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Heterologous Gene Expression and Secretion of the Anticoagulant Hirudin in a Methylotrophic Yeast *Hansenula polymorpha*

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A heterologous gene expression and secretion system using a methylotrophic yeast, *Hansenula polymorpha* was developed for the production of anticoagulant hirudin. Hirudin gene was expressed under the control of a strong and inducible methanol oxidase (*MOX* or *AOX*) promoter. The mating factor α pre-pro leader sequence of *Saccharomyces cerevisiae* was employed for hirudin to be secreted into the extracellular medium. Hirudin expression cassette was introduced into three strains of *H. polymorpha*, A16, HPB1 and DL1 which have different genetic backgrounds. This expression cassette was stably integrated into the host chromosomal DNA. Biologically active and mature hirudin was efficiently expressed and secreted into the extracellular medium. About 19 mg/L of hirudin was found in the culture supernatant in the case of a two-copy integrant of the strain HPB1 under suboptimal culture conditions.

In the production of recombinant proteins from yeasts, *Saccharomyces cerevisiae* has mostly been employed as a host. A few limitations arise, however, in utilizing this traditional yeast as a host for the heterologous gene expression: instability of recombinant strains (3), hyperglycosylation of secreted glycoproteins (10) and low secretion level and productivity (16). In these respects, a methylotrophic yeast, *Hansenula polymorpha* can be an alternative strain to overcome these limitations.

H. polymorpha is capable of utilizing methanol as the sole carbon and energy source (17, 22, 23). The mechanism of methanol utilization by *H. polymorpha* has been studied extensively (9, 27). The key enzyme for the initial pathway of methanol utilization in *H. polymorpha* is methanol oxidase (MOX), localized in peroxisome, which mediates the oxidation of methanol to formaldehyde. This enzyme is known to be expressed in up to 20~40% of the total cell protein when cells are grown on methanol (4, 25). The gene coding for methanol oxidase (*MOX*) was cloned and characterized (13). The *MOX* gene has a very strong and regulatable promoter which is induced by methanol and subject to catabolite repression by glucose (7). These characteristics of *MOX* promoter make *H. polymorpha* an attractive host for heterologous gene expression. Owing to the extensive studies of physiology and molecular biology of *H. polymorpha*, efficient transformation and integration procedures were developed. Several copies of heterologous gene were able to be introduced to the host chromosomal DNA via a non-homologous recombination (8, 18, 20, 24). Recently, there have been several successful applications of this system to the production of heterologous proteins such as HBsAg (12), glucoamylase (6) and α-galactosidase (5, 26).

In the present investigation, we tried to produce an anticoagulant hirudin from *H. polymorpha*. Hirudin is a protease inhibitor isolated from the salivary gland of the bloodsucking leech, *Hirudo medicinalis* (15). It is a potent thrombin-specific inhibitor which blocks thrombin-mediated conversion of fibrinogen to fibrin (28). We previously reported the expression and secretion of recombinant hirudin in *S. cerevisiae* using *GAL10* promoter, yeast mating factor α pre-pro leader sequence, and the chemically synthesized hirudin gene (21). In this paper, the gene expression and secretion of hirudin in *H. polymorpha* under the control of *MOX* promoter from *H. polymorpha* and mating factor α signal sequence from

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S. cerevisiae are reported.

MATERIALS AND METHODS

Strains and Plasmids

H. polymorpha HPB1(*ade2 leu2*), A16(*leu2*) and DL1 (*leu2*) were used as the host cells. These strains were originally isolated through MNNG mutagenesis from the wild-type *H. polymorpha* CBS 4732 (1). *E. coli* HB101 (*F⁻ hsdS20* (r^- m^-) *recA13 proA2 galK2*) or DH5α (*F⁻ laczΔM15 hsdR17* (r^- m^-) *gyrA36*) was used for the cloning of genes and propagation of plasmids. Plasmid pBluescript KS+ (Stratagene), which has a multicloning site, *E. coli* origin of replication and ampicillin resistance gene were used as the backbone of the general *H. polymorpha* integration vectors and for DNA sequencing. Plasmid pMOXΔRI (1) was used as the source of *H. polymorpha MOX* promoter and *MOX* transcriptional terminator, and pBα-HIR12 (21) as the source of the mating factor α pre-pro leader sequence and hirudin gene. YEp13 was used as the source of *S. cerevisiae LEU2* gene.

