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High-level production of Fc-fused kringle domain in *Pichia pastoris*

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Abstract Recently, as a new non-immunoglobulin-based protein scaffold, a human kringle domain was successfully engineered toward biologically functional agonists and antagonists. In this study, the fed-batch cultivation conditions were optimized for enhanced production of an Fc-fused kringle domain (KD548-Fc) in *Pichia pastoris*. Fed-batch cultivations were performed in 5-l laboratory-scale bioreactors, and in order to find the optimal conditions for high-level production of KD548-Fc, several parameters including the initial carbon source (glycerol) concentration, temperature, and pH were investigated. When cells were cultivated at pH 4.0 and 25 °C with 9.5 % glycerol in the initial medium, the highest production yield (635 mg/l) was achieved with high productivity (7.2 mg/l/h). Furthermore, functional KD548-Fc was successfully purified from the culture broth using a simple purification procedure with high purity and recovery yield.

Keywords Kringle domain · Fc-fusion protein · *Pichia pastoris* · Fed-batch fermentation

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Introduction

Due to their high activity and specificity, therapeutic antibodies have been invaluable tools for targeted therapy and diagnostics [2, 13]. However, several drawbacks of therapeutic antibodies, such as high manufacturing cost, low stability, and poor production yield in bacterial hosts, have limited their use. In the past decade, non-immunoglobulin-based protein scaffolds that have rigid frameworks and bind to specific targets with high affinity and specificity have been exploited as alternative platforms to antibody therapeutics [1, 7, 23, 24]. Compared with conventional antibodies, scaffold proteins have several favorable properties such as robustness, compact size, simple structure, and cost-efficient production in yeast or bacterial hosts. Furthermore, it has become clear that scaffold proteins will lead to the next generation of biopharmaceuticals and are significant in the pharmaceutical industry as well as in diagnostic systems.

Recently, as a new protein scaffold, a human kringle domain (KD) was successfully engineered toward biologically functional agonists and antagonists with high affinity and specificity [16]. The human kringle domain (KD) is composed of 80 amino acids and contains a rigid core structure with 7 flexible loop structures that are strongly sequence tolerant to mutation and thereby provide a potential protein scaffold toward distinct binding to various target molecules. Through yeast cell surface displays and high-throughput screening of a synthetic KD library, new KD variants, which could specifically bind to human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors as well as death receptors 4 and 5 (DR4 and DR5), were successfully isolated [16]. Among the isolated KD variants, one variant (KD548) had high binding affinity (K_D = 172 nM) to death receptor 5 (DR5) with high specificity. In addition, in order to increase the size,

serum half-life, and avidity-mediated affinity, the isolated KD548 was further engineered into a homodimeric Fc-fused form through C-terminal fusion to the Fc domain of human immunoglobulin G1. The KD548-Fc exhibited approximately tenfold higher affinity ($K_D = 58$ nM) to the target DR5 as well as approximately eightfold greater cytotoxicity to tumor cells when compared with its monomeric counterpart [16]. More recently, the mechanism of the antitumor activity of KD548-Fc that activates the Nox1 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate a superoxide anion, which leads to reactive oxygen species-mediated apoptotic cell death, was reported [20]. Collectively, the previous results clearly demonstrate that KD548-Fc is a potent inhibitor of tumor cell growth in vivo, and now optimization of culture conditions and purification procedures on a large scale are highly desired for further validation of the efficacy and commercialization of KD548-Fc.

Preparative-scale production of recombinant proteins with high efficiency is a key issue in protein characterization, further modification, and commercialization, being particularly important for proteins of human origin that may not be appropriately produced in bacterial systems [14]. In this respect, the methylotrophic yeast *Pichia pastoris* is an attractive eukaryotic host for production of recombinant proteins, particularly proteins that require posttranslational modification such as glycosylation [3, 19]. Compared with mammalian hosts, *P. pastoris* does not require an expensive growth medium, and therefore, the production cost can be significantly reduced, which must be considered in the design of the process. *Pichia pastoris* can also grow as fast as prokaryotic bacteria, and thus fermentation can be easily scaled up to meet greater demands for protein products [19]. In addition, through the well-established folding and secretion machinery, various proteins can be highly folded and efficiently secreted into the culture medium. Because *P. pastoris* does not secrete high levels of endogenous proteins, the purification of recombinant proteins secreted into the culture medium is much simpler than in other systems. Due to these advantages, *P. pastoris* has been successfully used for secretory production of various recombinant proteins with high production yields: antibodies from scFv (4.9 g/l) [5] to full-length IgG (1–1.6 g/l) [21, 25], the surface antigen of hepatitis B virus (6 g/l) [8], and staphylococcal protein A (8.8 g/l) [10], among others [18].

