J Ind Microbiol Biotechnol (2005) 32: 402–408
DOI 10.1007/s10295-005-0010-2

ORIGINAL PAPER

Yu-Jin Kim · You-Kwan Oh · Whankoo Kang
Eun Yeol Lee · Sunghoon Park

Production of human caseinomacropeptide in recombinant *Saccharomyces cerevisiae* and *Pichia pastoris*

Received: 31 January 2005 / Accepted: 14 June 2005 / Published online: 3 August 2005

Abstract Caseinomacropeptide is a polypeptide of 64 amino acid residues (106–169) derived from the C-terminal part of the mammalian milk k-casein. This macropeptide has various biological activities and is used as a functional food ingredient as well as a pharmaceutical compound. The gene encoding the human caseinomacropeptide (hCMP) was synthesized and expressed with an α-factor secretion signal in the two yeast strains, *Saccharomyces cerevisiae* and *Pichia pastoris*. The complete polypeptide of the recombinant hCMP was produced and secreted in a culture medium by both the strains, but the highest production was observed in *S. cerevisiae* with a galactose-inducible promoter. In a fed-batch bioreactor culture, 2.5 g/l of the recombinant hCMP was obtained from the *S. cerevisiae* at 97 h.

Keywords Caseinomacropeptide · Recombinant yeast · Secretion · Promoter · Fed-batch

Y.-J. Kim · Y.-K. Oh · S. Park (✉)
Department of Chemical Engineering and Institute for Environmental Technology and Industry, Pusan National University, 30 Jangjeon-dong, Geumjeong-gu, Busan, 609-735 Republic of Korea
E-mail: parksh@pusan.ac.kr
Tel.: +82-51-5102395
Fax: +82-51-5128563

W. Kang
Department of Chemical Engineering and Nano-Bio Technology, Hannam University, 133 Ojeong-dong, Daedeok-gu, Daejeon, 306-791 Republic of Korea

E. Y. Lee
Department of Food Science and Technology, Kyungsung University, 110-1 Daeyeon-dong, Nam-gu, Busan, 608-736 Republic of Korea

Introduction

Caseinomacropeptide (CMP) is a glycopeptide of 64 amino acid residues (106–169) derived from the C-terminal part of the mammalian milk k-casein. It is released by the action of chymosin during the primary phase of milk clotting. CMP is known to have various biological functions such as the growth promotion of beneficial bacteria including *bifidobacteria*, the regulation of food intake, the depression of platelet aggregation, and the inhibition of the adhesion of oral *actinomyces* to cell membranes and of the binding of *cholera* toxin to its receptors [1]. At present, bovine CMP (bCMP) is commercially produced from cow milk and mainly used in the food industry, mostly as a component of infant formula [2]. It has been suggested that the human CMP (hCMP) would be more desirable than bCMP because of its human origin. Nevertheless, the massive harvesting of human mother's milk for the production of hCMP is practically impossible, and hCMP is still not commercially available.

One way to produce hCMP in a large quantity is to use a recombinant microorganism containing the gene encoding hCMP. Up to now, the genes of various milk proteins such as β-lactoglobulin [3], β-casein [4], and lactoferrin [5] have been cloned and expressed in recombinant bacteria or yeasts. The complete amino acid sequence and the primary structure of hCMP have also been determined [6], but its production by a recombinant strain has not yet been attempted. Traditional baker's yeast *Saccharomyces cerevisiae* or methylotrophic yeast *Pichia pastoris* has been used most widely in producing recombinant milk proteins. They are nonpathogenic, can secret protein products into culture media and perform the post-translational modification that is essential for the biological activity of many eukaryotic proteins. *P. pastoris* has several other advantages such as the availability of strong and tightly regulated promoters, and the easy integration of heterologous DNA into the host chromosomal DNA [7–9].

In this study, the production of recombinant hCMP was investigated in two yeast strains, *S. cerevisiae* and *P. pastoris*. The gene for hCMP was chemically synthesized, amplified by PCR and cloned into expression vectors. One constitutive promoter (*GAP*) and two inducible promoters (*GAL*, inducible by galactose and *AOX*1, inducible by methanol), were used and compared for their efficiency in flask cultures. Bioreactor experiments were also conducted with *S. cerevisiae* containing a *GAL*-inducible system, which exhibited the best result in the flask experiments.