Media and Cultivation

For the general cultivation of auxotrophic *H. polymorpha* strains, cells were grown in YEPD medium (1% yeast extract, 2% peptone and 2% glucose) and all transformants were grown in YNB minimal selective medium (0.67 % yeast nitrogen base w/o amino acid, 0.07% of amino acid mixture lacking leucine and 2% glucose). To test the hirudin secretion activities of the transformants, cells were grown either in YNB medium containing 1% glycerol and 0.5% methanol as carbon sources, or in two types of rich media, Complex I (2% bacto-peptone, 1% yeast extract, 2% glycerol and 1% methanol) and Complex II (4% yeast extract, 0.5% casamino acid, 2% glycerol and 1% methanol). For the maximum gene expression, cells were first grown in the presence of glycerol and methanol for 24 hrs and then 1% of methanol was added to the culture media intermittently. Cultivations were made in baffled shake-flasks or test tubes at 37℃ with vigorous agitation.

Enzymes and Chemicals

Restriction endonucleases, Klenow fragment of *E. coli* DNA polymerase I and T4 DNA ligase were obtained from KOSCO Biotech. [^{35}S]dATPαS was from Amersham. DIG DNA labelling and detection kit, Taq DNA polymerase, calf-intestine alkaline phosphatase, RNase and Chromozym TH were from Boehringer Mannheim. Human thrombin, lithium chloride, PEG 3350 and formamide were purchased from Sigma Chemical Co. Standard recombinant hirudin(specific activity; 10,000 ATU/mg) was purchased from Accurate Chemical & Scientific Corporation. The GENECLEAN kit was obtained from BIO101 and was used according to the manufacturer's recommendations.

Recombinant DNA Techniques

General DNA manipulations were performed as described by Maniatis *et al.* (14). DNA fragments required for subcloning experiments were gel-purified using the GENECLEAN kit. *E. coli* transformation was done using the procedure described by Inoue *et al.* (11).

DNA Synthesis and Polymerase Chain Reactions (PCR)

Oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems model 391) and purified by OPC (oligonucleotide purification cartridge, Applied Biosystems). PCR was done in 100 μl volume with 20 ng of template DNA and 100 pmoles each of primer DNA. Amplification was done in a DNA thermal cycler (Ericomp Instrument) using the program set to denature DNA at 94℃ for 1 min, anneal at 50℃ for 2 min and extend at 70℃ for 3 min. This cycle was repeated for 25 times.

DNA Sequencing

DNA sequence of the junction between *MOX* promoter and secretion signal sequence created with PCR was determined by the dideoxy chain-termination method (19) using fragments subcloned into pBluescript.

Transformation of *H. polymorpha*

One colony of recipient strain was inoculated into 2~5 ml of YEPD medium and cultivated to reach a cell density of OD_{600} nm=0.3~0.4. Cells were harvested and washed once with TE (10 mM Tris-HCl; pH 7.4, 1 mM EDTA) buffer. Cell pellets resuspended in 10 ml of TE containing 0.1 M LiCl and 40 μl of 2-mercaptoethanol were incubated at 30℃ for 1 hr, pelleted by centrifugation at 6000×*g* for 10 min, and resuspended in TE-0.1 M LiCl solution to make the final concentration of competent cells about 1×10^9 CFU/ml. About 100~200 μl of competent cells were added to 10~20 μg of transforming DNA (10~50 μl) and incubated at 30℃ for 30 min. After incubation, the equal volume of 70% (W/V) PEG-3350 was added to the transformation mixture followed by incubation at 30℃ for 1hr. The preparations were heat-pulsed at 46℃ for 5-10 min and placed in a refrigerator at 4℃ for 10~15 hrs. The cell suspensions were plated onto a selective medium without washing and incubated at 37℃ for 4~7 days.

