point of water by LeIBP, or TH activity is not great enough to prevent freezing of cells, leading to cold damage to cells living in subfreezing environments, such as the Antarctic and the Arctic. Therefore, the RI activity seems to play a more important role in protecting the cell membranes from damage from extracellular freezing by controlling the growth of larger grains of ice (Raymond et al. 2007, 2008, 2009). The RI phenomenon can be suited for the application to the cryopreservation of cells. We examined the feasibility of improving the cryopreservation efficiency of human red blood cells by taking advantage of the RI property of the LeIBP (Lee et al. 2012b). Human red blood cells cryopreserved in the presence of LeIBP showed reduced post-thaw hemolysis and preserved similar cell size distribution to their nonfrozen counterparts. These data illustrate that RI activity of LeIBP can be utilized for cryopreservation application. For the further practical studies of cryopreservation and biotechnological applications, we need large quantities of LeIBP. Instead of improving production of other AFP system, we decided to pursue the mass production of rLeIBP because it has both TH and RI activities, because compared to other moderately active and hyperactive AFPs, it does not have cysteine residues, which sometimes cause protein misfolding and aggregation, and, more importantly, because it was proved to reduce the damage of red blood cells and other mammalian cells caused by freeze–thawing (Lee et al. 2012b; unpublished data). Hence, the aim of this study is to achieve the high production level of the recombinant LeIBP using *Pichia* system by optimizing the codon usage, the methanol induction temperature, pH, and feeding strategy. Here, we describe a simple fermentation strategy for the large-scale production of rLeIBP using a yeast expression system.

Materials and methods

Microorganism, plasmids, media, and materials

The methylotrophic *Pichia pastoris* X33 used for the expression of rLeIBP was purchased from Invitrogen (Carlsburg, CA, USA). The pPICZαA vector was used for the expression in *P. pastoris* system. *Escherichia coli* DH5α used for molecular biological work was obtained from Enzynomics (Daejon, Korea). Yeast extract–peptone–dextrose medium (YPD) contained 10 g glucose, 10 g yeast extract, and 20 g peptone per liter. Buffered minimal glycerol complex medium (BMGY) contained (per liter) 10 g yeast extract, 20 g meat peptone, 100 mM potassium phosphate buffer pH6.0, 13.4 g yeast nitrogen base without amino acids, 400 μg biotin, and 10 ml glycerol. Buffered minimal methanol complex medium (BMMY) had the same components as BMGY except for using 5 ml methanol substituted for 10 ml glycerol. Fermentation basal salts (FBS) medium consisted of 40 g glycerol, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, 26.7 ml H_3PO_4 (85 %) per liter. Trace metal solution contained 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $NaMo_2{\cdot}H_2O$, 0.02 g H_3BO_4, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin, and 5.0 ml H_2SO_4 per liter

Table 1 Comparison of recombinant AFPs

AFP	Origin	Expression host	Production condition[a]	Production level (mg/l)	Purification method	References
Type I	Winter flounder	*E. coli*	200 ml, M9, 30 °C	16	RP-HPLC	(Tong et al. 2000)
	Winter flounder	*E. coli*	1 l, LB, 37 °C	100	Ethanolic extraction and AS[c]	(Solomon and Appels 1999)
	Shorthorn sculpin	*E. coli*	1 l, LB, 37 °C	NS [b]	Ni-affinity and RP-HPLC	(Low et al. 1998)
Type II	Sea raven	*P. pastoris*	6 l, BMMY, 30 °C	30	Ni-affinity and anion exchange-FPLC	(Loewen et al. 1997)
		P. pastoris	5 l, FM, 30 °C	175	NS[b]	(d'Anjou and Daugulis 2001)
Type III	Ocean pout	*E. coli*	3 l, 37 °C	13	Size-exclusion, cation exchange-FPLC, and RP-HPLC	(Chao et al. 1993)
Type IV	Shorthorn sculpin	*E. coli*	MM, 37 °C	NS [b]	Ni-affinity and RP-HPLC	(Gauthier et al. 2008)
	Antarctic silverfish	*E. coli*	1 l, LB, 37 °C	NS [b]	Ni-affinity	(Lee et al. 2011)
	Black rockcod	*E. coli*	1 l, LB, 37 °C	NS [b]	Ni-affinity	
Plant AFP	Carrot	*E. coli*	1 l, LB, 37 °C	122	Refolding and RP-HPLC	(Zhang et al. 2004)
Insect AFP	Spruce budworm	*E. coli*	1 l, LB or MD, 37 °C	NS [b]	AS[c] and Refolding	(Tyshenko et al. 2006)
		P. pastoris	5 l, bioreactor, 30 °C	132	NS[b]	
Yeast IBP	*Leucosporidium* sp. AY30	*E. coli*	1 l, LB, 15 °C	24.5	Ni-affinity or ice affinity	(Park et al. 2012)
		E. coli	1 l, LB, 15 °C	19.3	Ice affinity	
		P. pastoris	2 l, BMMY, 28 °C	61.2	Ni-affinity and ice affinity	
		P. pastoris	500 l, FBS, bioreactor, 25 °C	300	Ion exchange and size-exclusion	This study

