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Sung-Hwan Woo · See-Hyoung Park
Hyung-Kwon Lim · Kyung-Hwan Jung

Extended operation of a pressurized 75-L bioreactor for shLkn-1 production by *Pichia pastoris* using dissolved oxygen profile control

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Abstract In this study, a dissolved oxygen (DO)-stat fed-batch process was conducted in a pressurized 75-L bioreactor, resulting in the production of the short version of human leukotactin-1 (shLkn-1) using *Pichia pastoris* as the host, with control of the DO-stat profile and an extension of the recombinant shLkn-1 production phase. By regulation of the exhaust-gas valve, we were able to maintain the vessel pressure at up to 120 kPa, in order to overcome DO limitations associated with the use of the DO-stat. The lowest DO value was adjusted by varying the feed pump speed, allowing us to control the DO-stat profile. This principle was successfully applied to both glycerol feeding during the growth phase and methanol feeding for the induction of shLkn-1. The extension of the methanol induction phase to a total of 192 h of culture time resulted in a shLkn-1 concentration of 2.5 g/L, and a total of 102 g of cumulative production. During this extended induction period, the C-terminal residue of shLkn-1 was truncated and this was confirmed by both reverse-phase HPLC and mass spectrometry.

Keywords shLkn-1 · *Pichia pastoris* · Dissolved oxygen · DO-stat profile control

S.-H. Woo · S.-H. Park · H.-K. Lim
Mogam Biotechnology Institute,
341 Pojung-Ri, Koosung-Eup,
Yougin, Kyonggi-Do, 449-913, Korea
E-mail: lhk@mogam.re.kr

K.-H. Jung (✉)
Department of Food and Biotechnology,
Chungju National University, 123 Geomdan-Ri,
Iryu-Myeon, Chungju, Chungbuk, 380-702, Korea
E-mail: khjung@chungju.ac.kr
Tel.: +82-43-8415246
Fax: +82-43-8415240

Introduction

Chemokines exhibit a variety of biological activities, including HIV inhibition, immunoregulation, leukocyte migration, and inhibition of hematopoietic stem cell division [17, 21]. Leukotactin-1 (Lkn-1) has been demonstrated to exert a protective effect on bone marrow stem cells during both chemotherapy and radiotherapy. The short version of human leukotactin-1 (shLkn-1) is a truncated form of Lkn-1, in which 26 amino acids have been deleted from the N-terminus [12].

The *Pichia pastoris* expression system is ideally suited to the industrial production of a host of heterologous proteins at commercially relevant concentrations [4] and has been used as a host for the abundant secretion of foreign proteins [1, 16, 19, 20]. However, the methanol concentrations in those experiments required careful monitoring, either directly by gas chromatography, or indirectly by dissolved oxygen (DO) concentration. A DO-stat strategy was used to produce rGuamerin and IGF-1 [2, 14, 18]. The control of methanol concentration was also employed in the production of human chymotrypsinogen B [6]. In the context of high-level expression processes, it is known that the entire process of protein production (cell growth, methanol induction) must be individually optimized for each foreign protein [4]. Recently, it was reported that the control of methanol feeding can be conducted while maintaining the monitoring and control of methanol concentration, using a methanol sensor or an off-gas analyzer [5, 7, 10, 15, 22, 23]. However, these results have generally been limited to small-scale bioreactors. Therefore, a practical method for the control of methanol concentration is required for effective production on industrial scales.

The objective of this study was to improve and optimize the shLkn-1 production process. In order to achieve large-scale shLkn-1 production, it was first required that the methanol concentration during induction be controlled. Residual methanol concentration was efficiently regulated by a DO-stat profile control, which

was able to modulate the speed of the feed pump, based on both the DO setpoint and the minimal DO value. Also, by controlling the DO-stat profile, the methanol induction phase could be extended for a long period of time. A 75-L bioreactor which had been fitted with a DO-stat for cell growth and methanol induction, was pressurized in order to increase the oxygen transfer rate (OTR). These approaches might constitute a practical method for the commercial production of shLkn-1, without the complicated control scheme.