Plasmid Stability

Transformants were grown overnight in YEPD medium with vigorous shaking and plated onto YEPD plates to obtain separate colonies. Plates with 100~200 colonies were replica-plated onto both non-selective and selective plates (*LEU*$^+$ auxotrophy) and these plates were incubated at 37℃ for 3 days. The level of plasmid stability was calculated by dividing the number of colonies

that appeared on selective plates with the number on non-selective plates.

Total DNA Isolation

H. polymorpha cells grown to the stationary phase (200 ml) were harvested and washed with distilled water. The cell pellet was resuspended in 5 ml of HP solution (0.1 M Tris-SO_4; pH 9.3, 0.05 M EDTA, 2.5% 2-mercaptoethanol) and incubated at 30℃ for 15 min. After centrifugation, cells were resuspended in 5 ml of lysis buffer [50 mM Tris-HCl; pH 8.0, 50 mM EDTA, 50 mM NaCl, 0.2 ml of 20% SDS and 40 μl of Proteinase K (10 mg/ml)] followed by incubation at 65℃ for 30 min and then 37℃ for 30 min. Lysed cells were transferred to a 500 ml flask and deproteinized with a phenol-chloroform-isoamyl alcohol (25 : 24 : 1) mixture by gentle mixing. After centrifugation at 5000×*g*, DNA in water phase was removed and precipitated with the equal volume of isopropyl alcohol. Precipitate was removed with pipet tips, washed twice with 70% and 96% ethanol, dried and dissolved in TE buffer. For some DNA preparations, deproteinization with phenol-chloroform was repeated.

Southern Hybridization

Southern hybridization of *Eco*RI-digested total DNA was carried out according to the method of Maniatis *et al.* (14). To score the integration copy number of hirudin expression cassette, a DNA fragment containing mating factor α signal sequence and hirudin gene was labelled with DIG-labelling kit and used as a probe.

Antithrombin Activity Assay

Determination of thrombin inhibitory activity in culture supernatants was carried out using chromogenic thrombin substrate, Chromozym TH, as described in the previous report (21). Antithrombin activities of culture supernatant were measured after removal of cells with centrifugation (6000×*g*, 5 min). Hirudin activity was measured in ATU (Anti Thrombin Unit) with one unit neutralizing 1 NIH unit of thrombin (Lot J, Sigma Chemical Co.) using commercial recombinant hirudin (specific activity; 10,000 ATU/mg) as a reference standard. The actual amounts of secreted hirudin in the culture supernatant were then calculated based on the specific activity of 13,000 ATU per mg protein which was previously determined with purified hirudin produced by *S. cerevisiae*.

RESULTS AND DISCUSSION

Construction of Hirudin Gene Expression Cassettes

As previously reported, hirudin gene was successfully expressed and excreted into the culture medium in *S. cerevisiae* using yeast mating factor α(MF-α) pre-pro leader sequence(ppL) as a signal sequence(21). Thus, the same construct containing ppL and hirudin gene was used for the construction of the hirudin gene expression cassette in *H. polymorpha*. To locate this construct under the control of *MOX* promoter from *H. polymorpha* without modifying the original sequence of *MOX* promoter, two DNA fragments were joined by the use of PCR. As shown in Fig. 1, PCR was performed in three steps. In the first step, a reverse primer (dAACAGCTATGACCATG) and *MOX*-19mer (dCATTTTGTTTTTGTACTTT) were added to the template pBMOX8 to recover a 1.6 kb *MOX* promoter fragment containing ATG translation initiation codon. In the second step, MF-α-32mer (dAAAGTACAAAAACAAAATGAGATTTCCTTCAA) and S10-46mer (dGCGGCGTCGACTTACTATCATTGCAAGTATTCTTCTGGGATTTCTT) were used for the recovery of a 0.49 kb fragment containing MF-α ppL and hirudin gene from the template pBα-HIR12. These two PCR products contained 16 bp complementary sequences. In the last step, these two fragments with a complementary sequence at each end were annealed and used as templates for the reverse primer and the S10-46mer. This resulted in the recovery of a 2.1 kb DNA fragment containing *MOX* promoter, MF-α ppL and hirudin gene.