[a] Working volume, culture medium and/or culture type, and temperature are described

NB nutrient broth, *MM* minimal medium, *MD* minimal dextrose, *LB* Luria–Bertani, *FM* fermentation medium, *BMMY* buffered methanol complex medium, *FBS* fermentation basal salts medium)

[b] Not specified

[c] Ammonium sulfate precipitation

(Lim et al. 2003). Restriction enzymes were from Bioneer (Daejon, Korea). All other chemicals were from Sigma (St. Louis, MO, USA), BD, and Merck (USA).

Codon optimization and construction of an expression vector

The construction of LeIBP-expressing vector was briefly described elsewhere (Park et al. 2012). In detail, to generate mature protein, 20 amino acids of putative signal peptide (MSLLSIITIGLAGLGGLVNG) were eliminated, and the coding region of the mature LeIBP gene was optimized according to the codon usage of *Pichia* using proprietary algorithms that replace rare codons, problematic messenger RNA structure, and various cis-elements in transcription and translation (Genotech Co., Korea). The restriction sites, *Xho*I and *Not*I, were introduced to the 5′ and 3′ ends of the optimized sequence, respectively, to facilitate subcloning into the yeast expression vector. The construction of LeIBP expression vector, transformation, selection of transformants were performed using the EasySelect Pichia Expression Kit (Invitrogen, Carlsburg, CA, USA) according to manufacturer's manual. The synthetic codon-optimized LeIBP gene was excised by digestion with *Xho*I and *Not*I and ligated into a yeast pPICZαA expression vector. The construct was designed to secrete the 242 aa mature rLeIBP into the culture media. The calculated molecular weight of the protein was 25.044 kDa.

Electroporation and selection of the transformants expressing rLeIBP

Approximately 2 μg of recombinant plasmid was linearized with *Sac*I restriction enzyme and used to transform the X33 strain of *P. pastoris* by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA). Transformants of *P. pastoris* were selected on YPD medium containing zeocin (100 μg/ml). To select transformants with the gene of interest integrated correctly into the genome, the genomic DNA of transformants was used as a PCR template with the pPICZαA expression vector-specific 5′ and 3′ AOX1 primers according to the manufacturer's instructions. Selected transformants grown on YPD plate containing zeocin were inoculated into BMGY medium and were cultured until OD_{600}=2. The cells were then transferred in BMMY medium and induced with 0.5 % methanol every 24 h. Small-scale cultures (5 ml) were centrifuged (4,500 rpm, 5 min); supernatant and cell fractions were stored at 4 °C until analysis for the presence of expressed rLeIBP protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and thermal hysteresis (TH) assay.

Laboratory-scale production of rLeIBP in *P. pastoris*

The laboratory-scale production of rLeIBP was performed to optimize the culture conditions such as pH and temperature using a 7-l Jar bottom magnet drive fermenter prior to pilot-scale production (KF-7L model, Kobiotech, Korea). Temperature was monitored by means of a thermocouple at the vessel base, and pH was controlled by the addition of acid or base by a peristaltic pump and monitored using an InPro® 3030 pH sensor (Mettler-Toledo GmbH, Switzerland). Dissolved oxygen was monitored by a sterilizable, polarographic dissolved oxygen electrode InPro® 6800 Series O2 Sensors (Mettler-Toledo GmbH, Switzerland). Fermentation was carried out with aeration at 15 l/min, mixing at 600 rpm, and dissolved oxygen levels maintained at more than 20–30 % of saturation. Antifoam was added to the fermentation media prior to inoculation and periodically. The effect of four culture temperatures (15, 20, 25, and 30 °C at pH5) on the recombinant protein production was first examined and then that of three pH values (pH5, 6, and 7) was investigated at the optimal induction temperature, in this case, 25 °C. Each pH value was maintained by the automatic addition of ammonium hydroxide solution.

The seed culture for fermentation was grown in a 1-l flask containing 300 ml of YPD medium with shaking at 250 rpm and 30 °C for 48 h. The seed culture was transferred into a 7-l fermenter containing 2.2 l of FBS medium supplemented with 4.4 ml of filter-sterilized trace metal solution per liter. The batch phase was carried out at the various temperatures (pH5) or pH values (25 °C) in a continuous steady-state mode until the initial glycerol was completely consumed as indicated by a sudden increase in the dissolved oxygen (usually for 24 h). Then, a DO-stat methanol fed-batch was started by adding 100 % methanol feed containing 12 ml/l of trace metal solution. The recombinant protein was concurrently induced in this phase. The methanol feed rate was set to 3.6 $ml h^{-1} l^{-1}$ of initial fermentation volume. Methanol feed was controlled as follows: Feeding was paused when the DO value dropped below 20 % while continued when the DO recovered to above the set point.

