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Rapid Selection of Multiple Gene Integrant for the Production of Recombinant Hirudin in *Hansenula polymorpha*

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Abstract For the rapid selection of higher recombinant hirudin producing strain in a methylotrophic yeast *Hansenula polymorpha*, a multiple gene integration and dose-dependent selection vector, based on a telomere-associated ARS and a bacterial aminoglycoside 3-phosphotransferase (*aph*) gene, was adopted. Two hirudin expression cassettes (HV1 and HV2) were constructed using the *MOX* promoter of *H. polymorpha* and the mating factor α secretion signal of *S. cerevisiae*. Multiple integrants of a transforming vector containing hirudin expression cassettes were easily selected by using an antibiotic, G418. Hirudin expression level and integrated plasmid copy number of the tested transformants increased with increasing the concentration of G418 used for selection. The expression level of HV1 was consistently higher than that of HV2 under the similar conditions, suggesting that the gene context might be quite important for the high-level gene expression in *H. polymorpha*. The highest hirudin producing strain selected in this study produced over 96 mg/L of biologically active hirudin in a 500-mL flask and 165 mg/L in a 5-L fermentor.

Keywords: hirudin, *Hansenula polymorpha*, G418 resistance, multicopy integration, *MOX* promoter

INTRODUCTION

The methylotrophic yeast, *Hansenula polymorpha* has been used widely as a host for the expression of heterologous protein [1]. As a facultative methylotroph, when the *H. polymorpha* is cultured on methanol as a carbon source, several key enzymes for methanol metabolism, such as methanol oxidase (*MOX*), dihydroxyacetone synthase (*DHAS*) and formate dehydrogenase (*FMDH*), are strongly expressed [2]. The *MOX* promoter has been intensively used for heterologous protein production in recombinant *H. polymorpha* strains [3-6]. A multiple gene integration system of *H. polymorpha* has an advantage for foreign gene expression [7] because the gene expression level is often dependent on the copy number and stability of the expression cassette [8]. Unfortunately, however, the selection procedures for the multiple integrant of *H. polymorpha* are usually laborious and time-consuming due to its low and unpredictable frequency of integration. For the facilitated control of integration copy number, a gene dosage-dependent selection method using the bacterial aminoglycoside 3-phosphotransferase (*aph*) gene of Tn903 that exhibits G418 resistance in yeast was reported [9]. The bacterial *aph* gene of Tn903 showed a proper performance when it was fused to a set of deleted glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoters of *H. polymorpha*. By using the weak *aph* expression cassette with a deleted *GAPDH* promoter (GAP61), the integration copy number could be controlled from 1 to 50 [9]. In a similar study we developed the vectors for the rapid selection of integrants with different plasmid copy number in *H. polymorpha* [10].

In this study, we isolated an optimal recombinant hirudin producing strain using the facilitated selection method by the gene-dose-dependent selection marker, *aph*. In addition, the expression levels of hirudin variant 1 (HV1) and 2 (HV2) which have 85-90% amino acid identities but have different amino acid sequences at nine residues [11] were compared to investigate the effects of different gene context on *H. polymorpha*.

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MATERIALS AND METHODS

Strains and Media

Auxotrophic strain *H. polymorpha* DL1-LU (*leu2 ura3*) derived from *H. polymorpha* DL-1 (ATCC 26012) was used as a host. *Escherichia coli* DH5α[F^- *lacZΔM15 hsdR17* (m^-) *gyrA36*] was used for the general transformation of genes. All transformants containing *LEU2* of *Hansenula* (*HLEU2*) were selected in the uracil containing minimal selective medium (2.0% [wt/vol] glucose, 0.67% [wt/vol] yeast nitrogen base without amino

acid, 100 μg/mL[wt/vol] uracil). G418 antibiotic selection was done with uracil containing minimal selective medium supplemented with different concentrations of G418 (Invitrogen, Carlsbad, CA, U.S.A.) from 0.5 to 4 mg/mL. To compare the expression level of hirudin of selected transformants, the cells were cultivated on YPM medium (1.0% [wt/vol] yeast extract, 2.0% [wt/vol] peptone, 2.0% [wt/vol] methanol) for 24 h at 37°C. The over expression of recombinant hirudin was done in 500-mL baffled shake flasks containing 50 mL production medium (2.0% methanol, 2.0% yeast extract, 0.1% tocopherol, 1.0% soybean oil) at 30°C with shaking of 130 rpm. The seed cultures were grown on minimal medium with uracil. Fermentation was carried out in a 5-L fermentor (NBS, Edison, NJ, U.S.A.) with 3 L of the production medium. Fermenter was inoculated with 150 mL of recombinant cells grown in the seed culture medium. The pH of the medium was controlled at 5.5 with 4 N NH_4OH and 4 N HCl during the cultivation.