In order to introduce this expression cassette into *H. polymorpha*, two types of integration vector were const-

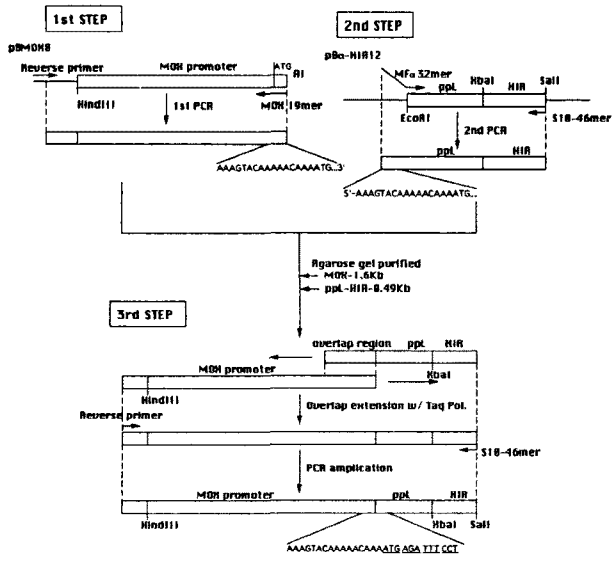

Fig. 1. PCR schemes for the fusion of a 3′ end of *MOX* promoter and initiation ATG codon of mating factor α pre-pro leader sequence (ppL) and hirudin gene. pBMOX8 containing *MOX* promoter, pBα-HIR12 containing mating factor α pre-pro leader sequence and hirudin gene were used for PCR template.

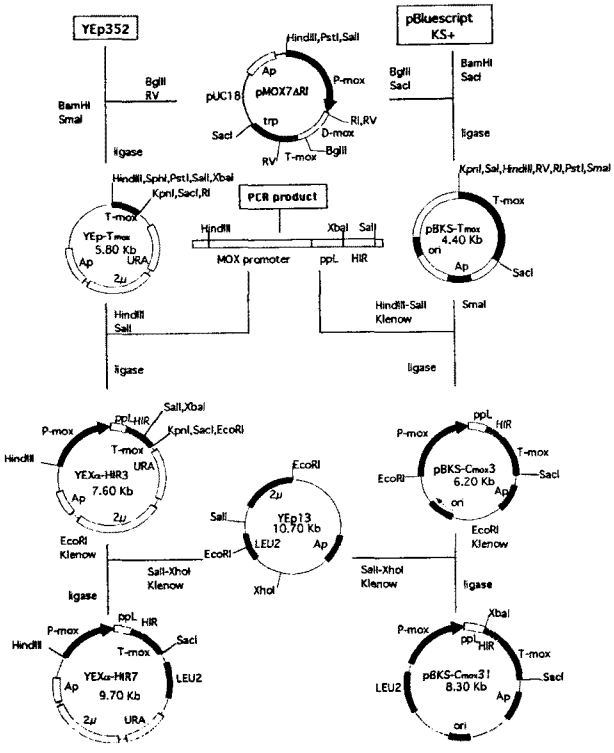

Fig. 2. Construction of *H. polymorpha* integration vectors, YEXα-HIR7 and pBKS-Cmox31.
Abbreviation: P-mox, *MOX* promoter; T-mox, *MOX* terminator, D-mox, deleted MOX structural gene.

