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Properties of the *Hansenula polymorpha*-derived constitutive *GAP* promoter, assessed using an HSA reporter gene

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Abstract

The glyceraldehyde-3-phosphate dehydrogenase promoter, P_{GAP}, was employed to direct the constitutive expression of recombinant human serum albumin (HSA) in *Hansenula polymorpha*. A set of integration vectors containing the HSA cDNA under the control of P_{GAP} was constructed and the elemental parameters affecting the expression of HSA from P_{GAP} were analyzed. The presence of a 5′-untranslated region derived from the HSA cDNA and the integration of the expression vector into the *GAP* locus were shown to improve the expression of HSA under P_{GAP}. Glycerol supported a higher level of HSA expression from P_{GAP} along with a higher cell density than either glucose or methanol. The growth at high glycerol concentrations up to 12% did not cause any significant repression of the cell growth. A high cell density culture, up to 83 g l^{-1} dry cell weight with a HSA production of 550 mg l^{-1}, was obtained in less than 32 h of cultivation in a fed-batch fermentation employing intermittent feeding with 12% glycerol. The *GAP* promoter-based HSA expression system showed a higher specific production rate and required a much simpler fermentation process than the *MOX* promoter-based system, demonstrating that P_{GAP} can be a practical alternative of the *MOX* promoter in the large-scale production of HSA from *H. polymorpha*.

Keywords: Human serum albumin; Glyceraldehyde-3-phosphate dehydrogenase promoter; Constitutive expression; High cell density culture; *Hansenula polymorpha*

1. Introduction

Human serum albumin (HSA) is a major protein in blood, constituting 60% of the total protein content of human plasma [1]. The protein is used clinically to treat hypoalbuminemia or traumatic shock, and is responsible for the maintenance of colloid osmotic pressure within the blood vessels. HSA solution is also used as a replacement fluid (plasma expander and compensator for blood losses). Recently, HSA has also been applied as a fusion partner to increase the half-life and retard the clearance rates of target proteins [2,3]. Due to its wide applications, several attempts at producing large quantities of recombinant HSA have been made in a number of heterologous systems, including several yeast systems such as *Saccharomyces cerevisiae* [4,5], *Kluyveromyces lactis* [6,7], *Pichia pastoris* [8] and *Hansenula polymorpha* [9].

The methylotrophic yeasts *P. pastoris* and *H. polymorpha* have recently emerged as attractive host organisms for the production of heterologous proteins, especially due to their favorable fermentation properties for industrial process development. In these yeasts, foreign genes have been typically expressed under the control of promoters derived from methanol-inducible genes, such as alcohol oxidase 1 (*AOX1*) and methanol oxidase (*MOX*) [10]. These promoters are also repressed by glucose and ethanol. A major advantage of this tight regulation of promoter activity is that foreign genes, whose products are toxic to the cell, can be readily maintained within cells by culturing the yeasts under repressive growth conditions, thus preventing the obstruction of cell growth. However, the use of methanol to induce heterologous protein expression may be inappropriate for the production of certain food products and additives. In addition, there is a potential fire hazard with the large volumes of methanol required in large-scale

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fermentations. Moreover, the optimal production of recombinant proteins under the control of methanol-inducible promoters generally requires a complicated fermentation process and a prolonged culture time.

In a search for a strong constitutive promoter, which could be a practical alternative to the methanol-inducible promoters, the glyceraldehyde-3-phosphate dehydrogenase promoter was shown to be comparable in strength to the methanol-induced *AOX1* promoter in *P. pastoris* [11,12]. Since the genes coding for glyceraldehyde-3-phosphate dehydrogenase are expressed constitutively and at high levels in yeasts, the *GAP* promoters from *S. cerevisiae* and *Cryptococcus neoformans* have also been used for heterologous gene expression [13,14]. Here, we report the use of the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}) from *H. polymorpha* to direct a high-level production of recombinant HSA. We analyzed the HSA expression patterns from P_{GAP} and developed a simple fermentation process for a high cell density culture of *H. polymorpha* which is practically suitable for the large-scale production of recombinant HSA.

2. Materials and methods

2.1. Strains and plasmids

The yeast strain used in this study was *H. polymorpha* DL1-L (*leu2*), a derivative of DL-1 (ATCC 26012). The plasmids pGAP7123 and 23P2/pT7 contain the 4.5 kb *H. polymorpha GAP* gene fragment and 0.8 kb promoter, respectively [15]. pBHSA11 and pYIP-HSA contain the P_{MOX}–HSA expression cassettes [9], which were used as the sources of the HSA cDNA fused with the *MOX* terminator. The selection markers *LEU2* and *G418*^{r}, and the autonomous replication sequence (ARS) were obtained from pGLG61-135 [9] and pCTEL135 [15], respectively.

2.2. Media and culture conditions

Recombinant yeast strains were cultivated in 250 ml baffled shake flasks containing 25 ml of YP medium (1% (w/w) yeast extract and 2% (w/w) peptone) supplemented with 2% (w/w) glucose (YPD), glycerol (YPG) or methanol (YPM). The cultures were grown in a rotary shaker with vigorous shaking (200 rpm) at 37°C. The cell growth was measured as the optical cell density at a wavelength of 600 nm using a spectrophotometer (Spectronic 21D, Milton Roy, USA), where an OD of 1 was equivalent to 0.32 g l^{-1} of dry cell weight of *H. polymorpha*. The fermentations were carried out in a 5 l fermenter (Ko Biotech, Korea). The main culture, with a 2 l starting volume, was inoculated with 100 ml of pre-culture, grown to mid-exponential phase, in YPG medium. During fermentation the culture pH was adjusted to 6.0 with 2 N HCl solution and 30% (v/v) of ammonia solution. The temperature was maintained at 37°C and the level of dissolved oxygen controlled above 20% with air and pure oxygen. For the fed-batch fermentations, a concentrated glycerol solution (80%, w/v) supplemented with yeast extract at a 3:2 weight ratio, was fed intermittently, after the consumption of the initial glycerol in the medium, at the end of the batch phase.