Transformation and Stabilization of *H. polymorpha*

H. polymorpha was transformed by the lithium acetate and DMSO method [12]. For stabilization procedure that was done for the selection of integrants, all transformants of *LEU2* prototrophs in minimal selective medium were pooled and inoculated into YPD broth (1.0% [wt/vol] yeast extract, 2.0% [wt/vol] peptone, 2.0% [wt/vol] dextrose) in a flask. After 24 hrs, 2% of culture broth was transferred to fresh YPD broth. This procedure was repeated until the cells attained 50 generations. After stabilization, multiple integrants were selected by plating the stabilized cells onto the minimal selective plate containing G418.

General DNA Techniques

General DNA manipulations were performed as described by Sambrook *et al.* [14]. DNA fragments required for subcloning experiments were purified by using QIAEXII Gel Extraction Kit (QIAGEN, Valencia, CA, U.S.A.). Total yeast DNA was isolated as described by Johnston [15]. *E. coli* transformation was performed according to the method described by Inoue *et al.* [13].

Southern Hybridization

Integration copy number and patterns were analyzed by Southern hybridization [14]. Total chromosome was isolated and digested with appropriate restriction endonucleases. After electrophoresis, the separated DNA was transferred onto a positively charged nylon membrane (S&S Nytran, U.S.A.). Probe DNA was labeled with Non-radioactive DIG-Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was carried out at 42°C using standard hybridization buffer with formamide (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 5% blocking reagent, 50.0% formamide).

Thrombin Inhibition Assay

Determination of thrombin inhibitory activity was done by ChromozymeTH (Boeringer Mannheim) degradation [11]. The amidolytic cleavage of ChromozymeTH by thrombin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was measured as the variation of absorbance at 405 nm with Thermomax microplate reader (Molecular Devices, U.S.A.). 0.6 NIH U/mL thrombin, diluted culture supernatant and 200 μM ChromozymeTH were loaded into 96-well assay plate in the ratio of 1:1:2. The reaction was monitored for 5 minutes. All components were diluted with thrombin reaction buffer (0.1 M Tris-HCl, 0.12 M NaCl, 0.01% Sodium azide, 0.1% Bovine serum albumin, pH 8.0). Commercial hirudin (Accurate Chemical & Scientific Corporations, Westbury, NY, U.S.A.) ranging from 0 to 0.6 ATU was used as a reference standard.

Quantification of Recombinant Hirudin

Quantification of hirudin in fermentation broth was done by analytical RP-HPLC (Beckman, U.S.A.) on a C8 column (5 μm particle size, 250 × 4.6 mm i.d., 120 Å, YMC Co., Kyoto, Japan). The column was initially equilibrated with 0.1% trifluoro acetic acid (TFA). After sample injection, it was operated isocratically with the same solvent for 5 min. The hirudin was eluted with a linear gradient of acetonitrile (15-30%) – 0.1% TFA, at 1 mL/min flow rate for 30 min. The absorbance was monitored at 220 nm using an UV detector. The concentration of hirudin in sample fluids was determined by comparing the sample peak areas to a purified hirudin reference standard.

RESULTS

Plasmid Construction

Two chemically synthesized hirudin genes (HV1 and HV2) which were fused to the *MFα1* leader (ppL) of *S. cerevisiae* were inserted between the *H. polymorpha* DL-1 *MOX* promoter and terminator, respectively [16]. These 3 kb hirudin expression cassettes were inserted into the *Bam*HI site of pGLG61 which contains *H. poymorpha ARS, HLEU2* and the *aph* gene fused to 61 nt *GAPDH* promoter. The resulting plasmids were named as pGLG-HV1 and pGLG-HV2 (Fig. 1).