ructed: YEXα-HIR7 with *S. cerevisiae* 2 μm gene and pBKS-C_{MOX}31 without *S. cerevisiae* 2 μm gene. As shown in Fig. 2, the PCR product was cloned into the plasmids pBKS-T_{MOX} and YEp-T_{MOX} which contained the *MOX* transcriptional terminator originated from pMOX7 ΔRI. To the resulting plasmids, pBKS-C_{MOX}3 and YEXα-HIR3, a *LEU2* gene of *S. cerevisiae* was introduced. The *LEU2* gene of *S. cerevisiae* is known to act both as a selection marker and a replication origin in *H. polymorpha* (8). Thus, a *XhoI-SalI* fragment of YEp13 containing *LEU2* gene was cloned into pBKS-C_{MOX}3 and YEXα-HIR3 and the resulting plasmids, pBKS-C_{MOX}31 and YEXα-HIR7, respectively, were obtained.

Introduction of Hirudin Gene Expression Cassettes into *H. polymorpha* Cells

As previously shown, transformation of *H. polymorpha* may result in different patterns of transformants depending on the type and state of transforming DNA (1). Transformation efficiency was higher with linear DNA fragments than with whole plasmid DNA in a circular form. Transformation with linear DNA fragments, however, resulted both in very unstable replication of the incoming DNA and in the integration of one copy of a selective gene (particulary *LEU2, TRP2, HIS3*) into different loci of *H. polymorpha* chromosome.

We first compared the transformation efficiencies of linear and circular forms of plasmid DNA using *H. polymorpha* HPB1 strain. Transformation was carried out with either undigested or *ScaI* digested YEXα-HIR7 plasmid DNA. Digestion with *ScaI* released a 6.2 kb fragment containing *MOX* promoter and terminator sequences, MF-α ppL sequence, hirudin gene and *S. cerevisiae LEU2* gene. YEp13 plasmid was also used as a control transforming DNA. Transformation efficiency and transformant patterns are summarized in Table 1. Transformation efficiency of linear plasmid was found to be higher than that of circular plasmid with both YEp13 and

Table 1. Comparison of transformation efficiencies and patterns of *H. polymorpha* HPB1 with different types of DNA

DNA	Phenotype of transformants	Number of transformants	Character of transformants	Group
YEXα-HIR7, 10 μg	*LEU*$^+$	1,000	Colonies with different sizes and irregular form	I
YEXα-HIR7 digested with *ScaI*, 5 μg	*LEU*$^+$	2,000	Most colonies small or very small, 1~2% of colonies large, round	II
YEp13, 10 μg	*LEU*$^+$	300	Colonies with different sizes and irregular form	–
YEp13 digested with *XhoI-SalI* 5 μg	*LEU*$^+$	5,000	Most colonies small, 0.1% of colonies large	–

YEXα-HIR7. The number of transformants obtained with YEp13 DNA fragment was higher than that with YEXα-HIR7. In the latter case, transformants were different in size with large colonies being predominant. When undigested plasmid DNA's were used for transformation, the transformation efficiency and pattern of transformants with YEXα-HIR7 were similar to those obtained with YEp13 plasmid.

About 200 transformants obtained with each type of YEXα-HIR7 DNA were restreaked on selective medium and tested for the level of hirudin activity. The colonies were cultured for 72 hrs in tubes with 2 ml of minimal medium containing glycerol and methanol as carbon sources. About 1% of transformants obtained with undigested plasmid and 15% of those obtained with *Sca*I-digested YEXα-HIR7 plasmid did not have any antithrombin activity. Most transformants exhibited only weak activity (below 1 mg/L), while only 3 transformants from the first group and 7 transformants from the second group produced more than 1 mg/L of hirudin under selective conditions. The hirudin expression was completely blocked in glucose-grown cells.