At the optimal temperature and pH (25 °C, pH6) for the production of rLeIBP determined in the above experiments, glycerol fed-batch phase was introduced after the glycerol batch phase to see the effect on the recombinant protein production. The DO-stat glycerol-methanol fed batch was initiated by supplying the glycerol feed medium [50 % glycerol (*w*/*v*) and 12 ml of trace metal solution per liter] at the feed rate of 18.18 $ml h^{-1} l^{-1}$ of initial fermentation volume until two to three times increase in cell density was achieved, which usually took about 9 h or longer. The methanol feed was followed 0.5 h after the glycerol feeding was done during which cells were allowed to consume added glycerol. The methanol was fed as described above to induce the recombinant protein production.

Pilot-scale fermentation of rLeIBP

The pilot-scale fermentation was carried out using fermenters (Kobiotech, Korea) at Chuncheon bioindustry foundation (Chuncheon, Korea) using the optimized culture conditions screened from the laboratory-scale production. The optimized conditions of temperature, pH, and feeding strategy were 30 °C, pH 6, glycerol fed-batch, respectively. The seed culture was grown in a flask containing 2 l of YPD at 250 rpm and 30 °C for 48 h and was inoculated to a 70-l fermenter containing 23 l of YPD medium at 30 °C and further grown for 12 h. The culture broth from the 70-l fermenter was transferred to a 700-l fermenter containing 230 l of growth medium. The fermentation operation was divided into two phases: (1) a growth phase in which glycerol was used as the carbon source and (2) the production phase at which DO-stat glycerol fed-batch and methanol induction phases were started as described above. The culture temperature was maintained at 30 °C during the growth phase and was reduced to 25 °C during the production phase. The agitation speed varied between 100 and 230 rpm, and the airflow rate was maintained between 200 and 700 l/min. The culture pH (pH6) was maintained by the addition of 28 % (*w*/*v*) ammonium hydroxide solution.

Separation and purification of rLeIBP

The fermentation broth was harvested by centrifugation at 8,000×*g* for 10 min and subsequently passed through 0.2-μm filter (Nalge Nunc, Rochester, NY, USA). The supernatant was then buffer-exchanged with 50 mM Tris–HCl (pH8) and concentrated on an Amicon Ultra Centrifugal filter device 10 kDa MWCO (Millipore, Bedford, MA, USA). The concentrated supernatant was applied to an ion exchange chromatography (QFF) column and then eluted with 50 mM Tris–HCl buffer (pH 8.0) containing salt gradient from 0 to 1 M NaCl. The elution fractions were analyzed by SDS-PAGE and were further separated on a Superdex 200 size-exclusion column (Amersham Pharmacia). The fractions were equilibrated with 50 mM Tris–HCl (pH8.0) buffer containing 150 mM NaCl and run at a flow rate of 1 ml/min.

Analytical methods

During all fermentation processes, 15 ml of culture medium was taken out periodically to measure the optical density, dry cell weight (DCW), protein concentration, and the TH

activity. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm with a UV/VIS spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences). The cell density was also determined by measuring the DCW. Cell density was expressed either/both the optical density or/and DCW. The ratio of DCW (g/l) to optical density was 0.264. The concentration of total secreted protein was determined according to the Bradford method using bovine serum albumin as a standard protein. The expression of rLeIBP was confirmed by SDS-PAGE, TH activity assay, and Western blot. The concentration of rLeIBP (amount of protein per medium volume) was calculated by multiplying the total secreted protein concentration by the percentage of rLeIBP, which was estimated by SDS-PAGE analysis or densitometry scanning.

Thermal hysteresis activity assay

TH activity was measured using a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand) as described previously (Lee et al. 2010). TH of the samples of crude culture media and the purified rLeIBP was measured directly or by diluting serially in distilled water. The concentrations of the rLeIBP used in this assay ranged from 0.0 to 17.75 mg/ml. The ice crystal morphology was recorded with a Canon Digital Camera connected to a light microscope mounted with a cold stage.