Materials and methods

Strains and plasmids

Table 1 summarizes the host strains and plasmids used in this study. *Escherichia coli* XL1-Blue (Stratagene, USA) was used for cloning and propagating genes. Host strains for recombinant hCMP were *S. cerevisiae* 2805 (*his*$^-$, *ura*$^-$) and *P. pastoris* GS115 (*his*$^-$). For *S. cerevisiae*, pYIGP (containing a constitutive *GAP* promoter) or pYEGα (containing a galactose-inducible *GAL* promoter) was used. For *P. pastoris*, pGAPZαA (containing a constitutive *GAP* promoter) or pPICZαA (containing a methanol-inducible *AOX* 1 promoter) was used.

Construction of hCMP expression plasmids

General DNA manipulations were performed following standard techniques [10]. The hCMP gene was chemically synthesized based on the amino acid sequence [6] and Genbank database (Accession No. M73628). Table 2 shows the primer sequences for the PCR amplification of the hCMP gene with the restriction sites included for further gene manipulations. The gene for the α-factor secretion signal sequence was placed before the hCMP structural gene so that the signal peptide was fused to the 5′ end of the hCMP and that the recombinant hCMP was secreted in the culture medium. In addition, the Kozak sequence was inserted into the position preceding the ATG initiation codon to facilitate an efficient translation in yeast [11]. For the construction of the inducible plasmid with pYEGα for *S. cerevisiae*, the hCMP gene was amplified using the primers hCMPECOαF (forward) and hCMPSALR (reverse), and digested with *EcoR* I and *Sal* I; whereas, for pYIGP, the gene was amplified using the primers hCMPECOαF (forward) and hCMPXBAR (reverse), and digested with *EcoR* I and *Xba* I. The resulting expression vectors were designated pYEGα-hCMP (inducible) and pYIGP-hCMP (constitutive), respectively. For *P. pastoris*, the hCMP gene was amplified by using hCMPECOF (forward) and hCMPKPNR (reverse), and digested with *EcoR* I and *Kpn* I for both pGAPZαA (constitutive) and pPICZαA (inducible). The resulting expression vectors were named pGAPZαA-hCMP (constitutive) and pPICZαA–hCMP (inducible).

Yeast transformation

The expression vectors amplified from *E. coli* XL1-Blue were transformed into yeast cells by electroporation (Gene Pluser, Bio-Rad, USA). The transformants of *S. cerevisiae* were selected and maintained in a minimal selective medium containing 0.67% yeast nitrogen base (without amino acid), 0.02% histidine, and 2% glucose. For *P. pastoris*, the plasmids were linearized before electroporation by digestion with *Bgl* II, which cut both pPICZαA and pGAPZαA vectors in front of the promoters. The linear pPICZαA-hCMP and pGAPZα-hCMP were integrated into the *AOX* 1 and *GAP* loci of the *P. pastoris* GS115 chromosome by a single crossover event. The transformants of *P. pastoris* were selected on YPD plates (1% yeast extract, 2% peptone, 2% glucose, and 1.5% agar) containing zeocin (100 μg/ml).

Media and culture conditions

Escherichia coli XL1-Blue harboring an expression vector was grown in a Luria–Bertani (LB) medium containing ampicillin (100 μg/ml) or zeocin (100 μg/ml) at

Table 1 *Escherichia coli*, yeast strains and plasmids used in this study

Strain or plasmid	Characteristics [a]	Reference or source
E. coli XL1-Blue	*recA endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relAlac*[F' *proAB lacI*^{q} *Z*(Δ) *M*15 *Tn*10 (*Tet*^{r})	Stratagene
S. cerevisiae 2805	*MAT*α *pep4::HIS3 pro1*-δ *can1 GAL2 his3* δ *ura3–52*	[14]
P. pastoris GS115	his4	Invitrogen
pYEGα	Inducible, *GAL* 10 promoter, *URA 3* marker, Ap^r	[17]
PYIGP	Constitutive, *GAP* promoter, *URA 3* marker, Ap^r	[17]
pPICZαA	Inducible, *AOX* 1 promoter, Zeo^r	Invitrogen
pGAPZαA	Constitutive, *GAP* promoter, Zeo^r	Invitrogen
pYEGα-hCMP	Inducible, *GAL* 10 promoter, *URA 3* marker, Ap^r	This study
pYIGP-hCMP	Constitutive, *GAP* promoter, *URA 3* marker, Ap^r	This study
pPICZαA-hCMP	Inducible, *AOX* 1 promoter, Zeo^r	This study
pGAPZαA-hCMP	Constitutive, *GAP* promoter, Zeo^r	This study

