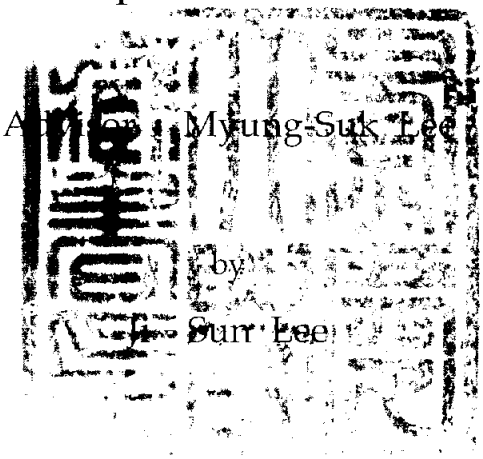
Expression of Human Prourokinase in Methylotrophic Yeast, *Hansenula polymorpha*

메탄올 자화효모인 *Hansenula polymorpha*에서 human prourokinase 발현



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Expression of Human Prourokinase
in Methylotrophic Yeast, *Hansenula polymorpha*

A Dissertation

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Abstract

Human urokinase-type plasminogen activator (uPA) is a serine protease which activates plasminogen to be able to catalyze the degradation of fibrin. It is considered the alternative treatment of thrombolytic disease because it does not have a danger of bleeding and production cost is less than the other treatments. There are two forms that are two chain form (tc-uPA or urokinase) and single chain form (sc-UPA or prourokinase). Recently, prourokinase has been reported as a more attractive treatment due to its fibrin specificity.

In this study, we have tried to express human prourokinase in methylotrophic yeast, *Hansenula polymorpha* which is an effective host for the production of heterologous protein. First of all, human prourokinase mature gene which was located in pGEM-T easy

vector was ligated in pBluescript KS II (+) and then mating factor α signal sequence from *Saccharomyces cerevisiae* was introduced into N-terminal prourokinase mature gene to increase the efficiency of secretion. Prourokinase containing signal sequence was inserted into pHIPX4, expression vector of *H. polymorpha*.

Transformation into *H. polymorpha* was accomplished by lithium chloride method and expression of prouokinase was confirmed by SDS-PAGE and western blotting. Fibrin plate test was performed in order to observe expressed prourokinase activity.

I . INTRODUCTION

The fibrinolytic enzymes system in human blood is responsible for the dissolution of blood clots (Zaworski *et al.*, 1989, Nagamine *et al.*, 1984). This system consists of three components ; 1) the proenzyme plasminogen, which can be activated by limited proteolysis to plasmin (which in turn catalyzes the degradation of the fibrin constituents of a blood clot), 2) plasmin inhibitors of which 2-antiplasmin is the most important component and 3) plasminogen activators. Physiological fibrinolysis is regulated by molecular interactions between plasminogen, plasminogen activators and fibrin and by interactions between plasmin and 2-antiplasmin. This regulation assures plasmin formation at the site of a blood clot and prevents systemic activation of plasminogen (Holmes *et al.*, 1985).

Clinical application of plasminogen activators has been found as the treatment of thrombolytic disease. These activators belonging to the class of serine proteases have been classified into two groups ; tissue type plasminogen activator (tPA) and urokinase-type plasminogen (uPA) (Moir and Davidow, 1991). Urokinase type has two forms, two chain (tc-uPA or urokinase) and single-chain (sc-uPA or prourokinase) which is the precursor of two-chain form. For more than two decades, tPA has been used as a thrombolytic

agent (Wang *et al.*, 2000(a); Tang *et al.*, 1997). However, due to side effects such as bleeding and high cost in production, the tc-uPA has been developed for thrombolysis (Okabayashi *et al.*, 1996).

More recently, sc-uPA, prourokinase has been developed for thrombolysis because of its fibrin specificity. Prourokinase is a classical zymogen and is inert in plasma at physiological concentrations. It has several unusual properties which distinguished it from other serine protease zymogens (Wang *et al.*, 2000(a)). These include its relatively high intrinsic catalytic activity, its reversible inhibition by diisopropylflourophosphate, its unique hypercatalytic transition state which forms during the conversion of prourokinase to urokinase, and its strong promotion by fibrin, which gives the intrinsic activity of prourokinase a catalytic activity against plasminogen equivalent to urokinase (Tang *et al.*, 1997).

The uPA has been prepared from human urine or kidney cell culture. However, in this case, the procedure is complicated and production cost is expensive.

Therefore, recombinant technology has been introduced as the way to produce uPA. Recombinant technology has been used to produce prourokinase from a variety of sources including *Escherichia coli*, mammalian cells and yeast (Wang *et al.*, 2000(b)). From the point of view in industrial production, expression in *Escherichia coli*

is not suitable, since the refolding of protein is not sufficient to produce large amounts of material. Further more, special caution is needed to avoid the contamination of endotoxin in final products when proteins are isolated from *E.coli* (Gellissen, 2000). The mammalian cell expression system is high in cost and low in yield and require stringent control procedures to detect infectious agents.