Results

Codon optimization, expression vector construction, and rLeIBP expression

To achieve a high-level expression of LeIBP in *P. pastoris*, the mature LeIBP sequence (Swiss-Prot/TrEMBL accession number C7F6X3) was optimized as shown in Fig. 1. A total of 114 codons were optimized based on the codon bias of *P. pastoris*, in which 197 nucleotides in the sequence were replaced. As a result, codon optimization significantly reduced the overall GC content of the gene from 62.6 to 47.6 %. The optimized sequence shared 73 % identity to the wild type. The codon-optimized mature LeIBP gene was subcloned into the pPICZαA vector, which contains an α mating factor signal sequence for secretory expression (Fig. 1b). Chromosomal integration of the LeIBP gene in *P. pastoris* X33 cells was confirmed. The expression of LeIBP, which was under the control of the AOX1 promoter, was induced with 100 % methanol once Zeocin-resistant cultures reached OD_{600}=4.0. Expression was analyzed by SDS-PAGE at 48 h after induction, and the clone showing the highest rLeIBP expression was used for further bioreactor studies.

Effects of temperature, pH, and glycerol-fed batch phase on the rLeIBP production

The initial characterization of the LeIBP, native or recombinant, showed that it was stable and active at wide range of pH and temperature. In particular, the activity of LeIBP remained 70 % after incubation for a week even at 30 °C (unpublished data). Hence, the general culture conditions of *P. pastoris* are not likely to affect the stability and activity of rLeIBP. Protein induction temperature and pH were investigated to obtain high cell densities as well as large quantities of target recombinant protein per fermentation volume, without the loss of its activity into consideration. The optimization experiment was carried out in a 7-l jar fermenter. To determine the optimal temperature during the methanol induction phase, the rLeIBP expression level was compared at various temperatures (15, 20, 25, and 30 °C at pH5). Recombinant protein expression was induced with methanol for a total of 144 h. As shown in Fig. 2a, cell density increased in the order of 15, 30, 25, and 20 °C, but the rLeIBP was maximally produced at 25 °C, reaching about 60 % of total secreted proteins in culture media. Typically, the production of rLeIBP was detected on SDS-PAGE 12 h postinduction, continuously increased and reached maximum expression level at 144 h (data not shown) at all induction temperatures. Based on cell density and rLeIBP production, 25 °C was the optimal temperature during the methanol-induction phase.

To find out the optimal pH for the production of rLeIBP, we performed fermentation at pH values of 5, 6, and 7. The growth temperature was kept at 30 °C during batch phase but lowered and maintained to 25 °C during induction phase. While at all three pH values, cells grew normally, and cell densities were not discernable between cultures; however, the expression level of rLeIBP was highest at pH 6 (Fig. 2b), which was identified as the optimal pH for rLeIBP production.

To further increase the cell densities and concomitantly the production of rLeIBP, we employed glycerol fed-batch phase between the batch and methanol-induction phases. For the initial cell growth in the batch phase, the temperature was set to 30 °C (pH6). Cell density reached ~25 g/l DCW at the end of the batch phase. During the glycerol fed-batch phase, the DCW reached ~55 g/l. After the fed-batch phase, the temperature of the fermentation vessel shifted to 25 °C. During methanol-induction phase, the cell density increased continuously and reached the highest DCW of 117 g/l after 60 h of induction and remained relatively constant until the termination of fermentation. However, the protein production was delayed about 24 h. The total secreted protein level increased to ~443 mg/l after 84 h of induction and remained constant, but the relative amount of rLeIBP increased continuously (Suppl. Fig. S1A). The maximal rLeIBP level was