Materials and methods

Strain

The shLkn-1 was expressed in *P. pastoris* GS115 (Invitrogen Co., Calif., USA), which was used as a host. The existing multiple cloning site of the pPIC9 plasmid vector (Invitrogen Co.) was modified in order to introduce the *stu*I restriction site. The 198-bp fragment encoding 66 amino acids of the recombinant shLkn-1 was then amplified by PCR from Lkn-1 DNA, using the following mutagenic primers: 5′-CACTTTGCTGCTGACTGC-3′ and 5′-GAATTCTTATATATTGAGTAGGGCTT-3′. The PCR product was then cloned in a frame with the α-factor signal peptide and a KEX2 recognition site. The constructed expression plasmid (pPM2-HF; Fig. 1) was then introduced into *P. pastoris* using the spheroplast transformation procedure, essentially as described by the *Pichia* expression kit manual (K1710-01; Invitrogen Co.). Gene-insertion events arose from a single cross-over located between the *his*4 locus in the chromosome and the *HIS*4 marker in the vector pPM2-HF, as previously described [13].

Fed-batch culture

The fed-batch culture consisted of three phases: a batch phase, a DO-stat fed-batch phase for high cell density,

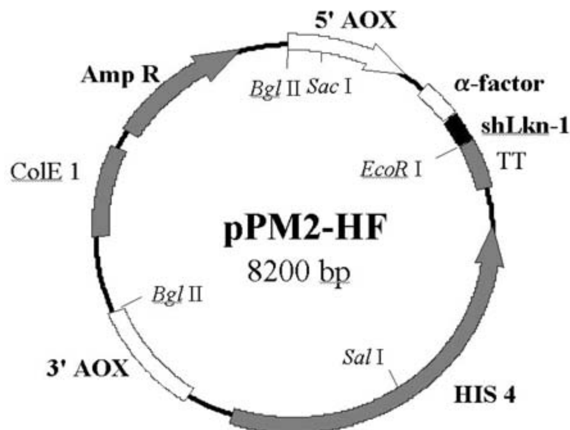

Fig. 1 Expression vector for shLkn-1

and a DO-stat fed-batch phase for methanol induction. The seed culture for a 75-L bioreactor was prepared using seven 1-L baffled flasks. An inoculum (1 mL) of frozen seed stock was added to each of these flasks containing 300 mL of YPD medium [containing, per liter: 10 g yeast extract (Difco, Mo., USA), 20 g bacto-peptone (Difco), 20 g glucose (Sigma, Mo., USA)]. The cultures were then incubated at 30°C, with shaking at 200 rpm, for 20–24 h. They were then transferred to the 75-L bioreactor, which contained 20 L of initial batch medium [containing, per liter: 40.0 g glycerol, 27.0 mL H_3PO_4, 0.9 g $CaSO_4$, 18.0 g K_2SO_4, 10.24 g $MgSO_4$, 4.13 g KOH, 30.0 mL NH_4OH, 4.4 mL yeast trace metal (YTM) solution]. YTM had the following composition (per liter): 2.4 g $CuSO_4 \cdot 5H_2O$, 0.09 g KI, 0.6 g $MnSO_4 \cdot H_2O$, 0.2 g $NaMo \cdot H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 15.0 g $ZnCl_2$, 63.5 g $FeSO_4 \cdot H_2O$, 0.2 g biotin, 10.0 mL H_2SO_4.

In this study, we used a 75-L bioreactor (Biostat D50-DCU 3; Braun Biotech International, Germany), all parameters of which were controlled by the DCU 3, a local control system designed to automate bioprocesses. This includes time-based profile controllers for operational variables (pH, temperature, air flow, stirrer speed, pressure, feed pump speed, DO level, anti-foam, etc.). The 75-L bioreactor was pressurized to compensate for the oxygen demands placed on it by cell growth. The temperature and pH of the system were controlled at 30°C and pH 5, respectively. The pH levels were maintained by the addition of either 30% phosphoric acid (Merck, Darmstadt, Germany) or ammonia solution (Riedel). Foam was controlled by the addition of 25% Antifoam 204 (Sigma). The initial agitation speed and aeration rate were 400 rpm and 1.0 vvm (20 L air/min to initial culture volume, 20 L), respectively. Throughout the entire culture period, the DO level was maintained above 20% air-saturation. When the glycerol present in the batch culture was depleted, glycerol feed medium [containing (per liter): 800.0 g glycerol, 0.45 g $CaSO_4 \cdot 2H_2O$, 9.00 g K_2SO_4, 5.12 g $MgSO_4$, 2.07 g KOH, 13.5 mL H_3PO_4 (85%), 24.0 mL YTM] was fed into the bioreactor, using the installed pump (313U/D; Watson Marlow) in a DO-stat-controlled manner, to allow for further cell growth. Once a dry cell weight (DCW) level of more than 100 g/L DCW had been achieved, the culture was starved for a period of 1 h, after which methanol induction was carried out in a DO-stat-controlled manner, using induction medium consisting of methanol and a 12 mL/L solution of trace metals.