2.3. Nucleic acid manipulation

General DNA manipulations were performed as described by Sambrook and Russell [16]. Yeast chromosomal DNA was prepared according to the method of Holm et al. [17], and the total RNA was isolated by the hot phenol extraction method of Elion and Warner [18]. Southern and Northern hybridizations were carried out as previously described [9]. The integration patterns and copy numbers of the transformants were determined by genomic Southern blot analysis using an image analysis system (GS-700, Bio-Rad, Hercules, CA, USA).

2.4. Construction of P_{GAP}–HSA expression cassettes I and II

To fuse the HSA cDNA devoid of its 5′-untranslated region (5′-UTR) directly to P_{GAP}, a polymerase chain reaction (PCR) was carried out in three steps. In the first step, the 0.8 kb P_{GAP} fragment containing the ATG initiation codon was amplified from pGAP7123 using the universal primer (5′-GTAAAACGAGGCCAGT-3′) and GAP-20-mer (5′-CATATTGTTTCTATATTATC-3′; the anticodon of the initiation codon is underlined). In the second step, the 247 bp fragment, corresponding to the N-terminal part of HSA cDNA, was amplified from pBHSA11 using two oligonucleotides, GAP/HSA-32-mer (5′-GATAATATAGAAACAATATGAAGTGGGTAACC-3′; the translation initiation codon of HSA cDNA is underlined) and HSA-*Afl*III 19-mer (5′-GCTGACTCATCAGCAACAC-3′). In the last step, these two PCR products, containing 20 bp complementary sequences at each end, were annealed and used as templates for the universal primer and GAP/HSA-32-mer to amplify the 1.1 kb DNA fragment of the *GAP* promoter fused with the N-terminal part of HSA cDNA. The final PCR product was inserted into the pT7Blue T-vector (Novagen, Madison, WI, USA), and then sequenced to check for any possible PCR error. The resulting plasmid, pT7Blue/P_{GAP}HSA247, was digested with *Bst*EII and *Kpn*I to generate the 0.8 kb DNA fragment containing the *GAP* promoter and the N-terminus of HSA cDNA, which replaced the 1.6 kb *Bst*EII/*Kpn*I fragment containing P_{MOX} and a part of the N-terminus of the HSA cDNA in the plasmid pBHSA11 [9]. The resultant plasmid, pBHSA16, contains the P_{GAP}–HSA expression cassette I, where the 3′-end of the *GAP* promoter was directly fused with the ATG start codon of the HSA cDNA. The P_{GAP}–HSA expression cas-

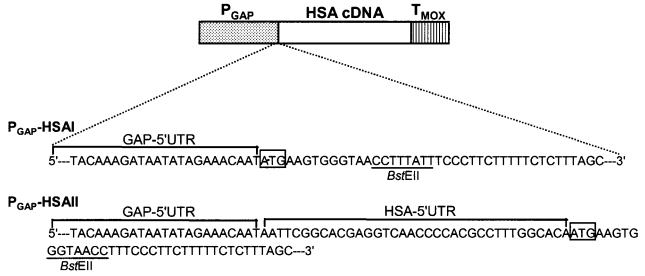

Fig. 1. P_{GAP}–HSA expression cassettes. Nucleotide sequences of the junction region, between the 3′-end of the *GAP* promoter and the 5′-end of HSA cDNA, in the expression cassettes are shown. The P_{GAP}–HSA cassette I was constructed with the HSA cDNA retaining its 5′-UTR. In the P_{GAP}–HSA cassette II, the HSA cDNA lacking its 5′-UTR was directly fused with the *GAP* promoter using a fusion PCR strategy.

sette II was constructed by replacing the 1.5 kb *Sal*I/*Eco*RI fragment of P_{MOX} in YIP-HSA [9] with the 0.8 kb *Sma*I/*Ssp*I fragment of P_{GAP} derived from the plasmid p23P2/pT7. The resultant plasmid, pYIP-HSA17, contains the expression cassette II where the HSA cDNA containing its 5′-UTR was connected with P_{GAP} (Fig. 1).

2.5. Construction of vectors containing P_{GAP}–HSA cassettes

The 3.3 kb DNA fragments, containing the P_{GAP}–HSA expression cassette I or the P_{GAP}–HSA expression cassette II, were inserted into the *Not*I site of pGLG61-135 [9], generating the integrative expression vectors pYHSA161 or pYHSA171, respectively (Fig. 2A and B). The vector pYHSA173 was derived from pYHSA171 by deleting the 1.5 kb *Eco*RI/*Sal*I fragment containing TEL135 and the *APH* gene (Fig. 2C). The vector pYHSA175 was constructed by replacing the 1.2 kb *Kpn*I/*Cla*I fragment containing the *HpLEU2* gene in pYHSA173 with the 1.6 kb *Kpn*I/*Cla*I fragment containing the defective *Hpleu2* gene [19] and TEL135, which was obtained from pCTEL135 (Fig. 2D).