It is known that the level of selective marker stability in yeast transformants reflects the type of transformants: autonomously replicating or integrative (18). The level of stability of *Leu*$^+$ phenotype was estimated for 15 transformed clones which belonged to different groups and produced different levels of hirudin. As shown in Table 2, clones producing hirudin generally showed higher stability of *Leu*$^+$ phenotype in Group II which were transformed with linear DNA. In Group I which were transformed with circular DNA, they did not show such a high stability. This result indicates that when compared with the circular forms, the linear forms of transforming DNA show themselves to be more readily integrated in the chromosome. It was also indicated that there were also some clones not in accordance with the relationship between hirudin activity and *Leu*$^+$ phenotype stability, probably due to the non-intactness of the integrated

Table 2. Characterization of selected *H. polymorpha* transformants

Number of colony	Group	Colony size*	Hirudin activity**	Stability of *Leu*$^+$ (%)
1-26	I	H	+	88
1-39	I	H	−	52
1-44	I	H	−	65
1-76	I	H	+	44
2- 1	II	L	++	100
2- 5	II	L		9
2-10	II	L	+	100
2-12	II	L	++	99
2-20	II	S	++	100
2-28	II	S	−	100
2-43	II	S	−	99
2-60	II	H	+	74
2-61	II	H	+	76
2-67	II	H	−	83
2-87	II	L	+	30

* H-heterogeneous in size, L-large, S-small.
** (++) Activity level above 1 mg/L, (+) Activity level below 1 mg/L, (−) No activity

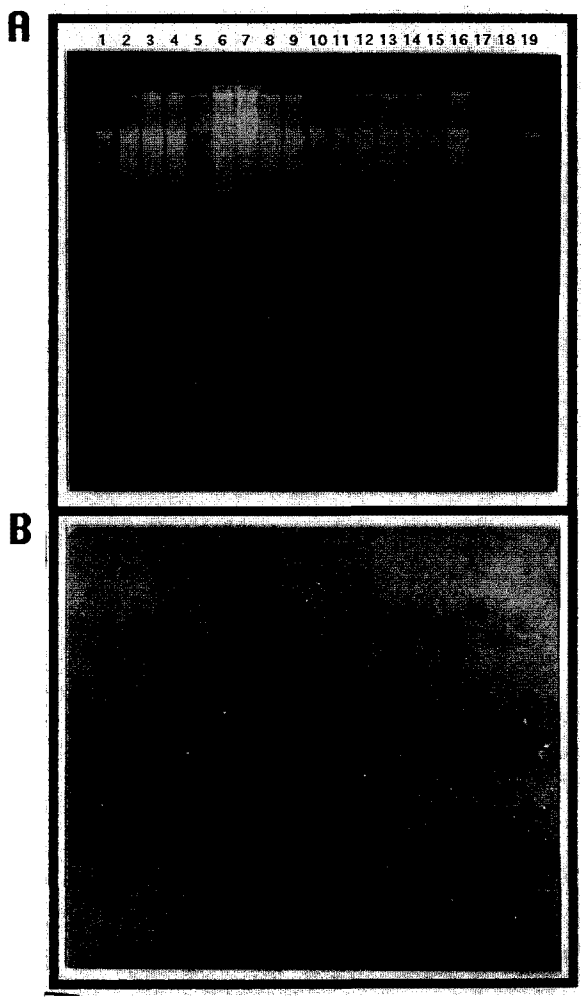

Fig.3. Southern hybridization of *H. polymorpha* genomic DNA digested to completion with *Eco*RI.
A: *Eco*RI-digested chromosomal DNA patterns of each transformant were analysed by agarose gel electrophoresis. B: Hybridization signals. Lane 1, 19; lambda *Hin*dIII digested DNA, lane 2; 1-26, lane 3; 1-39, lane 4; 1-44, lane 5; 1-76, lane 6; 2-1, lane 7; 2-5, lane 8; 2-10, lane 9; 2-12, lane 10; 2-20, lane 11; 2-28, lane 12; 2-43, lane 13; 2-60, lane 14; 2-61, lane 15; 2-67, lane 16; 2-87, lane 17; *Eco*RI digested YEXα-HIR7 and lane 18; *Sca*I digested YEXα-HIR7.

gene into the chromosomal DNA.