[a] *Tet*^{r} tetracycline resistance; *Ap*^{r} ampicillin resistance; *Zeo*^{r} Zeocin resistance

Table 2 Oligonucleopeptide primers used in this study for hCMP amplification

Yeast	Designation	Sequence	Specificity
S. cerevisiae	hCMPECOαF	5′ AATGAATTC **ACG** ATG AGA TTT CCT TCA 3′	pYEGα, pYIGP
	hCMPSALR	5′ AATGTCGAC TTA TGC CGT AGG TGG AGT AAC 3′	pYEGα
	hCMPXBAR	5′ AATTCTATG TTA TGC CGT AGG TGG AGT AAC 3′	PYIGP
P. pastoris	hCMPECOF	5′-AATGAATTC ATT GCC ATC CCC CCA AAG AAA AAT-3′	pPICZαA, pGAPZαA
	hCMPKPNR	5′-AATGCTACC TTA TGC CGT AGG TGG AGT AAC-3′	

Restriction sites underlined and Kozak sequence boldfaced

37°C. The *S. cerevisiae* cells with the constitutive plasmid were grown in a YPD medium, whereas the cells with the galactose-inducible plasmid were in a medium containing 2% yeast extract, 2% glucose, and 3% galactose. The recombinant *P. pastoris* cells with *AOX* 1 integration were cultivated in a YPG medium (1% yeast extract, 2% peptone, and 1% glycerol) and induced with methanol in the late exponential phase. Methanol was added intermittently up to 0.8% concentration (v/v) during the induction period. The *P. pastoris* cells with the constitutive plasmid were cultivated in a YPDG medium (1% yeast extract, 2% peptone, 2% glucose, and 1% glycerol).

Flask cultures were conducted at 30°C in 500 ml baffled flasks with a liquid working volume of 100 ml. The agitation speed was 200 rpm. A bioreactor culture was conducted with *S. cerevisiae* harboring the inducible plasmid pYEGα-hCMP in a 5 l bioreactor (initial working volume: 2 l). The operating conditions for the bioreactor were similar to the ones previously developed for the production of recombinant human lipocortin-I in *S. cerevisiae* [12, 13, 14] because the same host and promoter were employed. Inoculum was cultivated in 500 ml flasks and transferred to the bioreactor to be 5% (v/v) in the late exponential phase. Initially, the bioreactor was operated in a batch mode with a medium containing 2% yeast extract and 2% glucose. When the dissolved oxygen concentration rapidly increased due to the depletion of glucose, a concentrated growth medium (400 g glucose/l and 30 g yeast extract/l) was fed to the bioreactor until the cell concentration reached 50 g/l. Then, the growth medium was switched to a production medium (200 g glucose/l, 200 g galactose/l and 30 g yeast extract/l) and supplied until the end of the reactor operation [13]. The concentrations of glucose and galactose were measured twice a day, the theoretical feed rate was calculated and the concentrated medium was supplied to the bioreactor using a computer-controlled peristaltic pump. During the production phase, the glucose concentration was maintained below 1 g/l and the galactose concentration at approximately 15 g/l. The temperature and pH were maintained at 30°C and 5.0 – 5.5, respectively, throughout the bioreactor operation. The dissolved oxygen concentration was maintained above 10% of air saturation by controlling the aeration rate and the agitation speed, and by using oxygen-enriched air. Culture samples were taken at predetermined intervals and stored at 4°C before analysis.

Analyses

The cell concentration was measured by a spectrophotometer at 660 nm (Lambda 20, Perkin-Elmer, USA). Glucose, galactose, glycerol, and alcohols in the culture supernatant were analyzed at 50°C using a high-performance liquid chromatograph (1100 series, Agilent Technologies, USA) equipped with a Shodex-SH1011 packed column (ϕ 8 mm×300 mm, Showa Denko K.K., Japan) and a refractive index detector. An aqueous solution of 0.01 N H_2SO_4 was used as an eluent at 0.6 ml/min. The plasmid stability during the bioreactor culturing was calculated by the ratio of the number of CFUs (colony forming units) on a selective medium agar plate to those on a YPD agar plate.