2.6. Screening of H. polymorpha transformants expressing HSA

H. polymorpha was transformed according to the lith-

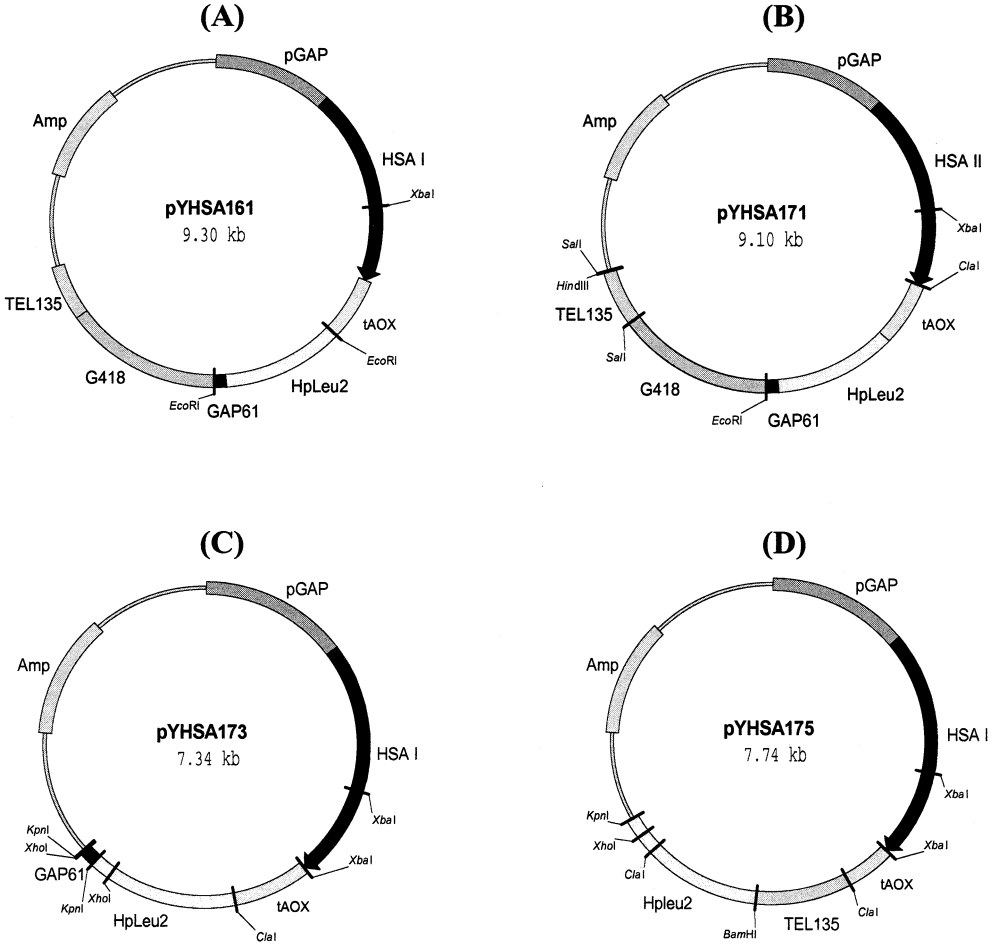

Fig. 2. Integrative vectors for the HSA expression in *H. polymorpha*. pYHSA161 (A) and pYHSA171 (B), containing the P_{GAP}–HSA cassettes I and II, respectively. Both vectors carry the telomeric TEL135 as an ARS, and *HpLEU2* and the G418^r cassette as selection markers [21]. The vector pYHSA173 (C) is identical to pYHSA171 with the exception of the lack of TEL135 and the G418^r selection marker. The vector pYHSA175 (D) is identical to pYHSA171 with the exception that it contains the defective *Hpleu2* gene [19] as a selectable marker instead of the *HpLEU2* and the G418^r cassette.

ium acetate and dimethyl sulfoxide (DMSO) method [20], and the obtained Leu$^+$ transformants harboring the pYHSA161 or pYHSA171 were stabilized as previously described [15]. For the expression of albumin, the transformants were first screened by colony immunoblotting. The Leu$^+$ transformants grown overnight on YPD agar were transferred onto nitrocellulose membranes, which were placed on fresh YPD agar plates for 1 day. The membranes were subsequently subjected to Western blot analysis as described previously [9].

2.7. Purification of recombinant HSA

The recombinant HSA, secreted into the culture supernatant of the fed-batch fermentation of *H. polymorpha*, was purified by the following steps. After heat treatment of the culture supernatant with 10 mM of 2-mercaptoethanol for 3 h at 60°C, the precipitants were filtered out using an ultrafiltration membrane (molecular-mass cut-off: 300 kDa, Amicon, USA). The permeate obtained was concentrated using an ultrafiltration membrane (molecular-mass cut-off: 30 kDa, Amicon, USA), and then subjected to hydrophobic interaction chromatography (HIC) using phenyl-Sepharose CL-4B resin (Pharmacia, Sweden) equilibrated with 50 mM phosphate buffer (pH 6.8). The recombinant HSA was eluted with a gradient increase of NaCl in the equilibration buffer at 5 ml min^{-1}, using an AKTA prime fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Sweden). The HIC-separated HSA was further purified by size exclusion chromatography using Sephadex G-75 resin (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 6.8). The pure recombinant HSA was eluted with a gradient increase of NaCl in the equilibration buffer at 0.08 ml min^{-1}. The high performance liquid chromatography (HPLC) profile of the purified recombinant HSA was compared with that of an authentic HSA (Sigma, USA) using TSK-gel G 3000 SWxL column (7.8×300 mm, Tosoh, Japan). The elution was carried out by supplying 50 mM of sodium phosphate buffer at a flow rate of 0.7 ml min^{-1} in the presence of 0.3 M NaCl, and was detected at a wavelength of 280 nm with an ultraviolet (UV) detector (Young-in, Korea). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 8% gels, according to the method of Sambrook and Russell [16], and the gels stained with Coomassie blue or silver to check the purity and quantification. For the N-terminal amino acid analysis of the recombinant HSA, the purified HSA was subjected to a Milligen/Biosearch M 6000 protein sequencer.