To analyze the integration pattern of these different types of transformant, Southern hybridization was performed using a DNA fragment containing mating factor α signal sequence and hirudin gene as a probe. As shown in Fig. 3, all the analyzed transformants showed signals ranging from 4 kb to 10 kb. All the clones seemed to contain one copy integration except the clones, 2-1, 2-10 and 2-12, in which two hybridization signals were observed. These two-copy integrants showed both higher hirudin level and *Leu*$^+$ phenotype stability compared with the single-copy integrants. Some of the single-copy integrants, however, did not produce any detectable amount of hirudin. This discrepancy may be related to the intactness of the integrated plasmid DNA in the chromosome. More careful examination by Southern hybridization is, therefore, required to look into this account.

Hirudin Gene Expression in Different Host Strains of *H. polymorpha*

To compare the secretion levels of hirudin among strains with different genetic backgrounds, we used three different *H. polymorpha* strains, HPB1, A16 and DL1. These strains were all *Leu*$^-$ auxotrophs of methanol utilizing (*MOX*$^+$) phenotype. It is known that multicopy integration of the *H. polymorpha* can be generated by performing repeated cultures of transformants in rich and minimal media (5, 18). This procedure is known as "stabilization". Prior to the occurrence of multicopy integration, some of the transformed plasmid should be replicated autonomously. For this purpose, it is more plausible to use the circular form of plasmid than the linear form. Therefore, each strain was transformed with undigested plasmid, pBKS-C_{MOX}31 (Fig. 2) for the future stabilization experiments.

Several transformants of each strain were chosen for the test of hirudin expression levels. There were not much differences in hirudin secretion levels among three strains (Table 3). Though the copy number of the transformants was not determined precisely, it was possible to estimate the copy number of these transformants as one or two by comparison with the previously obtained data on hirudin expression level and Southern blot. The hirudin secretion levels of each strain in different media were also compared. As shown in Table 3, there were small differences in hirudin secretion level among different strains. The hirudin levels were increased about 10~20 folds in all strains by replacing minimal media with complex media. Expression levels of hirudin seemed to be partially dependent on the cell growth. Maintenance of proper concentration of methanol for continuous induction greatly improved the hirudin levels, since all the strains used in this study can consume the methanol as energy source.

Table 3. Comparison of hirudin production levels in different host strains and media

Strain	Hirudin production levels and cell growth in different media [mg/L, (cell $OD_{600\,nm}$)]*		
	Minimal	Complex I	Complex II
A16	1.5(40.1)	17.5(107.8)	11.2(84.4)
HPB1	0.9(15.2)	12.7(89.4)	19.4(88.7)
DL1	1.4(20.2)	16.9(91.4)	15.9(94.3)

*Hirudin concentration in the culture supernatant and optical density were measured after 72 hrs of cell cultivation.

The recombinant hirudin secreted from each *H. polymorpha* strain was analyzed with SDS-PAGE and compared with purified control hirudin produced by *S. cerevisiae*. Culture supernatant of each recombinant strain containing hirudin gene were analyzed by SDS-PAGE together with those from control strains that did not contain hirudin gene. Fig. 4 shows that each recombinant strain containing hirudin gene showed a protein band with the same mobility as the partially purified hirudin control secreted by *S. cerevisiae*. In contrast, control cells lacking hirudin gene showed no band with similar mobilities. It was difficult to measure the amounts or activities of non-processed hirudin which might be accumulated inside the cells. This was due to the fact that antibody of hirudin was not available and N-terminal amino acid sequences were important to show anti-thrombin activity of hirudin. As a consequence, cell-free extracts of each strain were analysed by SDS-PAGE to check the possible accumulation of non-secreted hirudin inside the cell. As shown in Fig.4, no differences in protein profiles were noted between cell-free extracts of strains containing hirudin gene and control strains lacking hirudin gene.