In this study, the fermentation conditions were optimized for enhanced production of Fc-fused kringle domain variants in *P. pastoris*. As a target protein, Fc-domain-fused KD548 (hereafter denoted as KD548-Fc) was chosen because it has strong affinity to DR5 and can be used as a potential agonist of TRAIL [16]. In a 5-l laboratory-scale bioreactor system, the culture conditions including temperatures, glycerol concentration in the initial medium, and pH were optimized. For each condition, the cell growth rate, glycerol consumption, and production yield of KD548-Fc were analyzed and compared. After using the optimized culture conditions, the produced KD548-Fc was successfully purified and its strong activity confirmed.

Materials and methods

Yeast strain, plasmid, and flask cultivation

The Mut$^+$ strain of *P. pastoris* GS115 (Invitrogen, Carlsbad, CA) was used as the sole host for production of the Fc fusion protein. The gene encoding KD548-Fc was cloned into pPICZαA (Invitrogen) under control of a methanol-inducible alcohol oxidase promoter (AOX1) to yield pPICZα-KD548-Fc (Fig. 1). In this construct, the KD548-Fc gene was linked to the mating α factor (MF-α) secretion sequence for secretory production into the culture medium [4]. For flask cultivation, 1 ml of cells was inoculated into 200 ml of basal medium (glycerol 20 g/l, yeast nitrogen base 13.4 g/l, and biotin 400 μg/l) in a 1-l baffled flask, and the cells were cultivated at 30 °C and 150 rpm for 72 h.

Fed-batch fermentation

All fed-batch fermentations were performed in a 5-l jar bioreactor (BioCNS; Daejeon, Korea). A semidefined medium containing 9.4 g/l of potassium dihydrogen phosphate, 1.14 g/l of yeast trace metal (YTM) solution, 15.7 g/l of ammonium sulfate, 1.83 g/l of magnesium sulfate heptahydrate, 0.28 g/l of calcium chloride dehydrate, 0.4 mg/l of biotin, and 15 g/l of yeast extract was used [8]. The YTM solution comprised 207.5 mg/l of potassium iodide, 760.6 mg/l of manganese sulfate, 484 mg/l

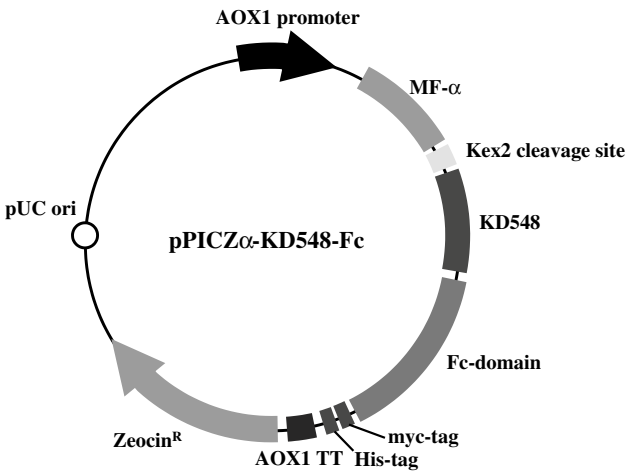

Fig. 1 Expression system of KD548-Fc in *P. pastoris*

of disodium molybdate, 46.3 mg/l of boric acid, 5.032 g/l of zinc sulfate heptahydrate, 12 g/l of ferric chloride hexahydrate, and 9.2 g/l of sulfuric acid. The initial volume of the culture was 2.2 l. In order to optimize the protein production, three different temperatures (20, 25, and 30 °C) and three different pH conditions (4.0, 5.6, and 7.2) were examined. The pH was controlled by adding 12.5 % (v/v) ammonia solution when the pH was lower than the set point. The dissolved oxygen (DO) was maintained above 40 % of saturated air throughout the fermentation. The DO was initially controlled by increasing the agitation speed from 200 to 1200 rpm, and then pure oxygen mixed with air was supplied. The total gas flow rate was maintained at 2 vvm throughout the fermentation. The fermentation process consisted of a batch phase and a fed-batch cultivation phase. During the batch phase, glycerol was used as the sole carbon source with three different concentrations (47.5, 95, and 142.5 g/l). After depletion of the initial glycerol in the batch phase, a sudden increase in the DO was observed and the culture was induced by feeding methanol with 1.4 % (w/w) YTM. Then, the methanol concentration was maintained below 1 % (w/w) of culture medium by adding 20 ml of methanol at every spike in the DO concentration. The methanol concentration in the medium was monitored by high-performance liquid chromatography (Shimadzu, Nakagyo-ku, Kyoto, Japan). The cell density was monitored using a UV–Vis spectrometer (OPTIZEN POP; Mecasys, Daejeon, Korea). The optical density at wavelength of 600 nm was measured for cell density monitoring.