3. Results

3.1. Effects of 5′-UTR and copy number on the HSA expression from P_{GAP}

The configuration of the 5′-UTR and the copy number of the expression cassette are major factors for determining the expression efficiency of heterologous genes. To investigate the effect of the 5′-UTR on the HSA expression from P_{GAP}, we constructed the P_{GAP}–HSA expression cassettes I and II, which were identical except for the configuration of the 5′-UTR (Fig. 1). The P_{GAP}–HSA expression cassettes I and II were inserted into the vector pGLG61-135 carrying the *APH* gene, which confers the G418 resistance as a dominant selectable marker [9], resulting in the integrative expression vectors pYHSA161 and pYHSA171, respectively (Fig. 2A and B). The stabilized transformants harboring pYHSA161 and pYHSA171 were plated on synthetic medium, containing different concentrations of G418. Primary screening for the HSA expression of the transformants was carried out by colony immunoblotting of the individual transformants (data not shown).

The Southern blot analysis of the selected transformants of pYHSA161 revealed that the copy number of the inte-

Table 1
Effects of the 5′-UTR and the copy number of integrative vectors on the HSA expression from the *GAP* promoter in *H. polymorpha* transformants

Vector	HSA 5′-UTR	Copy number[a]	HSA	
			mRNA[b]	protein (mg l^{-1})[c]
pYHSA161	−	1	−	–
	−	2	−	–
	−	3	±	<1
	−	9	++	1–2
	−	18	++	1–2
	−	27	++	1–2
pYHSA171	+	1	+	30–40
	+	2	++	60–70
	+	6	++	60–70

[a]The copy number was determined by the genomic Southern blot analysis using an image analysis system as described previously [9].
[b]Levels of HSA transcript after 16 h cultivation were analyzed by Northern blot analysis and presented as the relative value compared to the level observed in the transformant harboring single copy of the pYHSA171.
[c]The levels of intact HSA protein in the culture supernatants after 20 h cultivation were analyzed by densitometric tracing of the 66 kDa HSA band in Western blot with the authentic HSA as a standard [4]. Transformants were cultivated in a 250 ml baffled flask containing 25 ml YPD medium.

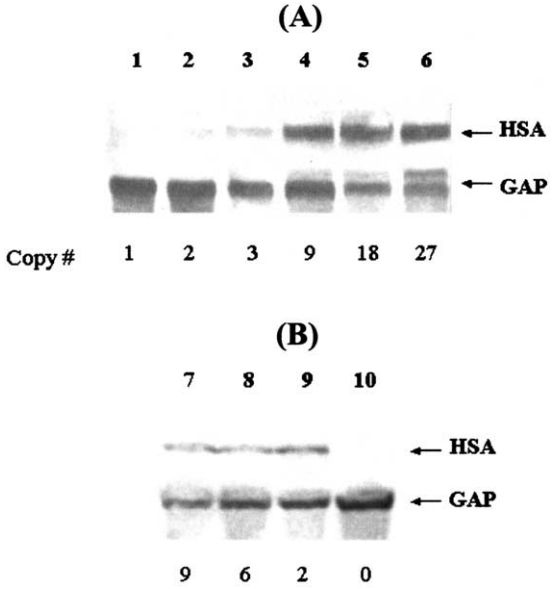

Fig. 3. Northern blot analysis of *H. polymorpha* transformants. Total RNAs were obtained from the transformants grown in the YPG medium for 16 h. Equal amounts of RNA (10 μg) were fractionated on 1.2% formaldehyde gels, and blotted to nylon membranes. The blot was hybridized with two probes made of the 1.8 kb *Bst*EII/*Hin*dIII DNA fragment of HSA cDNA and the 0.6 kb *Sca*I/*Eco*RI DNA fragment of the *GAP* structural region [9]. Colorimetric detection of the membranes was carried out with the reaction of anti-digoxigenin alkaline phosphatase. A: RNAs from the transformants harboring pYHSA161 in various copy numbers, from 1 to 27 (lanes 1–6). B: RNAs from the transformants harboring pYHSA171 from two copies (lane 9) and six copies (lane 8). Lane 7 contains the RNA sample from the transformant harboring nine copies of pYHSA161, in order to compare the signal intensities. Lane 10 contains RNA from the untransformed strain.

grated vector was proportionally increased, up to 27 copies, with increasing G418 concentration in the selection medium. In contrast, the highest integration copy number was just six in the transformants of pYHSA171 (Table 1). In the shake flask cultivation using YPD medium, the integrants of pYHSA161 showed extremely low levels of HSA expression below 2 mg l^{-1}, although the expression level was steadily enhanced with the increase of the copy number. In contrast, the integrants of pYHSA171 displayed a much higher level of HSA expression around 60 mg l^{-1}, which reached a plateau after two copies of the expression vector. These results suggested that the removal of the HSA 5′-UTR from the expression cassette negatively affected the HSA expression from P_{GAP}. These analyses also indicated that the effect of gene dosage, profoundly observed in the transformants containing the P_{GAP}–HSA cassette I, became obscure in the transformants containing the P_{GAP}–HSA cassette II.