Transformation and G418 Selection of Hirudin Producing Strains

For initial selection, transformants of two plasmids were plated on the uracil containing minimal selective medium for complementation of *LEU2* auxotrophy. Most of initial transformants grew poorly in minimal medium without leucine due to the unstable episomal replication of the transforming plasmid. To introduce the unstable episomal plasmid into the chromosomal DNA, the stabilization of transformants was done as described previously [9]. Complete stabilization of

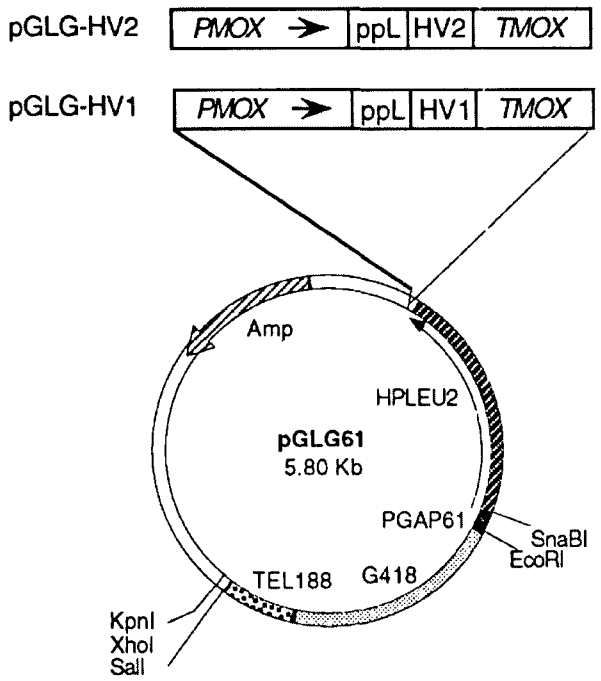

Fig. 1. Expression vectors of two hiudin variants. The hirudin expression cassettes were isolated with *Eco*RV–*Apa*I from pBDMOX-HV1 and pBDMOX-HV2. These 3 kb fragments were treated with Klenow enzyme and then inserted into the Klenow treated *Bam*HI site of pGLG61.

transformants was monitored by plating the cells on minimal selective plate and checking for an even and fast growth rate of colonies. After complete stabilization, to select the multiple gene integrants, 5×10^3 stabilized cells were plated on minimal selective plate with different concentrations of G418.

Several transformants randomly selected from each plate with different concentration of G418 were grown on YPM medium for comparison of hirudin expression level. The strains producing highest amounts of hirudin from each G418 concentrations are listed in Table 1. The hirudin expression level of transformants selected from the plates containing different concentration of G418 was in proportion to the concentration of G418. The hirudin expression level of *H. poymorpha* strains harboring HV1 gene was much higher than that of the strains harboring HV2 in all the range of G418 concentrations.

Copy Number Comparison of the Transformants

The correlation between hirudin expression level, plasmid copy number and integration locus was checked by Southern hybridization. Eight individual colonies listed in Table 1 were checked for their integration copy number and locus. The same amount of total genomic DNA isolated from each transformant was digested with *Eco*RI and separated in 0.8% agarose gel. Separated DNA was blotted with the *MOX* promoter as

Table 1. Comparison of hirudin expression level, plasmid integration copy number and locus of the transformants with two plasmids selected from different G418 concentrations

Plasmid	G418 concentration (mg/mL)	Strain	Hirudin concentration (mg/L)	Copy number & Integration Locus
pGLG-HV1	0.5	L0.5HV1-15	23	Single, *PMOX*
	1	L1HV1-13	27	Single, *PMOX*
	2	L2HV1-1	29	Multi, Telomere
	4	L4HV1-6	39	Multi, Telomere
pGLG-HV2	0.5	L0.5HV2-19	6	Single, *PMOX*
	1	L1HV2-8	9	Single, *PMOX*
	2	L2HV2-5	13	Multi, Telomere
	4	L4HV2-5	16	Multi, Telomere