In order to measure the recombinant proteins secreted in the culture broth, the culture sample was centrifuged at 5,000 *g* at 4°C for 10 min and the supernatant was subjected to SDS-PAGE (15%) electrophoresis. The amount of recombinant hCMP was estimated by a densitometry analysis (Gel Doc 2000, Bio-Rad, USA) after staining the protein band with silver nitrate, using bovine serum albumin as the standard. The total amount of protein in the culture broth was determined by the Bradford method [15].

For an N-terminal amino acid analysis of the recombinant hCMP, the culture supernatant fractionated on the 15% SDS-PAGE was transferred to a polyvinylidene difluoride membrane. The band corresponding to hCMP was excised and subjected to a protein sequencer (Procise 491, Applied Biosystems, USA).

Results

Expression of hCMP in *S. cerevisiae*

Figure 1 shows the growth of *S. cerevisiae* and the production of hCMP with constitutive (pYIGP-hCMP) and inducible (pYEGα-hCMP) promoters. The cell growth

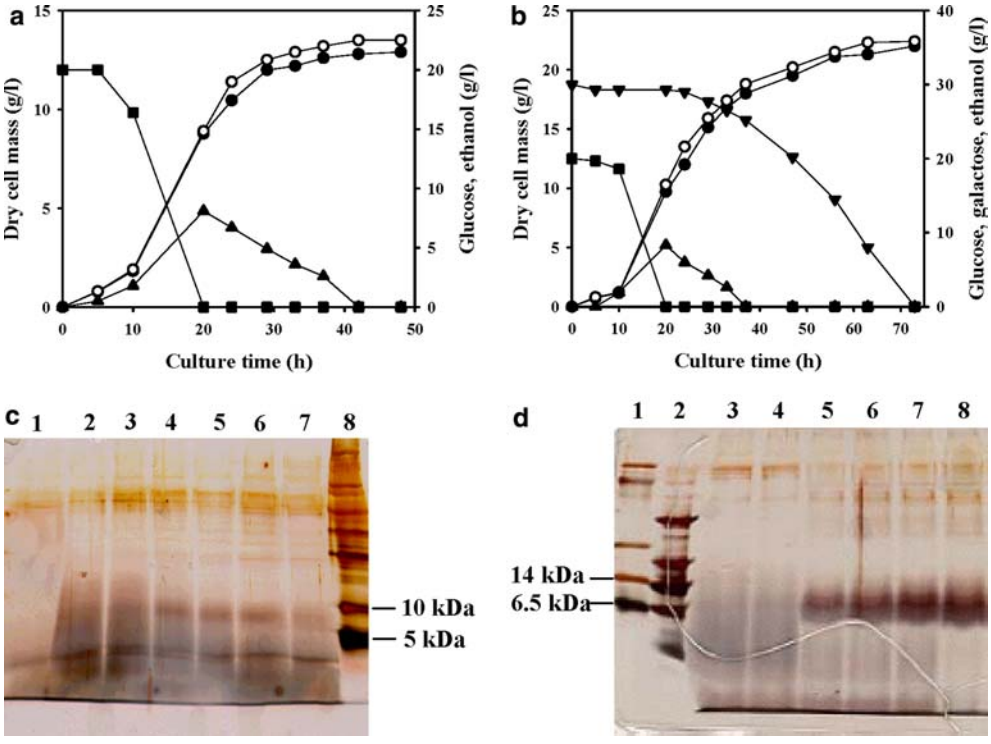

Fig. 1 Cell growth and hCMP production in flask culture of recombinant *S. cerevisiae* containing pYIGP-hCMP. (**a, c**) and pYEGα-hCMP (**b, d**). **a** and **b** cell growth of host (*open circle*) and transformant (*filled circle*), glucose (*filled square*), galactose (*filled inverted triangle*) and ethanol (*filled triangle*). **c** SDS-PAGE analysis: *lane 1*, host; *lane 2–7*, culture broth supernatant at 10, 20, 24, 33, 42 and 48 h; *lane 8*, molecular weight standard. **d** SDS-PAGE analysis: *lane 1* and *2*, molecular weight standard; *lane 3*, host; *lane 4*, before induction (20 h); *lane 5–8*, culture broth supernatant at 37, 47, 63 and 72 h