Purification of KD548-Fc

For purification of KD548-Fc, 100 ml of fed-batch culture broth was used. The cells were removed via centrifugation (4 °C, 6,000 rpm, 10 min), and the proteins in the supernatant were dialyzed against 1× phosphate-buffered saline (PBS; 2.7 mM KCl, 137 mM NaCl, 10 mM phosphate) with dialysis tubing (7,000 molecular weight cutoff) for 24 h. After dialysis with one buffer exchange, the KD548-Fc fusion protein was further purified using Protein A affinity column chromatography with fast protein liquid chromatography (AKTA Purifier 10 FPLC system; Amersham Pharmacia Biotech, Piscataway, NJ). The dialyzed protein samples were loaded onto the Protein A affinity column (GE Healthcare, Buckinghamshire, UK) equilibrated with 1× PBS. The bound proteins were eluted by applying the elution buffer (0.5 M arginine, pH 4.0), and the eluted proteins were directly neutralized using 0.5 ml of 1.5 M Tris-HCl buffer (pH 8.8) at each fraction. The flow rate was maintained at 1 ml/min, and the protein concentration in each fraction was monitored using a UV detector (UPC-900; GE Healthcare, Buckinghamshire, UK).

Preparation of DR5

For preparation of the antigen (extracellular domain of human DR5), *Escherichia coli* BL21 (DE3) containing pET21b-DR5 [15] was used in which the His tag-fused human DR5 gene was expressed under the T7 promoter. Cells were cultivated in 500 ml of Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g NaCl). When the cell density (OD_{600}) reached 0.6, the cells were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich Co., St. Louis, MO) to final concentration of 0.5 mM, with further cultivation at 25 °C and 200 rpm for 16 h. After centrifugation (6,000 rpm, 4 °C, 10 min), the cells were resuspended in 40 ml of PBS and disrupted multiple times using sonication (cycles of 10 s on-time and 10 s off-time at 20 % of maximum output, total exposure time 15 min) with high-intensity ultrasonic liquid processors (VC750; Sonics & Material Inc., Newtown, CT). The soluble protein fraction containing the His tag-fused DR5 was collected via centrifugation (10,000 rpm, 4 °C, 10 min) and mixed with 1 ml of TALON® Superflow metal affinity resin (Clontech Laboratories Inc., Mountain View, CA). After incubation at 4 °C for 1 h following washing with a buffer (50 mM phosphate-KOH, 300 mM NaCl, 5 mM imidazole, pH 8.0), the His tag-fused DR5 was eluted using an elution buffer (50 mM phosphate-KOH, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was applied to dialysis against 1× PBS, then the dialyzed proteins were stored at 4 °C.

SDS-PAGE analysis

After cultivation, the cells were removed via centrifugation (6,000 rpm, 4 °C, 10 min) and the supernatant containing the secreted KD548-Fc was stored at −70 °C for further analysis. The protein samples were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15 % acrylamide gels under reducing and nonreducing conditions (without β-mercaptoethanol in the SDS-PAGE sample buffer). After the gel electrophoresis, the gels were stained with Coomassie brilliant blue [50 % (v/v) methanol, 10 % (v/v) acetic acid, 1 g/l Coomassie brilliant blue R-250] for 30 min and destained using a destaining solution [10 % (v/v) methanol, 10 % (v/v) acetic acid].

Enzyme-linked immunosorbent assay (ELISA)

For the ELISA, 50 μl of purified DR5 (2 μM) dissolved in 50 mM of carbonate–bicarbonate buffer (pH 9.6) was coated on each well of a 96-well transparent plate (Nunc; Invitrogen Ltd., Carlsbad, CA) for 1 h at room temperature. After four washes with PBS-T (PBS containing 0.05 % Tween 20, pH 7.4), each well was blocked with

5 % (w/v) skim milk solution in PBS (PBS-M) for 1 h at room temperature. After four washes with PBS-T, the protein samples were serially diluted, added to the antigen-coated wells, and allowed to bind for 1 h at room temperature. After four subsequent washes with PBS-T, 100 µl aliquot with 1:2,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody dissolved in PBS-M was added to each well and allowed to react for 1 h at room temperature. After washing with PBS-T again, 50 µl of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (BD, Franklin Lakes, NJ) was added to each well for detection. The peroxidase reaction was stopped by adding 50 µl/well of 2 M H_2SO_4. The absorbance (Abs) at 450 nm was determined using an ELISA reader (Infinite M200 PRO; TECAN, Männedorf, Switzerland).