DO-stat profile control

During the DO-stat operations associated with cell growth and methanol induction, the DO-stat profile was manually controlled by adjusting the feed pump speed. As shown in Fig. 2, the feed pump speed was reduced when the minimal value of the DO profile reached a value below DO_{Low}, which was 10% below the DO

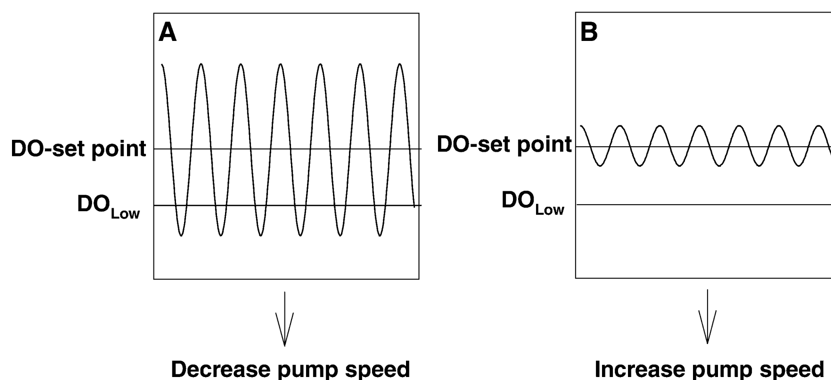

Fig. 2 Strategy for DO-stat profile control. The feed pump speed decreased when the lowest value of the DO-stat profile was below DO_{Low} (**A**) and increased when the lowest value of the DO-stat profile did not reach DO_{Low} (**B**)

setpoint. The feed pump speed was increased when this value exceeded DO_{Low}. Pump speed was regularly monitored and adjusted during the operation of the DO-stat. In this study, the DO setpoint was controlled in order to maintain air-saturation values in a range between 40% and 60%.

Mass spectrometry and N-terminal amino acid analysis

Samples were centrifuged (Avanti J-25; Beckman, USA) for 10 min at 15,000 rpm at 4°C, and filtered through a 0.45-μm membrane, prior to loading onto the chromatographic column of a Prep HPLC system. Reverse-phase Prep HPLC (Delta Prep 4000; Waters, Mass., USA) was conducted using a C18 column (Prep LC 25-mm module). Protein levels were monitored with a UV detector (Waters). In the final step of the operation, the collected fractions were evaporated (Rotavapor R-114, waterbath B-480; BUCHI, Switzerland) and lyophilized (Ilshin, Korea).

N-terminal sequencing was conducted with an automatic peptide sequencer (Procise 492; Perkin–Elmer, Mass., USA), and we conducted electrospray mass spectrometry using a Platform II (Micromass, UK), in order to measure the molecular weights of the recombinant shLkn-1 [11]. Nitrogen gas was used as a carrier gas, and 50% MeCN dissolved in water was employed as an elution solvent, at a speed of 0.04 mL/min.

Analyses

In order to determine DCW, culture broth samples (20 mL) were obtained in duplicate, centrifuged at 10,000 rpm for 5 min, and washed twice with deionized water. They were then dried and measured using an electronic moisture analyzer (model MA 40; Sartorius, Germany). The correlation between the DCW and optical density at 600 nm (OD_{600}) was as follows: DCW (g/L) = $0.32 \times OD_{600} + 5.21$. Methanol concentrations were assessed by gas chromatography (HP5890 series II; Hewlett Packard, Calif., USA) using a 10% degs column (1.8 m long, WHP 100/120, 19001a-L12). SDS-PAGE was conducted with Xcell SureLock (Invitrogen) and 4–12% Bis-Tris gel (Novex, USA). The production of shLkn-1 was assessed via analytical HPLC, using a C18 reversed-phase column (214TP54, 250.0×4.6 mm; Vydac).