The antithrombin activity and SDS-PAGE results indicate that biologically active and mature hirudin was secreted into the extracellular medium by correct processing of mating factor α pre-pro leader peptides and hirudin. The *S. cerevisiae KEX2* gene product, an endopeptidase, which cleaves the peptide bond next to Lys-Arg dipeptidyl sequence, is required for the correct processing of secretion signal and hirudin (21). This suggests that *H. polymorpha* also has such proteolytic activity or similar activities, and its processing capacity is not limiting in this case for hirudin secretion. However, there have been no reports yet concerning *KEX2*-like activities in *H. polymorpha*.

In conclusion, we have tested the feasibility of heterologous gene expression and secretion of hirudin by *H. polymorpha*. Biologically active and mature hirudin was efficiently secreted into the extracellular medium when induced with methanol. Though the multicopy integra-

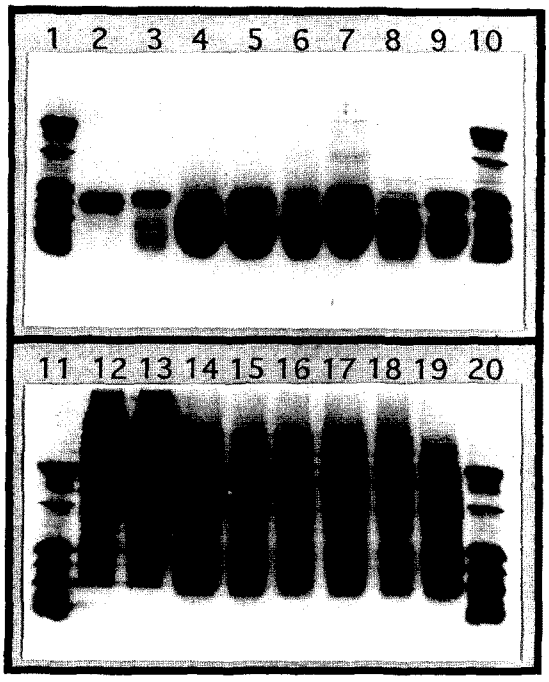

Fig. 4. SDS-PAGE analysis of Sep-Pak eluted extracellular medium and cell-free extracts.
A: protein profiles of Sep-Pak eluted extracellular medium. Extracellular medium was adjusted to 10% ACN/0.1% TFA and passed through a Sep-Pak C-18 cartridge (Waters). The bound hirudin was then eluted in 35% ACN/0.1% TFA. Lane 1, 10; standard molecular weight markers (43.0, 29.0, 18.4, 14.3, 6.2, and 3.0 kDas), lane 2; purified recombinant hirudin from *S. cerevisiae*, lane 3; Sep-Pak eluted medium of recombinant *S. cerevisiae*, lane 4; control medium of A16, lane 5; medium of recombinant A16, lane 6; control medium of HPB1, lane 7; medium of recombinant HPB1, lane 8; control medium of DL1, and lane 9; medium of recombinant DL1. B: protein profiles of cell-free extracts (CFE). Lane 11, 20; standard molecular weight markers, lane 12; CFE of control *S. cerevisiae*, lane 13; CFE of recombinant *S. cerevisiae*, lane 14; CFE of control A16, lane 15; CFE of recombinant A16, lane 16; CFE of control HPB1, lane 17; CFE of recombinant HPB1, lane 18; CFE of control DL1, and lane 19 CFE of recombinant DL1.

tion procedures were not performed yet, the expression levels of hirudin in *H. polymorpha* were comparable to those obtained with *S. cerevisiae* (manuscript in preparation). Also, a considerably less amount of contaminating proteins was secreted into the culture medium from the host cells of *H. polymorpha* compared with *S. cerevisiae*. Trials to increase the expression level of hirudin gene are currently being made, including stabilization after transformation, comparison of various signal sequences and optimization of culture conditions.

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