Results and discussion

Effect of initial glycerol concentration on cell growth and KD548-Fc production

During the fed-batch fermentation of *P. pastoris*, the induction point (cell density) can have an effect on the production yield of the target protein as well as on the cell growth; therefore, it is considered to be an important parameter in the optimization of fed-batch culture conditions [18]. During the batch cultivation, the glycerol in the initial medium, which is supplied as the sole carbon source in the batch mode, is consumed by the cells, and immediately after depletion of the initial glycerol, feeding solution containing methanol is supplied for cell growth in the fed-batch mode as well as induction of gene expression. Thus, the induction point of gene expression can be changed by controlling the glycerol concentration in the initial medium. In order to determine the optimal concentration of glycerol, three different concentrations of glycerol were examined: 4.75 % (47.5 g/l), 9.5 % (95 g/l), and 14.25 % (142.5 g/l). All other conditions (pH 5.6 and 30 °C) were maintained consistent. When the intermediate concentration of glycerol (9.5 %) was used, the initial glycerol was depleted at 23 h (Fig. 2a); at this time point, the cell density was OD_{600} = 150. Immediately after the glycerol depletion, the methanol-containing feeding solution was supplied, and the cells continued to grow up to OD_{600} = 304.8 at 58 h, then the cell density decreased (Fig. 2a). During the exponential growth phase, the specific cell growth rate (μ) was 0.099/h. Immediately after the methanol feeding, KD548-Fc began to be produced, and its content continued to increase during the cultivation (Fig. 2b). The maximum production yield (272 mg/l) of KD548-Fc was obtained at 55 h, and the productivity was

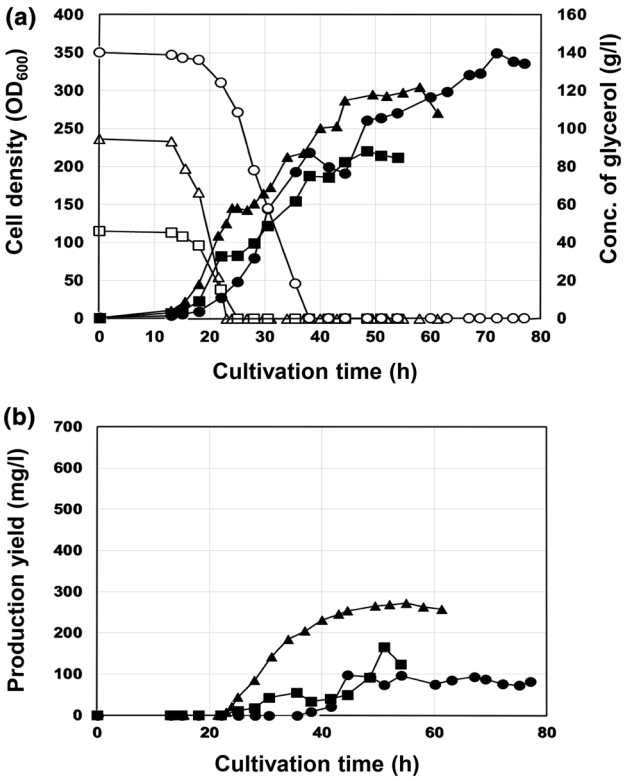

Fig. 2 Effect of glycerol concentration in the initial medium during the fed-batch cultivation: **a** time profiles of cell growth (*closed symbols*) and glycerol concentration (*open symbols*), and **b** time profiles of KD548-Fc production in culture supernatant. *Squares*, *triangles*, and *circles* correspond to different concentrations of glycerol in the initial medium: 4.75, 9.5, and 14.25 %, respectively. All fermentations were performed at 30 °C and pH 5.6

4.95 mg/l/h (Table 1). When the initial glycerol concentration was changed to 4.75 or 14.25 % (0.5-fold decreased or increased concentration compared with 95 g/l, respectively), the cell density for induction also changed to OD_{600} = 83.2 or 218.4, respectively (Fig. 2a). The growth rates in the exponential growth phase were very similar to that for the 9.5 % glycerol condition. After the depletion of the initial glycerol, methanol was added and KD548-Fc production began immediately (Fig. 2b). The maximum production yield for the high and low glycerol concentration was 98 and 166 mg/l, respectively, being lower than that (272 mg/l) for the intermediate glycerol concentration (Fig. 2b). Based on these results, the glycerol concentration in the initial medium was fixed at 95 g/l for all following fermentations.

Effect of pH on KD548-Fc production

In general, *P. pastoris* is able to grow across a relatively broad pH range (3.0–7.0), but production yields can be

Table 1 Maximum production yield and productivity for various fermentation conditions

Condition			Maximum production yield (mg/l)	Productivity (mg/l/h)
pH	Temperature (°C)	Initial glycerol conc. (g/l)		
5.6	30	95.0	272	4.95
5.6	30	47.5	166	3.25
5.6	30	142.5	98	2.20
4.0	30	95.0	440	6.82
7.2	30	95.0	58	0.63
5.6	25	95.0	445	4.45
5.6	20	95.0	533	3.53
4.0	25	95.0	635	7.20