Yeasts are often preferred for the production of plant or animal proteins, because in these, protein modifications typical for eukaryotes normally occur. These modifications (e.g. glycosylation, acylation, phosphorylation, formation of disulfide bond) are often essential for the function and stability of the protein (Weydemann *et al.*, 1995; Hiramatsu *et al.*, 1991). Moreover, yeast can be grown rapidly on simple media to high cell density and secreting expression of foreign proteins by yeast has advantages to express proteins over other expression systems (Gellissen, 2000).

Initially, *S. cerevisiae* has been the yeast species of choice for foreign protein production due to obvious reasons since its genetics were well developed and the organism is generally regarded as safe. Despite successful applications of this microorganism, specific disadvantages in the use of *S. cerevisiae* have also been encountered, e. g. the instability of the engineered strains, undesired hyperglycosylation and relatively low yields, due to the lack of strong promoters.

Methylotrophic yeasts have been described as particularly efficient hosts for high-level heterologous protein expression (Kang *et al.*, 1989). In contrast to *S. cerevisiae*, heterologous proteins produced in methylotrophic yeasts, as a rule, are not hyperglycosylation (Kang *et al.*, 2001(a); Narciandi *et al.*, 1995). A striking feature of methanol-grown yeasts is the excessive proliferation of peroxisomes. Studies on the physiology, biochemistry and ultrastructure of these yeasts revealed that methanol is oxidized by a hydrogen-peroxide producing alcohol oxidase (AOX), which is localized in peroxisomes (Kim *et al.*, 2000). In these organelles catalase, which decomposes the hydrogenperoxide, is also produced and dihydroxyacetonesynthase (DHAS), which catalyzes the first step in the assimilatory pathway, is present (Oh *et al.*, 1994). Other enzymes involved in methanol assimilation (enzymes of the xylulose -5-phosphate pathway) or dissimilation [formaldahyde dehydrogenase (FdDH) and formate dehydrogenase (FMD)] are cytosolic (Hollenberg, 1989). In such cells, alcohol oxidase (AOX) and dihydroxyacetone synthase (DHAS) may be controlled by very strong promoters. Moreover, the expression of the genes encoding formate dehydrogenase (FMDH) and formate dehydrogenase (FMD) are under control of strong, inducible promoters (Gellissen and Marten., 2001; Gellissen and Hollenberg, 1997).

Therefore, expression system of heterologous proteins using

these promoters has been developed in these days.

Especially, *H. polymorpha* has been recognized as a more attractive option comparison with the other methylotrophic yeasts (*Candida boidinii, Pichia pastoris*). *H. polymorpha* is more thermo-tolerant and capable to grow at higher rates on simple and defined media. The relatively high optimal growth temperature for *H. polymorpha* (37-43℃ vs. 30℃ for *C. boidinii, P. pastoris and S. cerevisiae*) may be favorable for the production of mammalian (including human) proteins (Lahtchev *et al.*, 2002). Besides, this yeast has the advantage that it allows a better cooling management and reduces the risk of contaminations in large scale fermentations. Furthermore, *H. polymorpha* has another strong point that this yeast species do not secrete significant amounts of proteins. Therefore, the secreted heterologous protein can be recovered in a relatively pure state from the culture medium (Gellissen, 2000).

Integrated transformations of *H. polymorpha* is usually mediated by non-homologous recombination; plasmids integrate at random sites within the *H. polymorpha* genome, which results in stable transformants carrying multiply integrated plasmids (Kang *et al.*, 2001(b)). Thus it is possible to obtain *H. polymorpha* transformants with multiple copies of cloned genes, which is important for increasing the level of heterologous gene expression (Kim *et al.*, 2000).

Secretion of a produced heterologous protein is favorable, because it facilitates the recovery of the protein from the culture fluid. To establish secretion, it is necessary to fuse the protein of interest to an N-terminal signal sequence that directs the protein to the secretory pathway (Wang *et al.,* 2000(b)). The various heterologous secretion signals are used in *H. polymorpha,* e.g. *Saccharomyces cerevisiae* mating αsignal sequence, *Schwanniomyces occidentalis* glucoamylase, *Kluveromyces lactis* killer toxin (Yebra *et al.,* 1999).

In this study, *H. polymorpha* has been chosen as the expression host for human prourokinase gene, and mating factor α signal sequence from *S. cerevisiae* was introduced into prourokinase mature gene for effective secretion of prourokinase.

Ⅱ. MATERIALS AND METHODS

1. Materials

1.1 Microorganisms and culture media

For construction of expression vector, *E. coli* DH5α was cultured in LB medium (bacto peptone 2.0 g, yeast extract 1.0 g, NaCl 1.0 g). Auxotrophic *H. polymorpha* strain A16 was obtained from Microorganism Processing Laboratory, KRIBB, South Korea. The strains and plasmids used are presented in Table 1.