To investigate the reasons for the extremely poor expression from the P_{GAP}–HSA cassette I, and the weak gene dosage effect in the P_{GAP}–HSA cassette II, Northern blot analysis of the obtained transformants was performed (Fig. 3). In the case of integrants harboring pYHSA161, the level of the HSA transcript from the P_{GAP}–HSA cassette I was extremely low in single- or two-copy integrants, although significantly higher levels were detected in the multiple integrants. Interestingly, in integrants bearing more than nine copies, the level of the HSA transcript increased no further, and the level of the *GAP* transcript from the chromosomal gene began to decrease. This suggests competition between the *GAP* promoters from the chromosomal gene and the transforming vector for some transacting factors that probably became limited in the presence of more than nine copies of P_{GAP}. Similarly, a remarkable decrease in the amount of *GAP* mRNA from the chromosomal gene was previously observed in the recombinant *S. cerevisiae* strain carrying the P_{GAP}-driven HBsAg construct [13]. In contrast to the integrants of pYHSA161, the transcript level of HSA from the integrants containing just two copies of pYHSA171 was high enough to be comparable to the maximal levels detected in the multiple integrants of pYHSA161. This indicates that the presence of the HSA 5′-UTR was required for the stability of the HSA mRNA expressed from P_{GAP}. Furthermore, the expression levels of the HSA protein in the multiple integrants of pYHSA161 were extremely low, despite the similar levels of HSA mRNA, compared to those of the integrants of pYHSA171. This suggests that the configuration of the 5′-UTR affected not only the transcript stability, but also the translation efficiency of the HSA mRNA expression from P_{GAP}.

3.2. Effect of integration site on the HSA expression

The integration site of the expression cassette is one of the important factors to be considered in optimizing expression systems for the production of recombinant proteins. The Southern blot analysis indicated that the vectors pYHSA161 and pYHSA171 were integrated mostly into the telomere locus, which was as expected from the presence of the telomeric ARS TEL135 [21]. To avoid the possible telomere positional effect on the expression of HSA [9], the targeted integrations of the P_{GAP}–HSA expression cassette I into the *GAP* and *LEU* sites were car-

Table 2
Effect of the integration site on the HSA expression from the *GAP* promoter in *H. polymorpha* transformants

Vector	Copy number[a]	Integration locus[a]	HSA productivity (mg l^{-1})[b]
pYHSA171	1	tel	30–40
pYHSA173	1	GAP	40–50
	2	GAP	80–90
	3	GAP	85–90
pYHSA175	1	LEU2	20–30
	2	LEU2	40–50

[a]The copy number and integration site were determined by the genomic Southern blot analysis.
[b]The levels of intact HSA protein in the culture supernatants after 20 h cultivation were analyzed by densitometric tracing of the 66 kDa HSA band in Western blot with the authentic HSA as a standard.

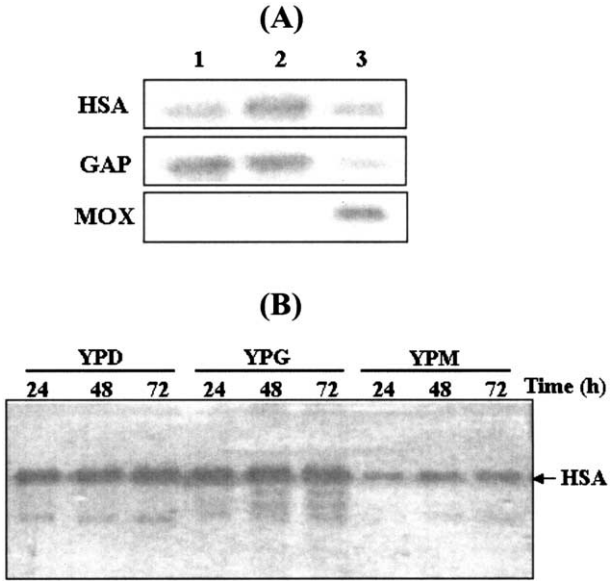

Fig. 4. HSA expression from the *GAP* promoter using different carbon sources. A: Northern blot analysis of the HSA transcript. The *H. polymorpha* transformant harboring a single copy of pYHSA173 integrated into the *GAP* locus was cultivated in YP medium, containing 2% dextrose (lane 1), 2% glycerol (lane 2) or 2% methanol (lane 3). The total RNA was obtained from cells in the exponential phase, and equal amounts of RNA (10 μg) were fractionated on 1.2% formaldehyde gels. B: Western blot analysis of the HSA protein. The *H. polymorpha* transformant was cultured for 72 h in YP medium, containing 2% dextrose (YPD), 2% glycerol (YPG) or 2% methanol (YPM), and the culture supernatants (3 μl) obtained at the indicated times were analyzed on SDS–polyacrylamide gels.

ried out using the integration vectors pYHSA173 (Fig. 2C) and pYHSA175 (Fig. 2D), respectively. To facilitate the homologous recombination at the *GAP* locus, pYHSA173 was linearized by digestion at the *Hpa*I site in the *GAP* promoter prior to its introduction into the host. In the case of pYHSA175, containing the defective *Hpleu2* marker, the integrants at the *LEU2* locus were selected from the transformants with a fast-growing phenotype on the selective medium, as a homologous single-copy integration into the chromosomal *LEU2* locus leads to restoration of the function of the *HpLEU2* gene [19]. The correct integration into the *GAP* or *LEU2* sites was confirmed by Southern blot analysis (data not shown). Comparison of the HSA levels in the culture supernatants of the selected integrants revealed that the integrants at the *GAP* locus displayed 150–200% higher levels of HSA expression than the integrants at other sites, those of the *LEU* and *TEL* loci (Table 2). This result implies that the *GAP* locus itself is the optimum site for the maximal performance of the *GAP* promoter used in the expression cassette.