affected by the pH of the cultivation [15, 18, 19]. Different proteins require different pH conditions depending on their stability, and therefore the optimal pH condition must be determined in order to provide better stability and production yields of KD548-Fc. For optimization of the pH condition, fed-batch fermentations were performed at two different pHs (pH 4.0 and 7.2), and the cell growth and protein production yields were compared with the results of previous fermentations performed at pH 5.6. In these fermentations, all other conditions including the temperature (30 °C), initial glycerol concentration (9.5 %), and DO (40 %) were the same. When the cells were cultivated at pH 4.0, the glycerol in the initial medium was depleted at approximately $OD_{600} = 150$, similar to the case of pH 5.6, but this required a slightly longer time (30 h) than at pH 5.6 (23 h) (Fig. 3a). After the depletion of the initial glycerol, the methanol feeding solution was supplied and then the cells continued to grow up to $OD_{600} = 358$ at 64.5 h, after which the cell density decreased gradually (Fig. 3a). During the exponential growth phase, the specific cell growth rate (μ) was 0.085/h, which was slightly slower than for the fermentation at pH 5.6. Immediately after the methanol feeding, KD548-Fc production began, and its content continued to increase during the cultivation (Fig. 3b). The maximum production yield (440 mg/l) of KD548-Fc was obtained at 64.5 h, and the maximum productivity was 6.82 g/l/h, being 1.4-fold higher than that for the fermentation at pH 5.6 (Table 1). When the cells were cultivated at a higher pH (7.2), they exhibited very poor cell growth compared with the lower pHs (4.0 and 5.6) (Fig. 3a). After a very long lag phase (more than 60 h), the cells began to grow and the initial glycerol was consumed (Fig. 3a). At 80 h, the cell density reached $OD_{600} = 118$, and the cells were induced by methanol. However, after this point, the cells did not grow further, and the cell density decreased gradually. After induction, KD548-Fc production began, but the maximum production yield (58 mg/l) was much lower than for the cultivations at lower pHs (Fig. 3b; Table 1).

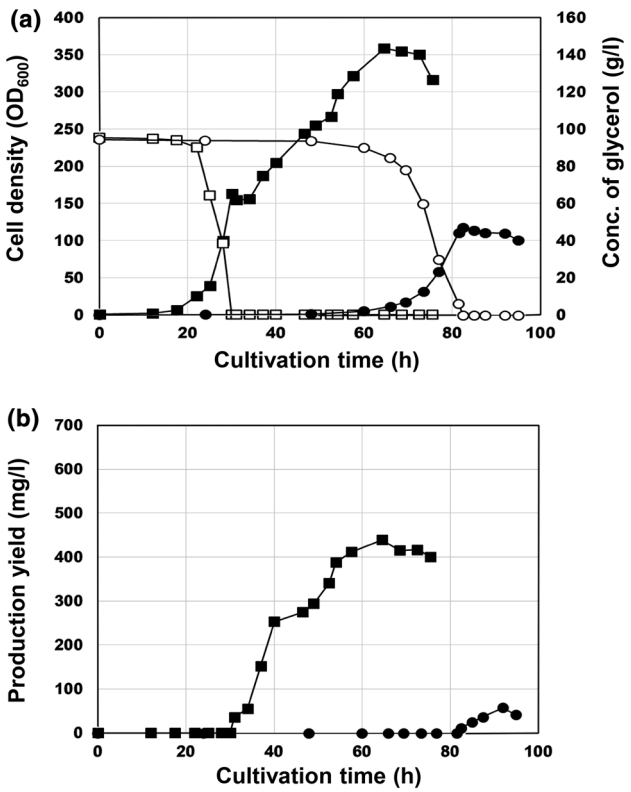

Fig. 3 Effect of pH during the fed-batch cultivation: **a** time profiles of cell growth (*closed symbols*) and glycerol concentration (*open symbols*), and **b** time profiles of KD548-Fc production in culture supernatant. *Squares* and *circles* correspond to pH 4.0 and pH 7.2, respectively. All fermentations were performed at 30 °C and 9.5 g/l glycerol

Effect of temperature on KD548-Fc production

Generally, secretion of recombinant proteins is significantly affected by culture temperature [6, 9, 11, 12, 17, 22]. In many cases, lower temperature has been preferred for efficient translocation and correct folding of the secreted protein; however, the decrease of the cell growth rate and

productivity at lower temperatures must also be considered. For optimization of the culture temperature, fed-batch fermentations were performed at two different temperatures (25 and 20 °C), and the production yields were compared with that of the previous fermentation at 30 °C (pH 5.6). At both low temperatures (25 and 20 °C), the cells exhibited a longer lag phase than for the cultivation at 30 °C (Fig. 4a). At 25 °C, the cell density began to increase after 50 h, and the cells grew up to OD_{600} = 350 at 100 h (Fig. 4a). The glycerol in the initial medium was depleted at 57 h, and then the methanol-containing feeding solution was supplied (Fig. 4a). The specific growth rate (μ) during the exponential growth phase was 0.066/h, which was slower than that of the cultivation at 30 °C. Immediately after supplying the feeding solution, KD548-Fc production began (Fig. 4b). The maximum content of KD548-Fc in the culture medium was 445 mg/l, which was 1.63-fold higher than that of the cultivation at 30 °C (272 mg/l). At 25 °C, the maximum productivity was 4.45 mg/l/h, being similar to that of the cultivation at 30 °C (4.95 mg/l/h) (Table 1).