Results

DO-stat profile control for cell growth and methanol induction

During fed-batch *P. pastoris* culture, high OTR were required, due to the high oxygen uptake rate (OUR) associated with the high cell density. In this study, we attempted to increase the OTR by increasing the vessel pressure of the 75-L bioreactor. This was achieved by adjusting the off-gas valve. From 12 h to 20 h (corresponding to the late phase of the batch culture and the early phase of the DO-stat fed-batch culture), the agitation speed, aeration rate, and vessel pressure were increased gradually according to a time-based profile. These values finally reached 750 rpm, 3 vvm, and 120 kPa, respectively, and the cell density achieved a level of about 30 g/L at 20 h (Fig. 3A, B). After the batch culture was finished, we conducted a DO-stat fed-batch culture, in which the DO-stat profile control was adopted and the DO setpoint and medium feed rate (per hour) were changed from 60% and 10 mL/L to 50% and 17 mL/L, respectively, at 22 h. As shown in Fig. 3B, the DO-stat profile could be controlled successfully, with no fluctuations. Before the beginning of the methanol induction phase, the cell density reached a level of about 100 g/L.

At 30 h, after 1 h of starvation, we began to feed the methanol induction medium into the bioreactor for the induction of shLkn-1, while manually controlling the DO level at approximately 60%. The hourly feed rate of the methanol induction medium was maintained in a range between 1 mL/L and 6 mL/L (Fig. 3C). Residual methanol concentrations were maintained at below 0.5% w/v (data not shown). The final cell and shLkn-1 concentrations were 123 g/L and 750 mg/L, respectively (Fig. 3B, D). The average specific shLkn-1 production rate (per hour) during the methanol induction phase was estimated to be about 0.14 g/g/h.

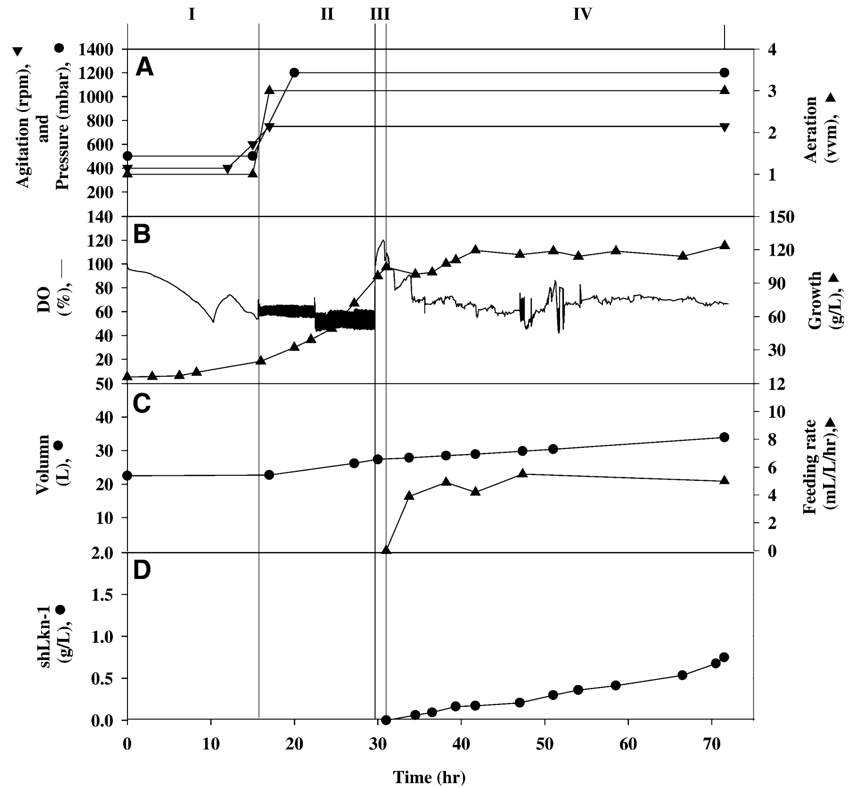

Fig. 3 Time-courses of fed-batch culture with DO-stat profile control for cell growth. I; Batch culture phase, II; DO-stat fed batch phase using glycerol, III; starvation phase, IV; methanol induction phase. **A**; Agitation speed, vessel pressure, and aeration rate, **B**; DO and cell density, **C**; culture volume and feeding rate of induction medium, **D**; shLkn-1 concentration