was not affected by the presence of the plasmid in either case (panels a, b). Higher cell growth was observed with the inducible plasmid because an additional carbon source of 3% galactose was supplemented (panel b). As seen in Panels c and d, a new protein band predicted to be the recombinant hCMP appeared in the SDS-PAGE analysis of the culture broth for both recombinants. The size of the new protein was estimated to be 7.0 kDa, which is in accord with hCMP based on the DNA sequence or reports in the literature [1, 6]. In the case of the hosts without the recombinant plasmids (panel c, lane 1, and panel d, lane 3), the putative hCMP bands were not observed. The N-terminus amino acid sequences of the putative hCMP were found to be Ile-Ala-Ile-Pro-Pro, which are identical to those deduced from the DNA sequences of hCMP. This indicated that the cloning of the hCMP gene in *S. cerevisiae* had been conducted successfully that and the complete polypeptide of the recombinant hCMP had been secreted in the culture broth by the action of the α-factor signal sequence.

Figure 1 also shows that the concentration of hCMP was higher with the inducible plasmid than with the constitutive plasmid. From the densitometry analysis, the amounts of hCMP in the culture broth at 48 h were estimated to be 50 – 60 mg/l with the *GAP* promoter (constitutive expression) and 120 mg/l with the *GAL* promoter (inducible expression), respectively. The concentration level increased with time, especially with the inducible plasmid. Since the *GAL* promoter is repressed in the presence of glucose, the production of hCMP in the inducible system started after 20 h (panel d, lane 4), after the glucose was depleted.

Expression of hCMP in *P. pastoris*

Figure 2 shows the growth of *P. pastoris* and the production of hCMP in *P. pastoris* with the constitutive (pGAPZαA-hCMP) and the methanol-inducible (pPICZαA-hCMP) promoters. The cell growth was not affected by the presence of the plasmid, and the expression level of the recombinant hCMP was almost the same in both strains. When separated in the SDS-PAGE, the culture broths exhibited a new protein band at 7.0 kDa as in the case of *S. cerevisiae*. The amino acid sequences at the N-terminus were also found to be Ile-Ala-Ile-Pro-Pro, indicating that the expression and secretion of the hCMP were completed successfully. The hCMP band was not observed in the host cell (panel c, lane 2, and panel d, lane 1) or in the recombinant with pPICZαA-hCMP before being induced by adding methanol (panel d, lane 2). The maximum concentration in *P. pastoris* was about 50 mg/l, which was much lower than that in *S. cerevisiae* with the *GAL* promoter.

Bioreactor operation

Figure 3 shows the results of the bioreactor fed-batch fermentation of *S. cerevisiae* with the inducible (pYEGα-hCMP) plasmid. The temperature and pH were maintained at 30°C and 5.0–5.5, because they are the optimal levels in the flask experiments (data not shown). The reactor was operated in a batch mode initially and switched into a fed-batch mode at 24 h. The fed-batch operation was divided into a growth phase and a