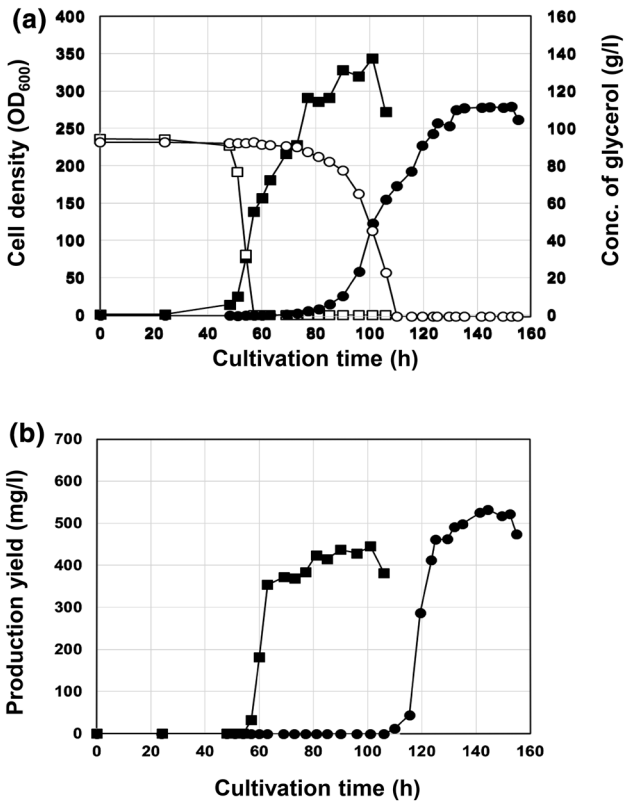

Fig. 4 Effect of temperature during the fed-batch cultivation: **a** time profiles of cell growth (*closed symbols*) and glycerol concentration (*open symbols*), and **b** time profiles of KD548-Fc production in culture supernatant. *Squares* and *circles* correspond to cultivation at 25 and 20 °C, respectively. All fermentations were performed at pH 5.6 and 9.5 g/l glycerol

When the fed-batch cultivation was undertaken at 20 °C, the cells exhibited a significantly longer lag phase growth (almost 75 h). The glycerol in the initial batch medium was depleted in 110 h, and then the methanol-containing feeding solution was supplied. The cells continued to grow up to OD_{600} = 285 with a specific growth rate (μ) of 0.055/h during the exponential growth phase; this was slower than those at higher temperatures (25 and 30 °C). At this temperature, a slightly higher production yield (533 mg/l) could be obtained than in the cultivations at higher temperatures (25 and 30 °C); however, the productivity (3.53 mg/l/h) was much lower compared with the results at the other temperatures due to the longer cultivation time.

Finally, fed-batch cultivation was performed at 25 °C and pH 4.0. At this pH condition, the lag phase (21 h) was significantly shorter than that of the cultivation at pH 5.6 and 25 °C; however, it was slightly longer than that of the cultivation at pH 5.6 and 30 °C (Fig. 5a). The cells could grow up to OD_{600} = 320 at 88 h, then the growth decreased. The cell specific growth rate during the exponential growth phase was 0.083/h. At this condition, the highest production yield (635 mg/l) could be obtained, and the productivity (7.2 mg/l/h) was also the highest (Table 1).

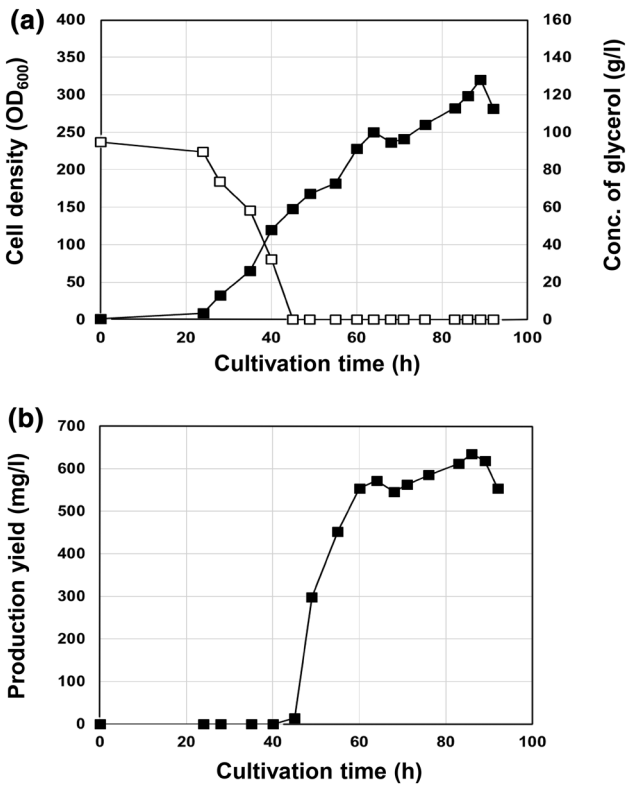

Fig. 5 Fed-batch cultivation at pH 4.0 and 25 °C with 9.5 % glycerol: **a** time profiles of cell growth (*filled squares*) and glycerol concentration (*open squares*), and **b** time profiles of production yield in culture