Extended production of shLkn-1 using DO-stat profile control

For the extended production of shLkn-1, DO-stat profile control was applied to the methanol induction process. As shown in Fig. 4, the induction process was extended to 192 h, with the culture broth being withdrawn a total of four times. During the batch and the DO-stat fed-batch culture phase, the agitation speed, aeration rate, vessel pressure (Fig. 4A), cell growth, and

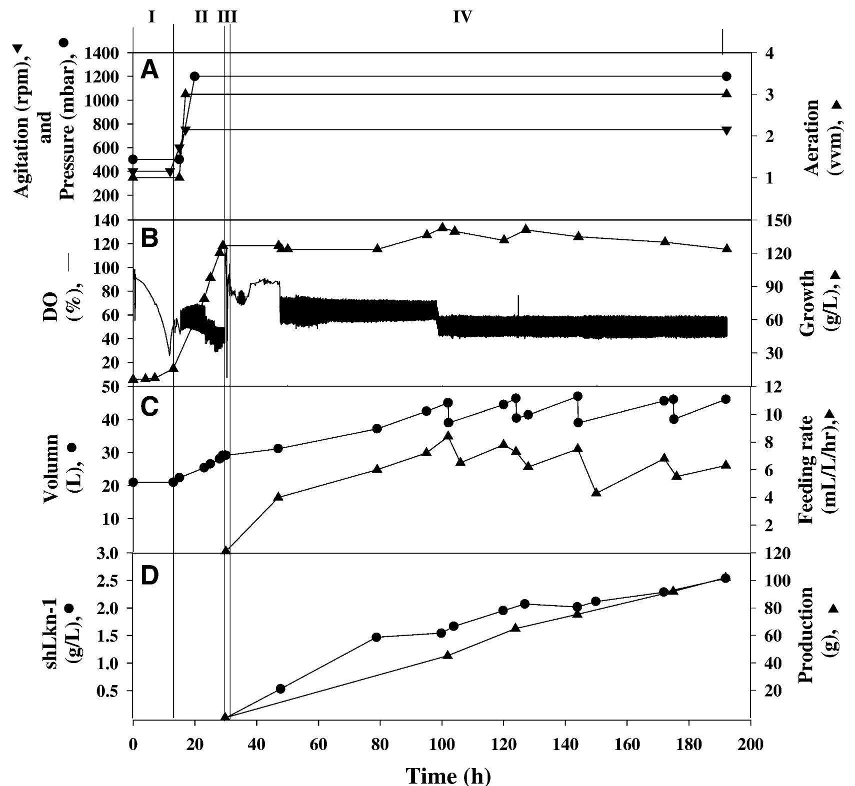

Fig. 4 Time-courses of fed-batch culture with DO-stat profile control for cell growth and methanol induction. I; Batch culture phase, II; DO-stat fed batch phase using glycerol, III; starvation phase, IV; methanol induction phase. **A**; Agitation speed, vessel pressure, and aeration rate, **B**; DO and cell density, **C**; culture volume and feeding rate of induction medium, **D**; shLkn-1 concentration and cumulative shLkn-1 production

DO (Fig. 4B), were determined to be similar to those observed in Fig. 3. The methanol induction phase was initiated using a DO-stat profile control, in which the DO setpoint was 60% at 50 h and, in order to increase the methanol supply, the DO setpoint was altered to 45% at 98 h (Fig. 4B). The DO profile was successfully controlled with no fluctuations, and the cell density achieved levels of over 142 g/L (Fig. 4B). The culture broth was withdrawn a total of four times, at 102, 124, 144, and 175 h, and the total volume withdrawn was approximately 70 L (Fig. 4C). The working volume was maintained between 39 L and 45 L during the process of methanol induction. The expression of shLkn-1 during the early induction phase, which persisted for 72 h, was about 1.5 g/L, a value considerably higher than the production levels displayed in Fig. 3D (see also Fig. 4D). In addition, the production of shLkn-1 was extended to 192 h. The shLkn-1 concentration and the total amount of shLkn-1 produced reached levels of approximately 2.5 g/L and 102 g, respectively (Fig. 4D). The residual methanol concentration was also maintained below the level of 0.5% w/v (data not shown). The shLkn-1 concentration, and total shLkn-1 production steadily increased during long-term induction. The specific shLkn-1 production rate (per hour) from the inception of production to the 79-h mark (a time-period corresponding to the early phase of methanol induction) was an average of 0.24 g/g, a value higher than that displayed in Fig. 3D. However, after 79 h, these hourly rates decreased to an average of 0.07 g/g/h.