3.3. *HSA expression from* P_{GAP} *in different carbon sources*

To investigate the effect of carbon sources on the expression of the HSA from P_{GAP}, the levels of mRNA and protein were measured in the transformants harboring a single copy of the pYHSA173 integrated at the *GAP* locus. The total RNA for the Northern blot analysis was prepared from exponentially growing cells ($OD_{600} = 0.5$), which were cultured in YP media containing 2% glucose (YPD), 2% glycerol (YPG) or 2% methanol (YPM). The steady-state levels of the HSA and *GAP* transcripts expressed under the control of P_{GAP} were significantly higher in the glycerol-grown cells, and lower in the methanol-grown cells (Fig. 4A), suggesting that the expression level of P_{GAP} varied depending on the carbon source used. The Western blot analysis also showed that the amount of secreted HSA was highest in the YPG medium, which was about 150 and 300% higher than those in the YPD and YPM media, respectively (Fig. 4B), although the higher final cell density in the YPG medium compared to the other media could partly contribute to the increased level of HSA that had accumulated in the culture supernatants. Therefore, glycerol seems to be an optimal carbon source both for the cell growth and the expression of HSA from P_{GAP} of *H. polymorpha*.

3.4. *Development of fermentation strategy for the HSA production from* P_{GAP}

To see how the activity of the *GAP* promoter was affected by the growth phase, the levels of the *GAP* and HSA transcripts were analyzed during the batch cultivation. It was observed that the activity of the *GAP* promoter was constitutive, but diminished significantly as the yeast cells entered the stationary phase (data not shown). This result suggested that maintaining an active growth phase during the fermentation could be one of the best strategies for the production of HSA from P_{GAP}. The

Table 3
The effect of initial glycerol concentration on cell growth, ethanol formation, and HSA production in the batch fermentation of *H. polymorpha* transformant

Conc. of glycerol (%)	OD_{600}	Glycerol (%)[a]	Ethanol (%)[a]	Specific growth rate (h^{-1})	HSA (mg l^{-1})[b]
3	114	0	0.022	0.44	70
6	144	0	0.039	0.39	150
12	180	0	0.03	0.36	200

[a]Residual concentrations of each substance in the culture supernatant were determined after 72 h batch fermentation.
[b]The levels of intact HSA in the culture supernatants after 72 h cultivation were estimated by densitometric scans of silver-stained polyacrylamide gels with the authentic HSA as a standard as described previously [9].

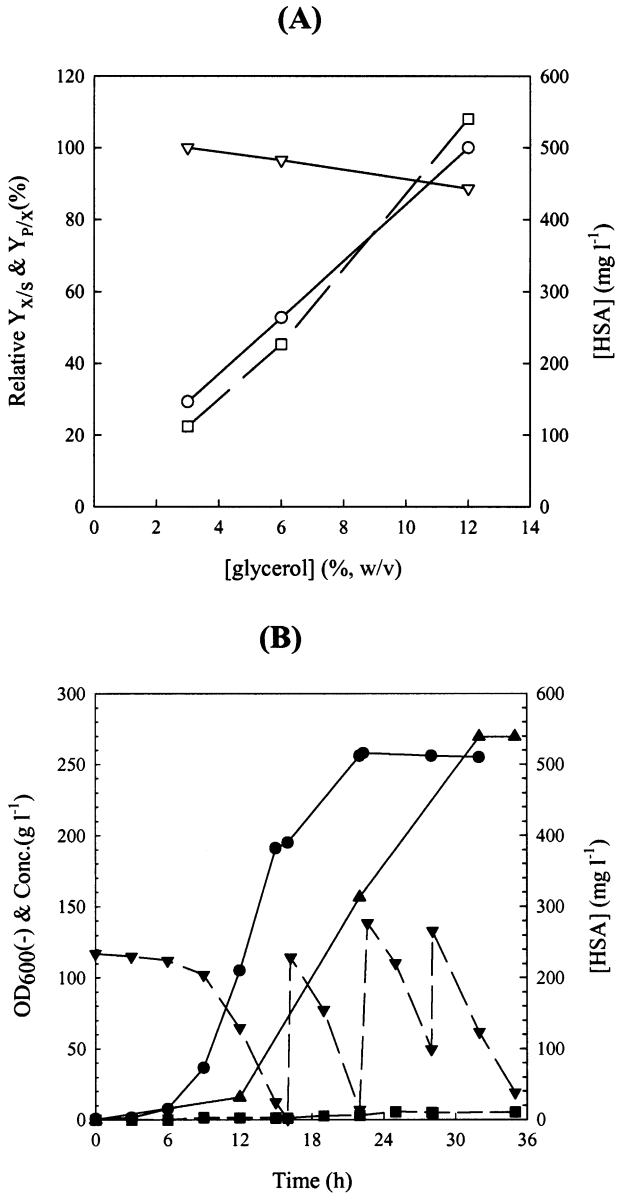

Fig. 5. Fed-batch fermentations of the recombinant *H. polymorpha* with intermittent glycerol feeding. The recombinant *H. polymorpha* harboring two copies of pYHSA173 was cultivated in 2 l YP medium, containing 3, 6 or 12% (w/v) glycerol. The culture was intermittently fed with the concentrated feeding medium containing 80% glycerol, supplemented with yeast extract (3:2 weight ratio of glycerol and yeast extract), resulting in a final glycerol concentration of 3, 6 or 12%. A: Final yields of cell mass and HSA production in the fed-batch fermentations at different glycerol feeding concentrations. ○, $Y_{P/X}$; ▽, $Y_{X/S}$; and □, HSA concentration. B: A fed-batch fermentation profile with 12% glycerol feeding. ●, cell density; ▼, glycerol concentration; ■, ethanol concentration; and ▲, HSA concentration.

use of a high concentration of glycerol has been proposed as a convenient approach, not only to maintain active growth phase for a longer period, but also to develop a simple fermentation process for a high cell density cultivation in other yeasts such as *P. pastoris* and *Yarrowia lipolytica* [22,23]. Thus, the effects of the glycerol concentration were examined on the cell growth and HSA expression from P_{GAP} in the batch fermentations of *H. polymorpha*. When the *H. polymorpha* transformant of pYHSA173 was cultured in YPG medium with initial glycerol concentrations of 3, 6 or 12% (w/v), the recombinant strain showed similar specific growth rates regardless of glycerol concentration. Even at the high level of 12% glycerol, no residual glycerol concentration was observed after 72 h of cultivation, and the final ethanol concentration accumulated in the culture medium was only at a trace level. Moreover, the expression of HSA appeared to continue longer in the medium containing higher concentrations of glycerol, leading to higher levels of HSA accumulation in the culture supernatants (Table 3).