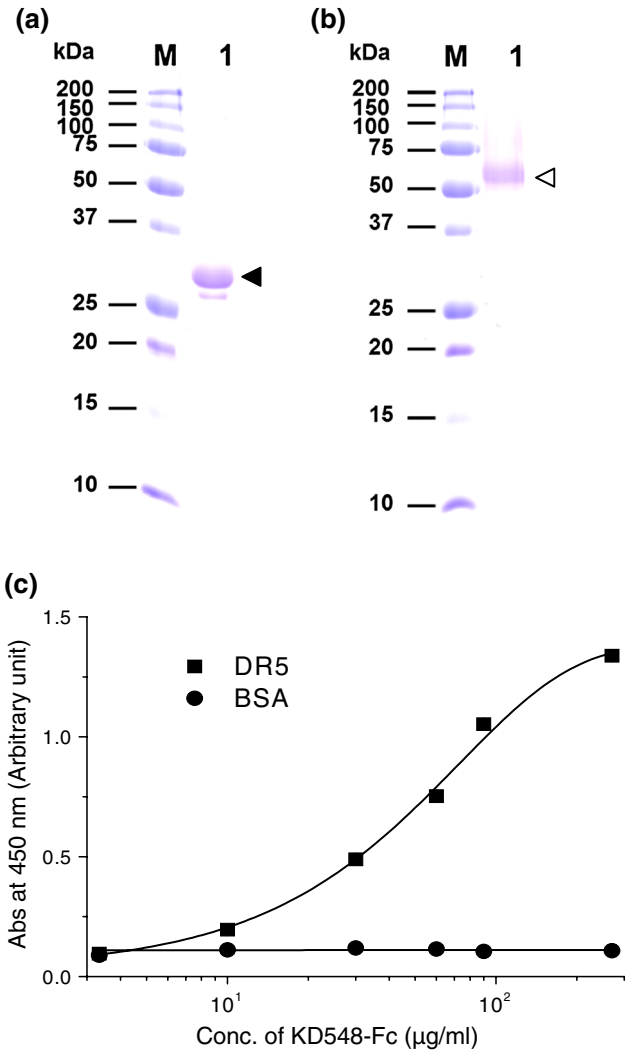

Fig. 6 SDS-PAGE analysis of KD548-Fc purification under (**a**) reducing and (**b**) nonreducing conditions. Lanes M and 1 represent protein size markers and the protein samples eluted from Protein A affinity column chromatography. *Closed* and *open arrowheads* indicate reduced and nonreduced KD548-Fc, respectively. **c** ELISA data of purified KD548-Fc. Bovine serum albumin (BSA)-coated wells were used as negative control (*filled square*, DR5; *filled circle*, BSA)

Purification of KD548-Fc

For purification, 100 ml of culture broth from the fed-batch fermentation under the optimal conditions (9.5 % glycerol, cultivation at 25 °C and pH 4.0) was used. After centrifugation, the supernatant was dialyzed against PBS, and the KD548-Fc was purified using Protein A affinity column chromatography as described in "Materials and methods" section. From 100 ml of culture broth, 10.4 mg of KD548-Fc could be purified with high purity (93 %) and recovery yield (approximately 40 %) (Fig. 6a). According to the SDS-PAGE analysis under the nonreducing condition, it was also confirmed that the purified KD548-Fc was present in dimeric form (Fig. 6b). The binding activity of the purified KD548-Fc against DR5 was analyzed using ELISA. It was clearly observed that the purified KD548-Fc could bind to DR5 with high specificity and affinity (Fig. 6c).

Conclusions

In this study, the fed-batch cultivation conditions were optimized for enhanced production of KD548-Fc. Under the optimized conditions (cultivation at pH 4.0 and 25 °C with 9.5 % glycerol in the initial medium), the maximum production yield of KD548-Fc was as high as 635 mg/l with high productivity (7.2 mg/l/h). In addition, functional KD548-Fc could be successfully purified using a simple purification procedure with high purity. These results clearly demonstrate the suitability of *P. pastoris* for large-scale production of functionally assembled Fc-fused kringle domain, having significant potential for application of kringle domain in immune diagnostics as well as disease treatment. To date, the production yield achieved in this work is the best result in the production of Fc-fused recombinant proteins in *P. pastoris*, and it is believed that the proposed strategy for optimization of fed-batch fermentation could be successfully used for production of Fc-fused proteins and other recombinant proteins in *P. pastoris*.