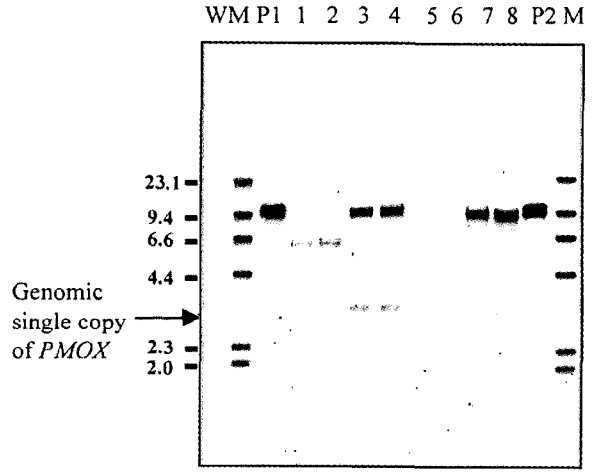

Fig. 2. Southern blot analysis of the integration copy number and integration locus in transformants. All genomic DNA was digested with *Eco*RI and digoxigenin-labeled 0.8 kb *MOX* promoter was used as a probe. Lanes: M, digoxigenin-labeled lambda *Hind*III size marker; W, wild type *H. polymorpha* DL-1; 1, L0.5HV1-15; 2, L1HV1-13; 3, L2HV1-1; 4, L4HV1-6; 5, L0.5HV2-19; 6, L1HV2-8; 7, L2HV2-5; 8, L4HV2-5; P1, pGLG-HV1; P2, pGLG-HV2.

probe (Fig. 2). Genomic single copy of the *MOX* promoter was identified as a band around 3 kb in untransformed wild-type cell. Interestingly, two (lane 1, 2 and 5, 6) among four transformants of each vector showed 6 kb single band instead of 3 kb genomic single copy of the *MOX* promoter. This integration was supposed to be occurred as a single copy in the *MOX* locus via homologous recombination of the plasmids. In contrast, 3 kb band for genomic single copy of the *MOX* promoter remained intact in the other two (lane 3, 4 and 7, 8) of four tested tansformants of each vector. They also showed an additional dark band around 10 kb which was the same size with the transforming vector. It suggested the multiple tandem integration of a transforming vector into a single locus (telomere), but not in the

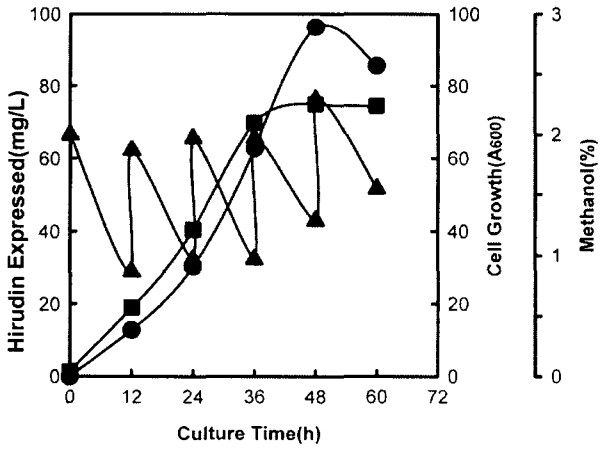

Fig. 3. Expression of hirudin variant HV1 from *H. polymorpha* L4HV1-6 in a shake flask. Hirudin (●); methanol (▲); cell growth (■).

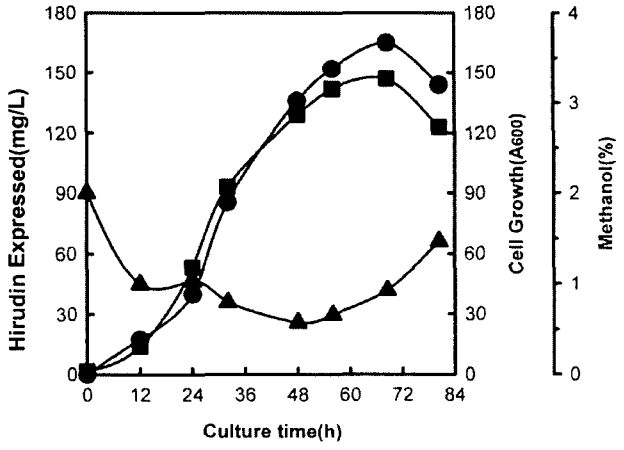

Fig. 4. Expression of hirudin variant HV1 from *H. polymorpha* L4HV1-6 in a 5-L fermentor. Hirudin (●); methanol (▲); cell growth (■).

MOX locus. Most of tandemly repeated integration of the vector pGLG61 containing a telomeric *ARS* exclusively occurred near the end of chromosome, namely telomere [17]. *Bal*31 sensitivity test of a band generated by the integration demonstrated that the multiple tandem integration of this study also might occur near telomere (data not shown).