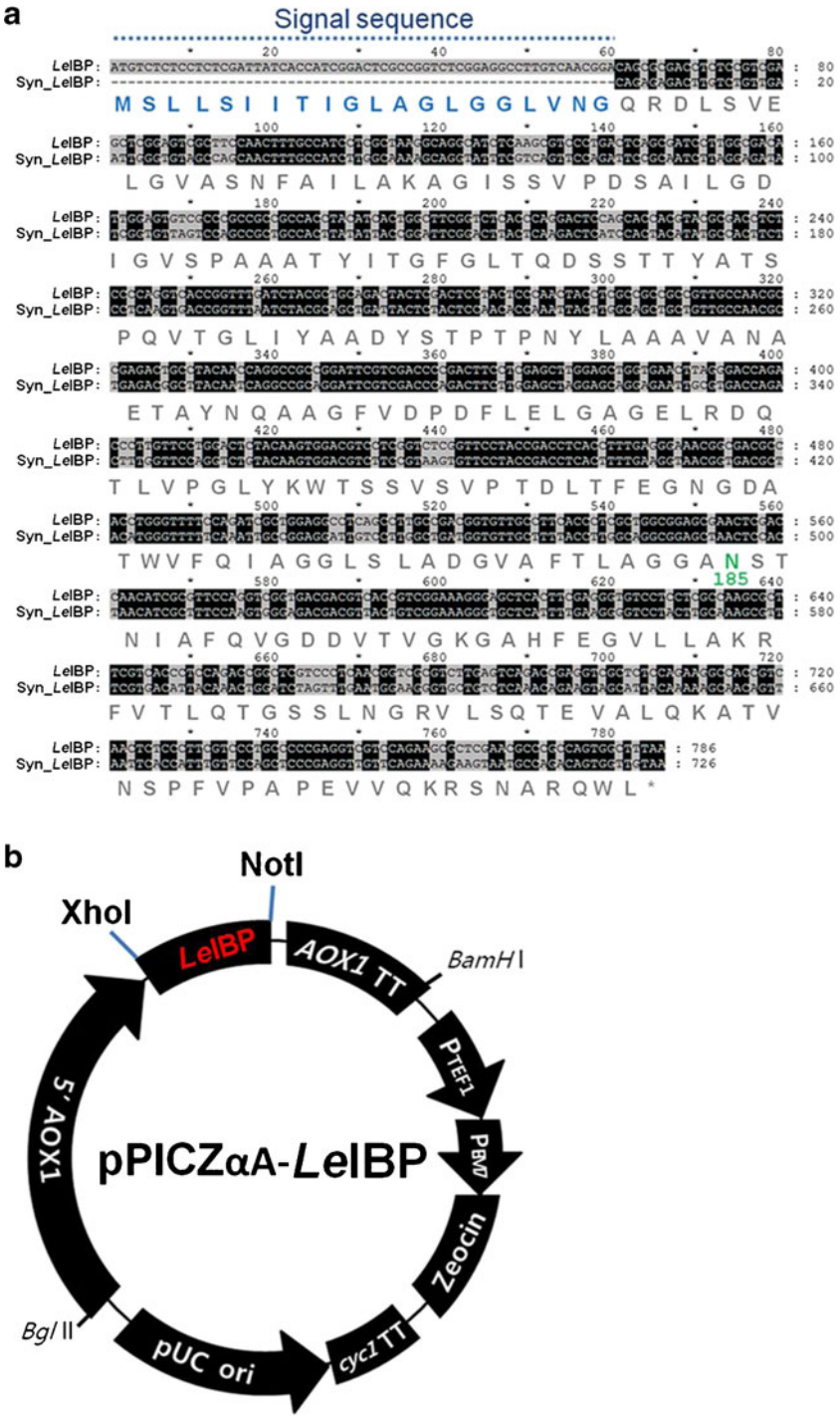

Fig. 1 Codon optimization of LeIBP for expression in *Pichia*. **a** Nucleotide sequence alignment of wild-type and codon-optimized LeIBPs. The altered nucleotides are marked with a *gray background*. The deduced amino acid sequence is given below the DNA sequence. The Asn 185, which can undergo N-linked glycosylation, is colored in *green*. **b** Plasmid map of the pPICZαA-LeIBP expression construct in *Pichia pastoris*

61 % of the total secreted protein (Suppl. Fig. S1B). Under these conditions, the yield of expressed rLeIBP based on SDS-PAGE and scanning densitometer was ~272 mg/l. The introduction of glycerol fed-batch further increased not only cell densities but also recombinant protein production compared to the only methanol induction mode. In our laboratory-scale experiments, the optimal conditions for the production of rLeIBP were induction temperature of 25 °C, pH6, and glycerol fed-batch mode.

Pilot production of rLeIBP

Scale-up production of rLeIBP by *P. pastoris* X33 was examined in a 700-l fermenter containing an initial working volume of 250 l using the optimized culture conditions obtained from 7-l laboratory scale fermentation. Glycerol and methanol were the carbon sources. Figure 3a shows the time profiles of fed-batch *P. pastoris* X33 cultures using 20 kg of glycerol and 180 l of methanol. The cell density reached ~107 g/l; the total