Truncation of shLkn-1 during the extended induction

During the extended induction, we verified shLkn-1 production in the culture supernatant using SDS-PAGE and analyzing major single bands (Fig. 5). However, smeared impurities were observed around the bands. According to the results of reverse-phase HPLC analysis, we determined that these impurities increased directly with the levels of shLkn-1 production (Fig. 6). The shoulder peaks (peaks II, III) on the left-hand side of the major peak (peak I) also gradually increased throughout methanol induction; and two shoulder peaks could be clearly observed at 192 h (Fig. 6D). In order to identify the peaks that were observed in the reverse-phase HPLC analysis, the final culture supernatant was separated into two fractions (α, β) using prep-HPLC (Fig. 7). Then, we carried out N-terminal amino acid sequencing and mass spectrometry for the two fractions. All of the N-terminal sequences were found to be identical to the correct N-terminal sequence (histidine–phenylalanine; data not shown). According to the mass spectrometry data, we determined that fractions α and β were composed of two mass peaks (6,998.74, 7,086.61; Fig. 8A) and one mass peak (7,201.63; Fig. 8B), respectively. The mass peak of fraction β (Fig. 7) was almost identical to that of authentic shLkn-1 (7,212 Da; theoretical mass value) and so fraction β was confirmed as authentic shLkn-1. Finally, it was presumed that, as the masses of peaks (1) and (2) in Fig. 8A were smaller than the mass of the peak shown in Fig. 8B and all of the N-terminal sequences had been conserved, peaks (1) and (2) in Fig. 8A must correspond to shLkn-1 derivatives that were truncated at the one or two residues contained in the C-terminal of shLkn-1.

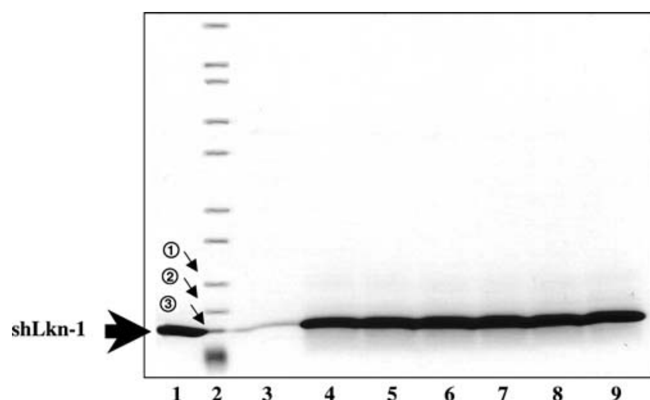

Fig. 5 SDS-PAGE of culture supernatants from Fig. 4. *Lane 1*: shLkn-1 standard, *Lane 2*: Size markers (Mark 12™; Novex, USA) (*Arrows* ①–③ show 21.5, 14.4, 6 kDa, respectively), *Lanes 3–9*: Culture supernatants at 29, 79, 95, 120, 144, 172, and 192 h. Sample loading volume was 20 μL. The gel was stained with Coomassie brilliant blue R-250 for 60 min and then destained in a solution consisting of 10% (v/v) methanol and 10% (v/v) acetic acid