The integration loci of the transformants were described in Table 1. The integration locus of the plasmid in the transformants picked from the plates with low concentration of G418 (0.5 and 1 mg/mL) was the *MOX* region whereas that from high concentration of G418 (2 and 4 mg/mL) was near the end of chromosome. These results imply that the multiple gene integration that is necessary for the cell to be resistant to high concentration of G418 always occurred near the chromosomal end. The highest copy number of integration was roughly estimated about 10 by comparing the signal intensity of Southern blot between the samples and single copy integrant as a reference [9]. Even though the hirudin expression level did not increase in proportion to its copy number, the multiple integration of expression vector resulted in high level expression of hirudin. The integrated copy numbers in *H. polymorpha* strains each harboring HV1 and HV2 appeared to be similar in the medium with same concentrations of G418. Considering the all conditions, *e.g.* G418 concentration, plasmid copy numbers and integration locus, for the expression of hirudin variants HV1 and HV2 are almost same, the difference in expression level of hirudin between the two variants might comes from the difference of gene context in the variants.

Overexpression of Hirudin

The highest amount of hirudin producing strain L4HV1-6 was grown in a 500-mL shake flask and in a 5-L fermentor in order to investigate its capability of the hirudin production. Methanol was used as sole carbon source. In this case, methanol acted not only as an inducer for the *MOX* gene but also as a substrate for cell growth. Shake flask cultivation was started with 50 mL production medium initially containing 2% methanol in a 500-mL baffled flask. When the initially supplied methanol was depleted, 0.5 mL of methanol was added into the 50 mL of production medium every 12 h after 48 hours of cultivation. The maximum hirudin concentration reached to 96.4 mg/L at 48 hours of cultivation (Fig. 3) and this amount was 2.5 fold higher than that obtained using the simple YP-methanol medium without additional methanol feeding as shown in Table 1. Expression level of hirudin by the strain L4HV1-6 was examined in a 5-L fermentor. Methanol concentration was maintained between 0.5 and 2.0% by the controlled feeding of methanol. The maximum expression level of the hirudin was 165 mg/L at 68 hours of cultivation (Fig. 4).

DISCUSSION

Previously, we found that the integration copy number of pGLG61 containing a bacterial *aph* gene under control of a 61 bp *GAPDH* promoter of *H. polymorpha* was linearly dependent on the G418 concentration [9]. Interestingly, the multiple plasmid integration events exclusively occurred near end of chromosome via homologous recombination due to the telomeric origin of *ARS* used in the vector [17]. In this study, we added an additional homologous sequence, the *MOX* promoter and terminator into the vector pGLG61. Even though the homologous single copy integration into the *MOX* locus was also found in the transformants selected from the plate containing low concentration of G418, multiple homologous integration of the trans-formants selected with high concentration of G418 occurred most exclusively near end of chromosome. To survive in high concentration of G418, cells should have enough num-

ber of G418 resistance gene copies in the chromosome. But homologous recombination into MOX locus could not support such multiple integration. The multiple integration of a transforming vector was completely dependent on the function of telomeric *ARS* used in the vector. Therefore, a vector containing a telomeric *ARS* could greatly facilitate the selection of multiple gene integrants for the development of industrial recombinant protein producing strain in *H. polymorpha*.

Gene-dosage often plays a key role on the expression of heterologous protein [8]. However, the expression level of HV1 and HV2 was quite different inspite of the same copy number and integration locus. The amino acid and DNA sequence of them are different and this was the only reason of expression level difference. The previous reports about the expression of hirudin variants were studied about only one variant at one time even though two of them were tested in one organism.

Several cases about the correlation between DNA sequence and expression level of its protein in *E. coli* [18, 19] and *S. cerevisiae* [20,21] were reported but the reports limited to one variant protein. It was suggested that the GC content of a gene related with the gene expression level in *H. polymorpha* [3]. The difference of GC content might be quite important for the codon usage. The GC content of two hirudin genes, HV1 and HV2 is 48.2 and 41.5%, respectively. The GC content of the MOX gene, which is highly expressed in *H. polymorpha*, is 53.5%. Thus it is conceivable that the difference in expression level between HV1 and HV2 is due to the difference of GC content of them. In addition, the amino acid sequence difference in two hirudin variants might affect on the expression level, in the aspects of protein secretion and proteolytic degradation. Further optimization of hirudin expression system in molecular level and downstream processing, especially fermentation, is under progress to produce recombinant hirudin in industrial scale.

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