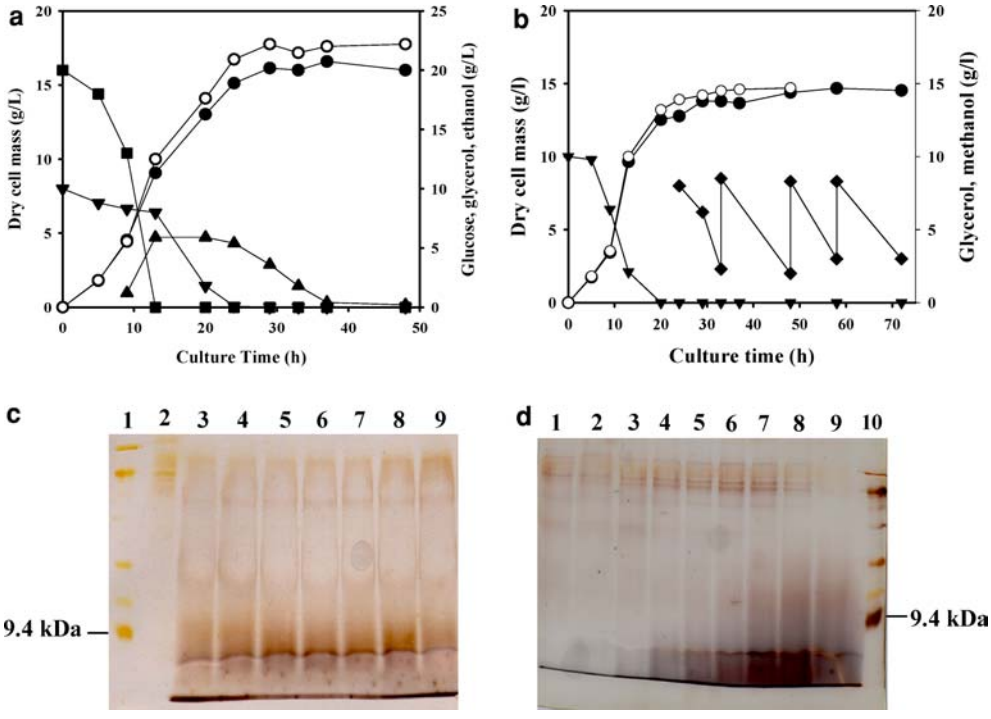

Fig. 2 Cell growth and hCMP production in flask culture of recombinant *P. pastoris* containing pGAPZαA-hCMP (**a, c**) and pPICZαA-hCMP (**b, d**). **a** and **b** Cell growth of host (*open circle*) and transformant (*filled circle*), glucose (*filled square*), glycerol (*filled inverted triangle*), ethanol (*filled triangle*) and methanol (*filled diamond*). **c** SDS-PAGE analysis: *lane 1*, molecular weight standard; *lane 2*, host; *lane 3–9*, culture broth supernatant at 5, 9, 13, 20, 29, 37 and 48 h. **d** SDS-PAGE analysis: *lane 1*, host; *lane 2*, before induction; *lane 3–9*, culture broth supernatant at 5, 9, 13, 24, 34, 40 and 48 h after methanol induction; *lane 10*, molecular weight standard

subsequent production phase. During the growth phase, a concentrated nutrient medium lacking galactose was added, and the cell concentration increased to 50 g/l at 42 h. During the production phase, the medium containing 200 g galactose/l was added, and a high galactose concentration of 15 g/l was maintained in the culture medium. The glucose concentration was controlled below 1 g/l during the production phase to avoid catabolite repression on the *GAL* promoter. During the fed-batch operation, the feed rate was estimated by the following equation:

$$F = \frac{\mu X V}{(S_f - S) Y_{X/S}}$$

where F, feeding rate (l/h); μ, specific cell growth rate (h^{-1}); X, cell concentration (g/l); S, carbon source concentration (g/l); S_f, carbon source concentration in the feed (g/l); $Y_{X/S}$, cellular yield coefficient based on carbon source consumption (g cell/g carbon source). The parameters used in above equation were determined in flask cultures based on glucose as follows (data not shown): For the growth medium, $Y_{X/S}$ and μ were 0.6 g cell/g glucose and 0.1/h; for the production medium, $Y_{X/S}$ and μ were 0.6 g cell/g glucose and 0.09/h. Pure oxygen was supplied from 40 h since the air could not meet the high oxygen demand of densely grown yeast cells. With this nutrient feeding strategy and the supply of the pure oxygen, continuous cell growth was observed and a high final cell concentration of 114 g/l was obtained at 97 h. Plasmid stability was over 95% during the growth phases and at about 90% at the end of the production period. The hCMP was not observed before the induction, but once induced by galactose, it appeared and increased steadily, attaining about 2.5 g/l at 97 h. This corresponded to a 20.8-fold increase over the concentration obtained in the flask culture.

Discussion

In this study, the production of recombinant hCMP was investigated in two yeast strains, *S. cerevisiae* and *P. pastoris*. The gene for encoding hCMP was chemically synthesized, cloned into plasmids under the control of either a constitutive or inducible promoter, and successfully expressed in recombinant yeast cells. The complete peptide of hCMP was produced and secreted in a culture medium with the aid of the α-factor signal sequence.

The comparison of the four expression systems constructed in this study indicates that *S. cerevisiae* with the *GAL* promoter is the most efficient. Several glalactose-inducible promoters such as *GAL* 1, *GAL* 7, and *GAL* 10 have been extensively exploited in the search for the tightly regulated and high-level production of recombinant proteins in *S. cerevisiae* [16], and it is generally accepted that they are more efficient than constitutive ones [17]. The production in *P. pastoris* was as low as 50 mg/l for both the constitutive and the methanol-inducible promoters. Considering that *P. pastoris* usually proves to be a better host in yielding a higher concentration than *S. cerevisiae* of many recombinant proteins [7–9], this result was somewhat unexpected. The expression level of recombinant proteins is affected by a variety of genetic and environmental parameters such as the host, the promoter, a foreign gene, culture conditions, among others. The