In the batch fermentations, a high concentration of glycerol, up to 12%, supported cell growth efficiently with the sustained expression of HSA, suggesting the feasibility of a feeding strategy of highly concentrated glycerol in fed-batch fermentations. To develop a simplified fed-batch fermentation process for the P_{GAP}–HSA system, fed-batch fermentations were carried out with the intermittent feeding of a medium containing 80% glycerol, which resulted in a final concentration of 3, 6 and 12% glycerol. As shown in Fig. 5A, the fed-batch fermentation with the intermittent feeding of 12% glycerol resulted in the highest HSA production. The recombinant *H. polymorpha* was cultivated up to 83 g l^{-1} dry cell weight, and over 500 mg l^{-1} of recombinant HSA was obtained in less than 32 h of cultivation (Fig. 5B). Compared to the previous HSA production system based on the *MOX* promoter [9], the P_{GAP}–HSA system showed a higher specific production rate and a much simpler fermentation process (Table 4). Although the maximal level of HSA obtained in the P_{MOX}-based system exceeded the level produced by the P_{GAP} system, the time taken to reach peak product concentration was comparatively much longer with the P_{MOX}–HSA system. Moreover, quite a delicate feeding procedure was required to achieve a high level of induction from the *MOX* promoter. Taken together, the results obtained in this study demonstrate that P_{GAP} could be a practical alternative of P_{MOX} in directing the high-level expression of recombinant proteins in *H. polymorpha*.

3.5. *Analysis of the purified recombinant HSA expressed from* P_{GAP}

Most of the recombinant HSA expressed from P_{GAP} was secreted extracellularly, and less than 10% of the total HSA expressed was detected in the intracellular fraction of the recombinant *H. polymorpha* (data not shown). The intact 66 kDa HSA was secreted as the predominant protein species in the culture supernatant of the fed-batch cultivation (Fig. 6A, lane 4), although some HSA degradation products were also generated in the P_{GAP}–HSA system, as previously observed in the P_{MOX}–HSA system [9]. The recombinant HSA in the culture supernatant was purified to homogeneity through a series of purification

steps, including heat treatment (lane 5), HIC (lane 7) and size exclusion chromatography (lane 8). The 45 kDa degradation product of the HSA was efficiently removed after HIC using a phenyl-Sepharose column. The analytical HPLC chromatogram of the purified HSA showed only a single sharp peak, with an identical retention time to that of authentic HSA (Fig. 6B). The purity of the purified HSA protein was >98%, as judged by peak quantitation from the analytical HPLC. The purified protein was subjected to N-terminal amino acid sequencing which confirmed that the first 22 amino acids, DAHKSEVAHRFKDLGEENFKA, were identical to those of authentic HSA [1]. These results demonstrated that the inherent leader sequence of the HSA was correctly processed, and the recombinant HSA expressed from the *GAP* promoter was not subjected to any aberrant post-translational modification, such as *N*- or *O*-linked glycosylation, during the secretion process from *H. polymorpha*.

4. Discussion

One of the major considerations in developing the large-scale production of recombinant proteins is to set up cost-effective, simple fermentation processes. In the expression system using a methanol-inducible *MOX* promoter, the relatively long fermentation times and complicated feeding procedures are the major limitations for its industrial applications. Moreover, methanol is an explosion hazard in large-scale processes, as well as a toxic compound. As a practical alternative for the P_{MOX}-based recombinant HSA production system, a P_{GAP}-based recombinant HSA production system has been developed. During the course of optimizing the HSA expression cassette, removal of the HSA 5′-UTR in the P_{GAP}–HSA expression cassette was observed to cause remarkable instability and an extremely poor translational efficiency of the HSA transcript, leading to low levels of HSA expression from the *GAP* promoter. This is in contrast to the previous observation where the removal of the HSA 5′-UTR in the P_{MOX}–HSA expression cassette improved the expression efficiency by about 5-fold at the translational level [9], suggesting that the effect of a 5′-UTR configuration on expression level is specific to each expression cassette. Nevertheless, as in the case of the P_{MOX}–HSA cassette, the gene dosage effect on the HSA expression became much weaker after optimizing the P_{GAP}–HSA cassette. Thus, the HSA expression reached a maximum level with the two-copy integration of the P_{GAP}–HSA expression cassette.