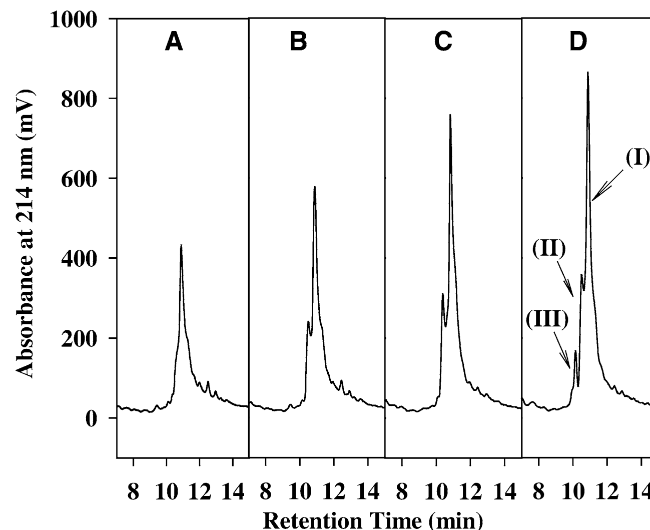

Fig. 6 Reverse-phase HPLC analysis of culture supernatants. **A** 49 h, **B** 104 h, **C** 150 h, **D** 192 h. The mobile phase consisted of solvent A (0.1% trifluoroacetic acid; TFA) and solvent B (99.9% MeCN, 0.1% TFA). A gradient was developed by increasing the fraction of solvent B from 25% to 35% over 10 min at uniform flow rate of 0.7 mL/min. Proteins were monitored by absorption at 214 nm

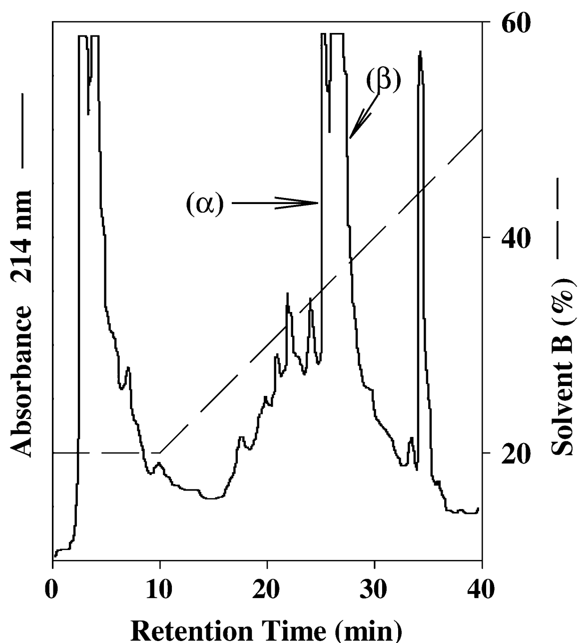

Fig. 7 Chromatogram of the prep-HPLC culture supernatant obtained at 192 h. Fractions were collected by reverse-phase prep-HPLC in a Prep LC 25-mm module C18 column using solvent A (0.1 % TFA) and solvent B (99.9% MeCN, 0.1% TFA). The gradient profile was maintained at 20% MeCN for 10 min after injection and then linearly increased to 50% during the next 30 min and finally to 95% for the last 5 min at a uniform flow rate of 17 mL/min. Separation was monitored by absorption at 214 nm