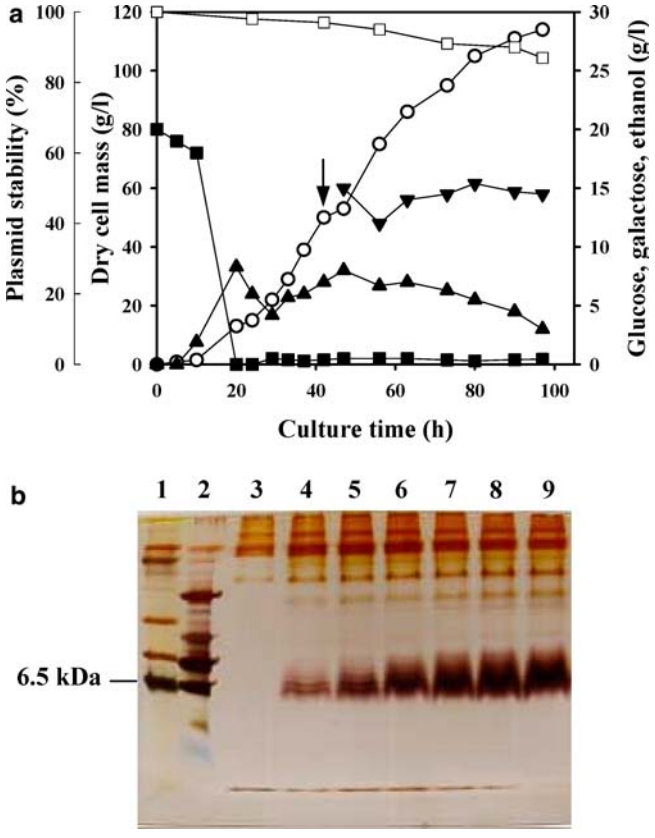

Fig. 3 Bioreactor fed-batch fermentation of recombinant *S. cerevisiae* containing pYEGα-hCMP. *Arrow* indicates the time when the growth medium was changed to the production medium. **a** cell growth (*open circle*), plasmid stability (*open square*), glucose (*filled square*), galactose (*filled inverted triangle*) and ethanol (*filled triangle*). **b** SDS-PAGE analysis: *lane 1* and *2*, molecular weight standard; *lane 3–9*, culture broth supernatant at 37, 47, 56, 63, 80, 90 and 97 h

present result might reflect the poor optimization in the construction of the *P. pastris* expression system and/or the culture conditions of the recombinant rather than the intrinsic low performance of *P. pastoris* in producing recombinant hCMP. Since recombinant *S. cerevisiae* showed a satisfactory result, however, no further effort to improve the *P. pastoris* system was made in the present study.

A high hCMP concentration of 2.5 g/l was obtained in a fed-batch bioreactor operation. The rationale of the feeding strategy is maintaining the cell growth rate at a high level during the production phase by supplying glucose along with galactose. By properly controlling the concentrations of glucose and galactose and their ratio in the culture medium, the maintenance of the high production activity of the recombinant protein to the end of the fermentation process was possible. This feeding strategy was essentially the same as that developed for the production of human lipocortin-I (MW = 36 kDa) in a recombinant *S. cerevisiae-GAL* promoter expression system [13]. The strategy developed for human lipocortin-I was equally effective for the production of hCMP. The degradation of hCMP was not significant when determined by protein quantification and electrophoretic analyses. The production of recombinant proteins over several grams of protein/l is not difficult in *E. coli*, but it is still challenging in *S. cerevisiae*. Considering the low molecular weight of hCMP (7.0 kDa), the present result of 2.5 g/l is remarkable.

For the commercial applications, practical equivalence between natural hCMP and recombinant hCMP should be verified. This is primarily related to the amino acid sequence of the peptide, which was investigated in the present study. Another aspect of practical equivalence is the glycosylation of the peptide, because it affects biological activity and sometimes results in side effects, including immune response, during administration. The antiobesity activity and glycosylation pattern of recombinant hCMP are under investigation.

Acknowledgements This study was supported by the Brain Korea 21 project and the Brain Busan 21 project, one of the authors, Dr. Y-K. Oh, was supported by 2004 postdoctoral study grant of Pusan National University.

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