The *GAP* promoter is known as one of the strongest constitutive promoters in yeasts. However, the constitutive P_{GAP} of *P. pastoris* has been shown to vary significantly, depending on the carbon source used for cell growth [11,12]. Similarly, the carbon source- and phase-dependent activity of the *S. cerevisiae GAP* promoter has also been reported [24,25]. In this study, the activity of the *H. polymorpha* P_{GAP} was observed to vary depending on the carbon source and growth phase. These data emphasize the importance of studying the promoter activity in the context of the cell physiology, in order to develop efficient fermentation processes. It was notable that *H. polymorpha* showed growth without any apparent growth retardation, ethanol accumulation and inhibition of the *GAP* promoter activity, even at high concentrations of glycerol up to 12%. This allowed us to develop a simple intermittent feeding strategy, with concentrated glycerol, to achieve a high cell density of the *H. polymorpha*. The easy cultivation of recombinant *H. polymorpha* to a high cell density with a comparable yield to that from the *MOX* promoter surely strengthens the potential of P_{GAP} as a practical alternative to P_{MOX}. The P_{GAP}–HSA system also shows a higher yield, and a much simpler fermentation process, compared to the other previously reported HSA system, which was based on the constitutive *PMA1* promoter [26], where the high cell density fermentation was carried out with the controlled feeding of carbon source to keep carbon at growth-limiting concentrations for a set nominal growth rate.

Furthermore, the *GAP* promoter-driven constitutive expression of HSA can be easily adapted to a continuous culture strategy, which permits the cells to be maintained in the exponential phase, thus sustaining the maximum activity of P_{GAP}. Recently, the feasibility of the *GAP* ex-

Table 4
Comparison of the *MOX* and *GAP* promoter-based HSA production systems

Promoter	Fed-batch mode	HSA yield[b] (g l^{-1})	Cell density[c] (DCW-g l^{-1})	Culture time (h)	Specific HSA production rate[d] (mg l^{-1}·h^{-1}·(DCW-g l^{-1})$^{-1}$)
MOX[a]	gradual feeding change from glycerol to methanol	1.3	81	96	0.17
GAP	intermittent feeding of glycerol	0.55	83	32	0.21

[a]Values on the P_{MOX}–HSA production system were obtained from the previous study [9].
[b]The levels of intact HSA in the culture supernatants were estimated by densitometric scans of 66 kDa HSA band in SDS–PAGE with the authentic HSA as a standard.
[c]Dry cell weight (DCW) was calculated by converting the value of optical cell density (OD) at 600 nm, in which OD 1 was equivalent to 0.32 g l^{-1} of dry cell weight of *H. polymorpha*. Cell density and HSA level were the values at the fermentation time to achieve the maximum level of HSA.
[d]Specific HSA production rate was the average rate calculated for the fermentation time, which was defined as the period required to reach the maximum expression level of HSA.

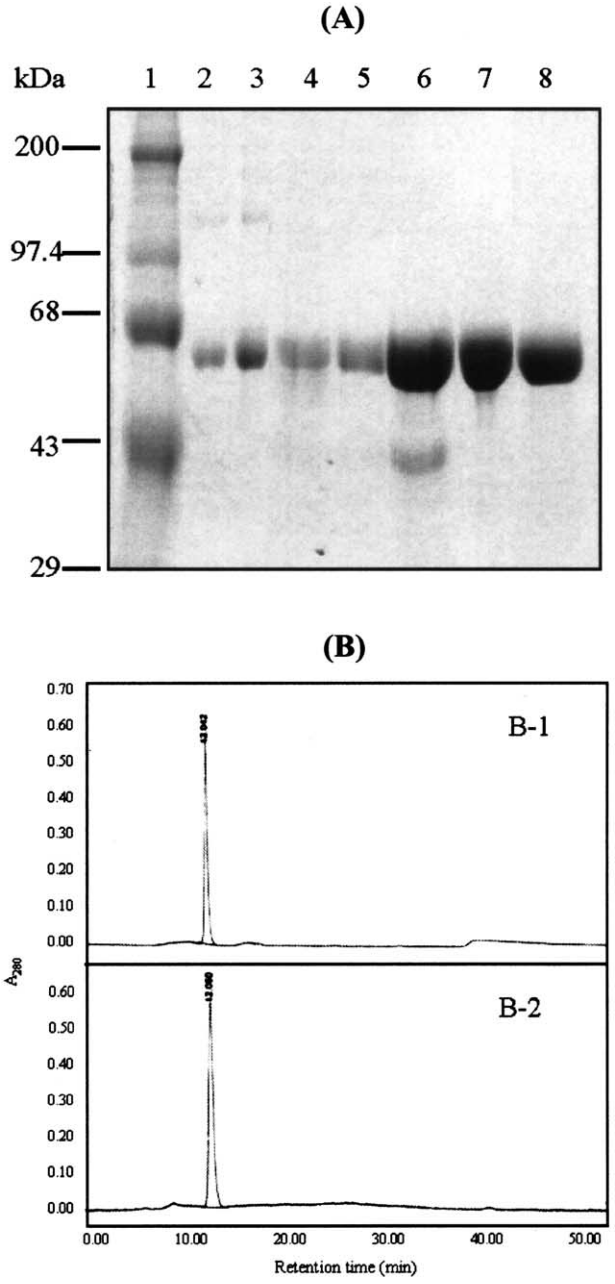

Fig. 6. Analysis of the purified recombinant HSA. A: SDS–PAGE. Lane 1, molecular-mass standard marker; lanes 2 and 3, authentic HSA (500 ng and 1000 ng, respectively); and lane 4, the supernatant of the fed-batch culture. Lanes 5–8, samples after heat treatment (lane 5), ultrafiltration (molecular-mass cut-off: 30 kDa) (lane 6), phenyl-Sepharose column (lane 7), and Sephadex G-75 column (lane 8). B: Elution profiles of the purified recombinant (B-1) and authentic HSA (B-2) on gel permeable HPLC.

pression system to a continuous culture strategy has been demonstrated in *P. pastoris* systems for the production of the recombinant human chitinase [26] and the hepatitis B surface antigen [27]. This feature of the *GAP* expression system may extend the versatility of the *H. polymorpha* system, and will contribute significantly to the development of cost-effective methods for the large-scale production of recombinant therapeutic proteins.

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