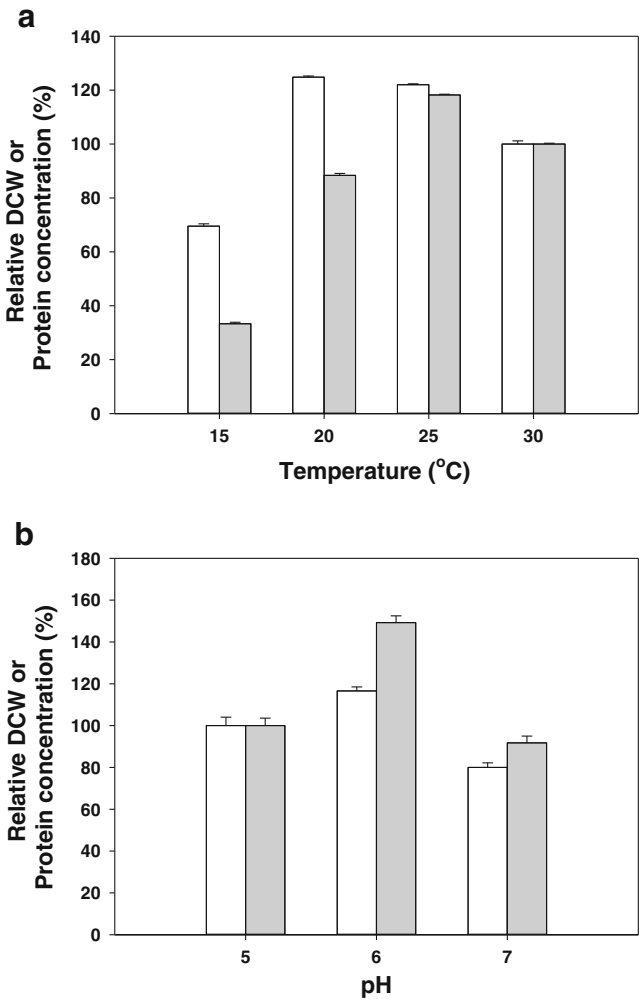

Fig. 2 Optimization of the rLeIBP expression condition in *P. pastoris* during methanol induction. Cells were grown in a 7-l jar fermenter with batch-type fermentation at 30 °C, after which various culture temperatures (**a**) and pH values (**b**) were applied during a 144-h methanol induction. At the end of culture growth, the dry cell weight (*white bar*) and total secreted protein (*gray bar*) were measured

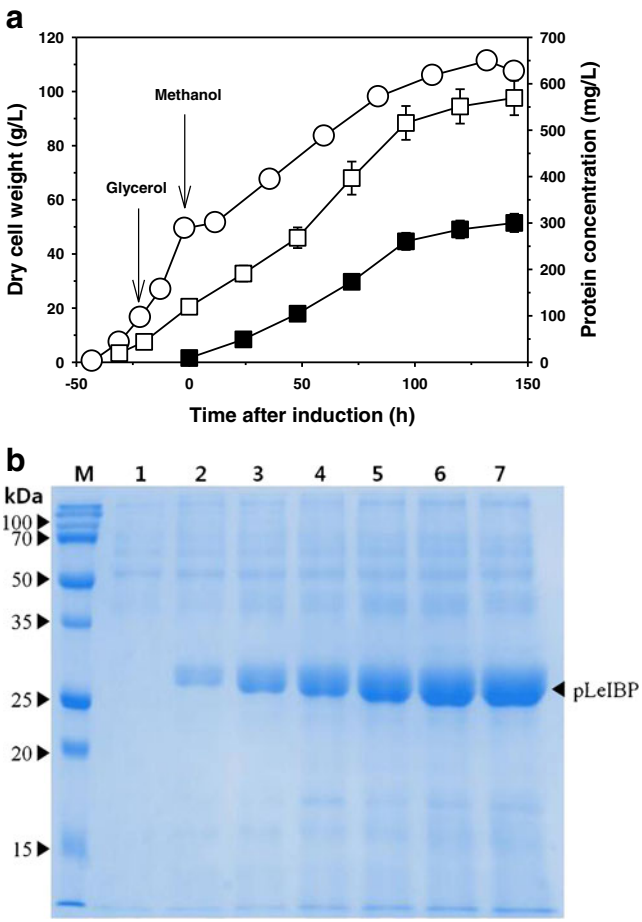

Fig. 3 Pilot-scale production of rLeIBP in the 700-l fermenter. **a** Time profile of cell growth by dry cell weight (*open circle*), production of total secreted protein (*open square*), and production of rLeIBP (*solid square*). **b** rLeIBP expression was visualized using 12 % SDS-PAGE by running the supernatants obtained from the 700-l fermenter. *Lane M* size marker, *lane 1* preinduction, *lanes 2–7* after 24, 48, 72, 96, 120, and 144 h postinduction, respectively

secreted protein level was 560 mg/l. During the glycerol-feeding phase, the cell density showed more than 3-fold increase from 16 to 52 g/l, whereas the total secreted protein concentration increased by only 50 %, that is, from 80 to 120 mg/l. During the induction period, the total protein concentration increased 4.6-fold, while the cell density increased only by 2-fold. The expression level of rLeIBP was ~52 % of the total secreted protein at the end of the fermentation (Fig. 3b). The expressed rLeIBP concentration in the 700-l fermenter was about 300 mg/l, which was slightly higher than that observed in the 7-l fermenter.