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References

1. Banta S, Dooley K, Shur O (2013) Replacing antibodies: engineering new binding proteins. Annu Rev Biomed Eng 15:93–113
2. Beck A, Wurch T, Bailly C, Corvaia N (2010) Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 10:345–352
3. Daly R, Hearn MT (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18:119–138
4. Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 93:31–39
5. Damasceno LM, Pla I, Chang HJ, Cohen L, Ritter G, Old LJ, Batt CA (2004) An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. Protein Expr Purif 37:18–26
6. Gasser B, Saloheimo M, Rinas U, Dragosits M, Rodriguez-Carmona E, Baumann K, Giuliani M, Parrilli E, Branduardi P, Lang C, Porro D, Ferrer P, Tutino ML, Mattanovich D, Villaverde A (2008) Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. Microb Cell Fact 7:11

7. Gebauer M, Skerra A (2009) Engineered protein scaffolds as next-generation antibody therapeutics. Curr Opin Chem Biol 13:245–255
8. Gurramkonda C, Adnan A, Gabel T, Lunsdorf H, Ross A, Nemani SK, Swaminathan S, Khanna N, Rinas U (2009) Simple high-cell density fed-batch technique for high-level recombinant protein production with *Pichia pastoris*: application to intracellular production of hepatitis B surface antigen. Microb Cell Fact 8:13
9. Hackel BJ, Huang D, Bubolz JC, Wang XX, Shusta EV (2006) Production of soluble and active transferrin receptor-targeting single-chain antibody using *Saccharomyces cerevisiae*. Pharm Res 23:790–797
10. Hao J, Xu L, He H, Du X, Jia L (2013) High-level expression of staphylococcal protein A in *Pichia pastoris* and purification and characterization of the recombinant protein. Protein Expr Purif 90:178–185
11. Inan M, Aryasomayajula D, Sinha J, Meagher MM (2006) Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. Biotechnol Bioeng 93:771–778
12. Inan M, Chiruvolu UV, Eskridge UKM, Vlasuk GP, Dickerson K, Brown S, Meagher M (1999) Optimization of temperature–glycerol–pH conditions for a fed-batch fermentation process for recombinant hookworm (*Ancylostoma caninum*) anticoagulant peptide (AcAP-5) production by *Pichia pastoris*. Enzyme Microb Technol 24:438–445
13. Jeong KJ, Jang SH, Velmurugan N (2011) Recombinant antibodies: engineering and production in yeast and bacterial hosts. Biotechnol J 6:16–27
14. Khow O, Suntrarachun S (2012) Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pac J Trop Biomed 2:159–162
15. Lee HW, Lee SH, Lee HW, Ryu YW, Kwon MH, Kim YS (2005) Homomeric and heteromeric interactions of the extracellular domains of death receptors and death decoy receptors. Biochem Biophys Res Commun 330:1205–1212
16. Lee CH, Park KJ, Sung ES, Kim A, Choi JD, Kim JS, Kim SH, Kwon MH, Kim YS (2010) Engineering of a human kringle domain into agonistic and antagonistic binding proteins functioning in vitro and in vivo. Proc Natl Acad Sci USA 107:9567–9571
17. Li Z, Xiong F, Lin Q, d'Anjou M, Daugulis AJ, Yang DS, Hew CL (2001) Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Expr Purif 21:438–445
18. Lin H, Kim T, Xiong F, Yang X (2007) Enhancing the production of Fc fusion protein in fed-batch fermentation of *Pichia pastoris* by design of experiments. Biotechnol Prog 23:621–625
19. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249–270
20. Park KJ, Lee CH, Kim A, Jeong KJ, Kim CH, Kim YS (2012) Death receptors 4 and 5 activate Nox1 NADPH oxidase through riboflavin kinase to induce reactive oxygen species-mediated apoptotic cell death. J Biol Chem 287:3313–3325
21. Potgieter TI, Cukan M, Drummond JE, Houston-Cummings NR, Jiang Y, Li F, Lynaugh H, Mallem M, McKelvey TW, Mitchell T, Nylen A, Rittenhour A, Stadheim TA, Zha D, d'Anjou M (2009) Production of monoclonal antibodies by glycoengineered *Pichia pastoris*. J Biotechnol 139:318–325
22. Shi X, Karkut T, Chamankhah M, Alting-Mees M, Hemmingsen SM, Hegedus D (2003) Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. Protein Expr Purif 28:321–330
23. Skerra A (2000) Engineered protein scaffolds for molecular recognition. J Mol Recognit 13:167–187
24. Skerra A (2007) Alternative non-antibody scaffolds for molecular recognition. Curr Opin Biotechnol 18:295–304
25. Ye J, Ly J, Watts K, Hsu A, Walker A, McLaughlin K, Berdichevsky M, Prinz B, Sean Kersey D, d'Anjou M, Pollard D, Potgieter T (2011) Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. Biotechnol Prog 27:1744–1750