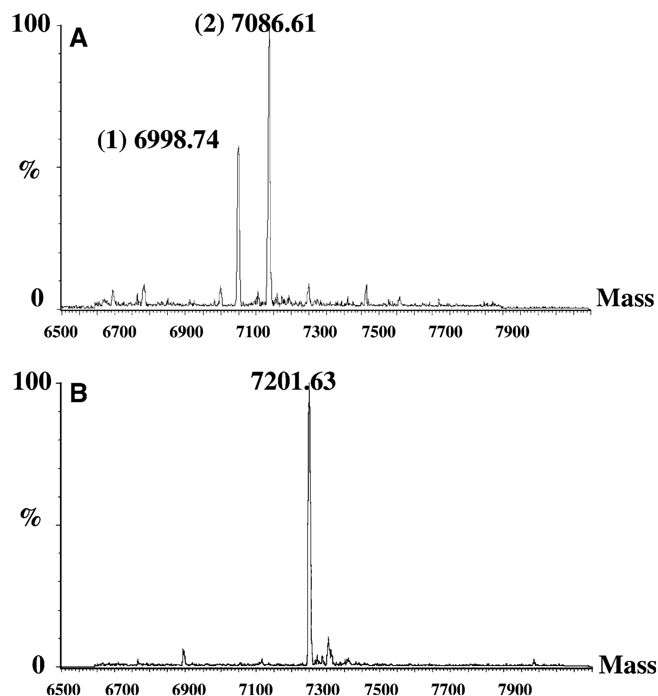

Fig. 8 Mass spectrometries of fractions from prep-HPLC (Fig. 7). **A** Fraction α, **B** fraction β, Electrospray ionization mass spectrometry (ESI-MS) was performed for the initial examination of the various peaks from the prep-HPLC separation. Data acquisition and processing were controlled by the Micromass MassLynx Window ver. 3.0 data system running on a PC platform. Fractions were freeze-dried and reconstituted in 100 μL of 50% MeCN in deionized water for injection into the ESI-MS system. Identification of purity within HPLC fractions were facilitated by the use of an electrospray ion-trap mass spectrometer fitted with a static nanospray source. Ions were generated using nano-ESI from 50:50 H_2O:MeCN solutions

Discussion

In our recent report regarding a repeated fed-batch process for the long-term production of rGuamerin [14], it was demonstrated that the OUR requirement of *P. pastoris* could be fulfilled by increasing the OTR, which was accomplished via supplementation with pure oxygen. In this study, in order to increase the OTR, we adopted an operational strategy involving the increase of vessel pressure via adjustments of the off-gas valve on the 75-L bioreactor. As the oxygen requirements associated with methanol metabolism are known to be about three times greater than those of glycerol metabolism [8], the maintenance of suitable OTR during methanol induction is crucial to the generation of heterologous proteins. In the case of small-scale fermentation, in order to achieve a high cell concentration, a rather high proportion of pure oxygen must be mixed with inlet air, to maintain desirable pO_2 levels. However, in large-scale fermentation, pure oxygen cannot be used, due to the relatively high cost. Therefore, instead of pure oxygen supplementation, we opted to increase vessel pressure. As a result, the operation proceeded successfully throughout the entire 192-h period. The operations for both cell growth and methanol induction were successfully accomplished with no irregularities and/or fluctuations of the DO-stat profile, which occur fairly frequently in conventional DO-stat fed-batch cultures under pressurized conditions. Through the DO-stat profile control protocols employed in this study, a stable DO-stat was maintained at the higher OTR values induced by pressurization of the vessel, especially during the methanol induction phase.

Mut^+ strains of *P. pastoris* exhibit profound sensitivity to both high and low residual methanol concentrations [3]. Therefore, in order to maintain an optimal residual methanol concentration, methanol concentrations were measured periodically, either on- or off-line [6]. Also, control schemes were recently introduced which involved the direct control of methanol concentration in the culture broth, in which the methanol concentration was maintained at a concentration between 0.4% and 3.0% [18]. The methanol concentrations were crucial indicators for the determination of the feeding rates of the induction media. However, methanol concentration measurements were tedious in the large-scale process and might ultimately prove impractical. Using our DO-stat profile control scheme, the residual methanol concentration could be maintained between 0.3% and 0.5% (data not shown). Thus, the DO-stat profile control could be considered an alternative strategy for the control of methanol concentrations

within a narrow range during methanol induction. This technique might also feature significantly in a methodology for the practical application of a large-scale production process.

For the first 49 h of the early phase of methanol induction (Fig. 4D), the specific shLkn-1 production rate was determined to be higher than the values reported previously [13]. However, the specific production rates decreased gradually during the extension of the induction phase. This phenomenon has been reported in several other studies [8, 9, 22]. Reductions in the specific shLkn-1 production rate after 79 h (Fig. 4D) were probably due to increases in the production of C-terminal-truncated derivatives of shLkn-1, as shown in Figs. 6, 7. Based on the differences in mass between the authentic and derivative shLkn-1 (Fig. 8), we deduced that the mass peak at 7086.61 (Fig. 8A) corresponded to a shLkn-1 derivative, in which one C-terminal amino acid, L-isoleucine, could be truncated. Another mass peak at 6998.74 (Fig. 8A) was deduced as a shLkn-1 derivative, which corresponded to another derivative, in which two C-terminal amino acid residues, L-isoleucine and L-serine, were sequentially truncated. Consequently, a proper harvesting time and/or preventing the production of the C-terminal truncated derivatives are both prerequisites for successful high-quality shLkn-1 production, especially during extended operation.

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