Purification and TH activity of rLeIBP

The supernatant of culture medium containing the secreted rLeIBP from the 700-l fermenter was collected, concentrated, and subjected to the chromatography. The soluble rLeIBP was purified by two-step purification process involving anion exchange chromatography and gel filtration (Table 2). The procedure yielded 78.8 mg of rLeIBP that was approximately 95 % pure based on SDS-PAGE and densitometry scanning. The purified rLeIBP was further concentrated with a Centriprep filter device (10-kDa cutoff) and stored at −20 °C prior to use. The recovery and purity of LeIBP at the different purification steps are summarized in Table 1. N-terminal sequencing of the rLeIBP identified the first five amino acids as Gln–Arg–Asp–Leu–Ser, which matches mature LeIBP amino acid sequences.

The TH activity of rLeIBP was measured periodically during all the fermentation experiments. The TH value of rLeIBP in this study was 0.4 °C or so, which is very close to those previously obtained from other recombinant LeIBPs (Park et al. 2012). In our typical fermentation experiments the amount of expressed rLeIBP in the culture media

Table 2 Purification steps of rLeIBP

Purification steps	Total volume (ml)	Total protein (mg)	Recovery (%)	Purity (%)
Concentrated supernatant	300	1360	–	55
Anion exchange chromatography (Q-Sepharose FF)	225	110	8.1	85
Size exclusion chromatography (Superdex 200 HR)	150	78.8	5.8	95

increased in proportion to the induction time period, but the TH activity of the culture media reached a plateau of 0.4 °C quickly (Fig. 4a). This is not because rLeIBP was aggregated, precipitated, or misfolded but because the TH activity results from the noncolligative property of IBPs. In the optimized 7-l fermentation the culture media showed about 70 % of maximal TH activity 24 h after induction and leveled out 48 h after methanol induction. The TH value of 700-l fermentation showed almost the same pattern as that of the laboratory scale (Fig. 4a). We also recorded how rLeIBP affect the shape of ice crystals. The development of ice crystal facet was affected by the presence of LeIBP as described elsewhere (Lee et al. 2010). As shown in Fig. 4b, the culture media containing low amount of rLeIBP (24 h postinduction) from both 7- and 700-l fermentations produced a distinct hexagonal shape of ice crystal, while large amount of rLeIBP-containing culture media (lower panel of Fig. 4b) created bipyramidal shape of ice crystal with limited growth on the basal plane. These data strongly support that like native LeIBP, the rLeIBP was stable and active during long period of fermentation.

Discussion

The present study reports, for the first time to our best knowledge, the highest expression level and large-scale production of recombinant IBP in *P. pastoris* system.

In our previous report, we showed that even the native LeIBP sequence was not problematic for the heterologous expression in *E. coli* using pCold system. However, this does not guarantee its successful expression in *P. pastoris*. Hence, the codon-optimized mature LeIBP gene was designed, synthesized, and subcloned into the pPICZαA vector for the expression in *P. pastoris*. (Lee et al. 2012b; Park et al. 2012). The flask-scale experiment showed that the codon optimization translated into successful expression of rLeIBP and produced 61.2 mg from 1 l BMMY culture media.

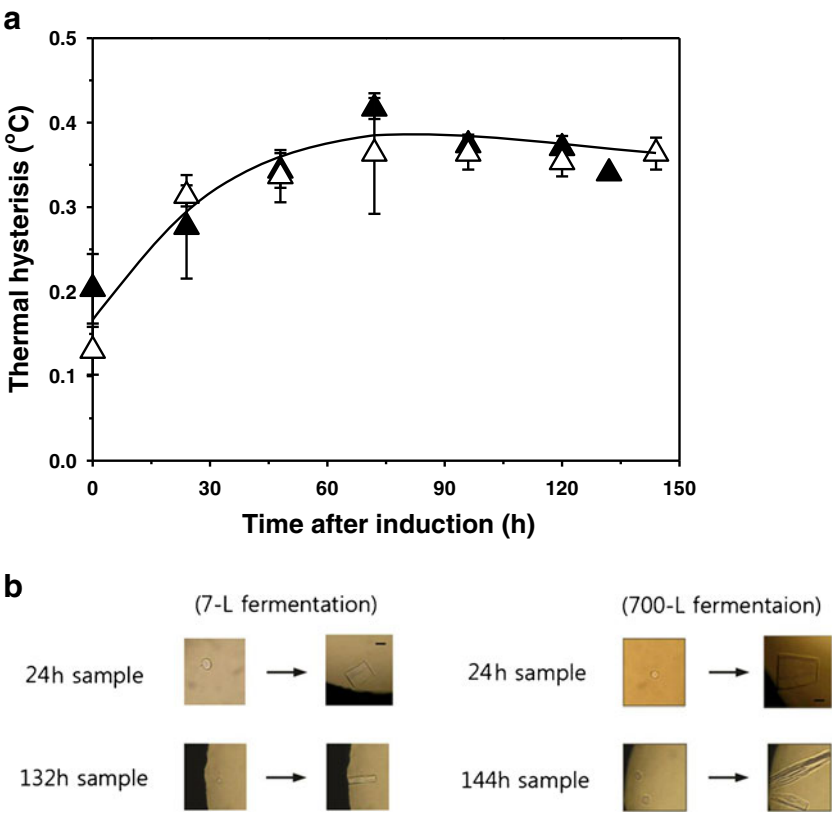

Fig. 4 Demonstration of rLeIBP antifreeze activity. **a** Thermal hysteresis activity (*solid triangle*, 7-l fermenter; *open triangle*, 700-l fermenter). **b** Ice morphology modification at different culture condition

The optimized culture conditions were investigated in laboratory-scale fermentation. Since the typical growth temperature, duration of fermentation, and pH of *P. pastoris* barely affected the activity of rLeIBP (data not shown), we just focused on increasing the production of the recombinant protein by adjusting induction temperature, pH, and feeding strategy. The methylotrophic *P. pastoris* grows optimally at 30 °C, but the optimal growth temperature does not ensure the maximal production of target recombinant protein. A number of reports demonstrated that lowering the growth temperature could increase the recombinant product yield tens to hundreds fold, by increasing cell viability and at the same time reducing proteolytic degradation (Hong et al. 2002; Li et al. 2001; Wang et al. 2009). In particular, the yield of herring type II AFP was improved by lowering the growth temperature (Li et al. 2001). In our case, lowering the induction temperature down to 20 °C increased cell densities moderately, but production of the recombinant protein was slightly better at 25 °C. The lower temperature was likely to increase cell viability but may slow down the protein synthesis in the rLeIBP case. Another advantage to lowering the temperature is to reduce proteolytic activities. However, we could not detect any severe proteolytic degradation of the rLeIBP at all temperatures. Overall, the induction temperature did not affect the production of rLeIBP dramatically with 25 °C being marginally optimal. The production of recombinant protein is also known to be affected by varying pHs (Clare et al. 1991; Zhu et al. 1995). The values range from pH3 for the production of insulin-like growth factor I and cytokine growth blocking peptide (50 mg/l) (Koganesawa et al. 2002) to pH6 and 7 for the production of recombinant mouse epidermal growth factor (Clare et al. 1991) and anticoagulant peptide (rAcAP-5) (Inan et al. 1999), respectively. In our case, the cell growth and the concomitant rLeIBP production were also affected by pH. The order of high cell density was of pH6, 5, and 7. The rLeIBP production was significantly higher at pH6 than at pH 5 and 7 (Fig. 2b). This is probably due to the reduced proteolytic activity at pH6 (Sinha et al. 2005)

Because the amount of recombinant protein secreted into the medium increases often proportionately with cell density, a high cell density during fermentation is generally desirable (Pal et al. 2006; Romanos et al. 1992). We included glycerol fed-batch phase before methanol induction under optimized culture conditions of 25 °C, pH6. This step gave 10 % increase in cell density and ~100 mg/l increase in total protein production. These optimized conditions were applied to the scale-up fermentation. The amounts of rLeIBP in the 7- and 700-l fermenters were 272 and 300 mg/l, respectively. These are the highest levels of IBP production reported so far, and exceed the levels of 30–175 mg/l sea raven type II AFP (Loewen et al. 1997; d'Anjou and Daugulis 2001) and 132 mg/l *Choristoneura fumiferana* AFP (Tyshenko et al. 2006). This study demonstrates that large-scale production of rLeIBP using the yeast expression system led to 37-fold improvement in LeIBP production compared with the native protein production from *Leucosporidium* sp. (Park et al. 2012).

The TH activity of rLeIBP in this study was almost the same to those reported elsewhere (Park et al. 2012), meaning that the correctly folded active protein was produced. This is further confirmed by matrix-assisted laser desorption/ionization–time of flight (Suppl. Fig. S2), circular dichroism (Suppl. Fig. S3), and PNGase F treatment (Suppl. Fig. S4). The PNGase F cleavage showed that the rLeIBP was N-glycosylated like native LeIBP, but had a little bit different pattern as shown Suppl. Fig. S2. However, glycosylation itself is not critical for the function (Park et al. 2012). The rLeIBP remained active throughout the fermentation duration (6 days), which could be advantageous in obtaining large quantities of the recombinant protein. Because the final yield of rLeIBP was low, further optimization of large-scale purification scheme for the rLeIBP will be considered.

In summary, we have successfully demonstrated that optimization of codon usage, and culture conditions can improve the production of recombinant LeIBP. The simple fermentation strategy outlined here may provide a practical basis for the large-scale production of other IBPs. Now that supplying large quantities of IBP has been available, it may pave the way for biotechnological application using IBPs.

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