For general cultivation, these cells were grown in YPD medium (1.0% yeast extract, 2.0% bacto peptone and 2.0% glucose) and all transformants were grown in YNB minimal selective medium (0.6% yeast nitrogen base without amino acid and 2.0% glucose). To express human prourokinase, the transformants were grown in complex medium (2.0% bacto peptone, 1.0% yeast extract, 2.0% glycerol and 1.0% methanol).

1.2 Chemicals and primers

Restriction enzymes used in this study were purchased from Bioneer Co. (South Korea). Ampicillin, kanamycin, glass bead, fibrinogen, thrombin, goat anti-rabbit IgG were obtained from Sigma (USA) and urokinase antibody was gained from Santa Cruz Biotechnology (USA). The oligonucleotides used for PCR are showed in Table 2.

Table 1. Strains and plasmids used in this study

Strains and plasmids	Genotype or phenotype	Ref.
E. coli DH5α	F-Ψ80d/lacZ△M15U169, *deo*R, *rec*A, *end*A	Invitrogen
Hansenula polymorpha A16	LEU2 auxotroph	KRIBB (Korea)
pGEM T easy	Cloning vector : Amp^r, *lacZ*, 3.0kb	Promega
pGEM T easy with human prourokinase		Yonsei University (Korea)
pBluescript KS II(+)	Cloning vector : Amp^r, *lacZ*, 2.9kb	Stratagene
pPIC9	Expression vector : AOX1, His4	Invitrogen
pHIPX4	Expression vector : Kan^r, AOX, LEU2	Groningen University (Netherlands)

Table 2. Oligonucleotides used in this study

Primers	Sequence
MF-F	5′-GCG AAG CTT ATG AGA TTT CCT TCA-3′
MF-R	5′-TCT TTT CTC GAG AGA TAC CCC TTC-3′
UK-F	5′-GCG CTC GAG AAA AGA AGC AAT GAA CTT C-3′
UK-R	5′-TAT GGT ACC GTC GAC TCA GAG GGC CAG-3′

2. Methods

2.1 Construction of recombinant plasmid containing human prourokinase

The plasmid pGEM-T easy vector including human prourokinase gene, which was obtained from Prof. Kwang-Hoi Jung in Yonsei university (Korea), was amplified by two primers; UK-F and UK-R (Table 2). The amplified human prourokinase was digested with Xho I and Kpn I, then purified and ligated with pBluescript KS II(+) that was pretreated with the above enzymes.

The plasmid pPIC9 including *S. cerevisiae* signal sequence was amplified by two primers; MF-F and MF-R (Table 2). Then, amplified signal sequence was inserted into the Xho I and Kpn I site of pBluescript KS II (+) including prourokinase mature gene. Thereafter, this signal sequence was incoporated to introduce into the first codon of human prourokinase mature gene. Finally, the human prourokinase containing signal sequence was restricted with HindIII and Sal I and ligated in *H. polymorpha* expression vector, pHIPX4 (Fig. 3) which is provided from Prof. Marten Veenhuis in Groningen University, Netherlands.

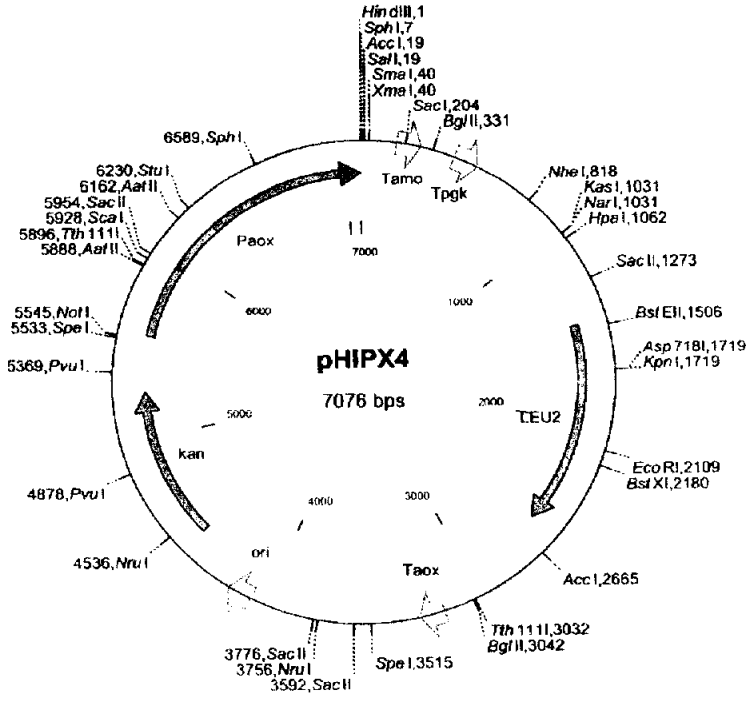

Fig. 1. The map of *Hansenula polymorpha* expression vector, pHIPX4.

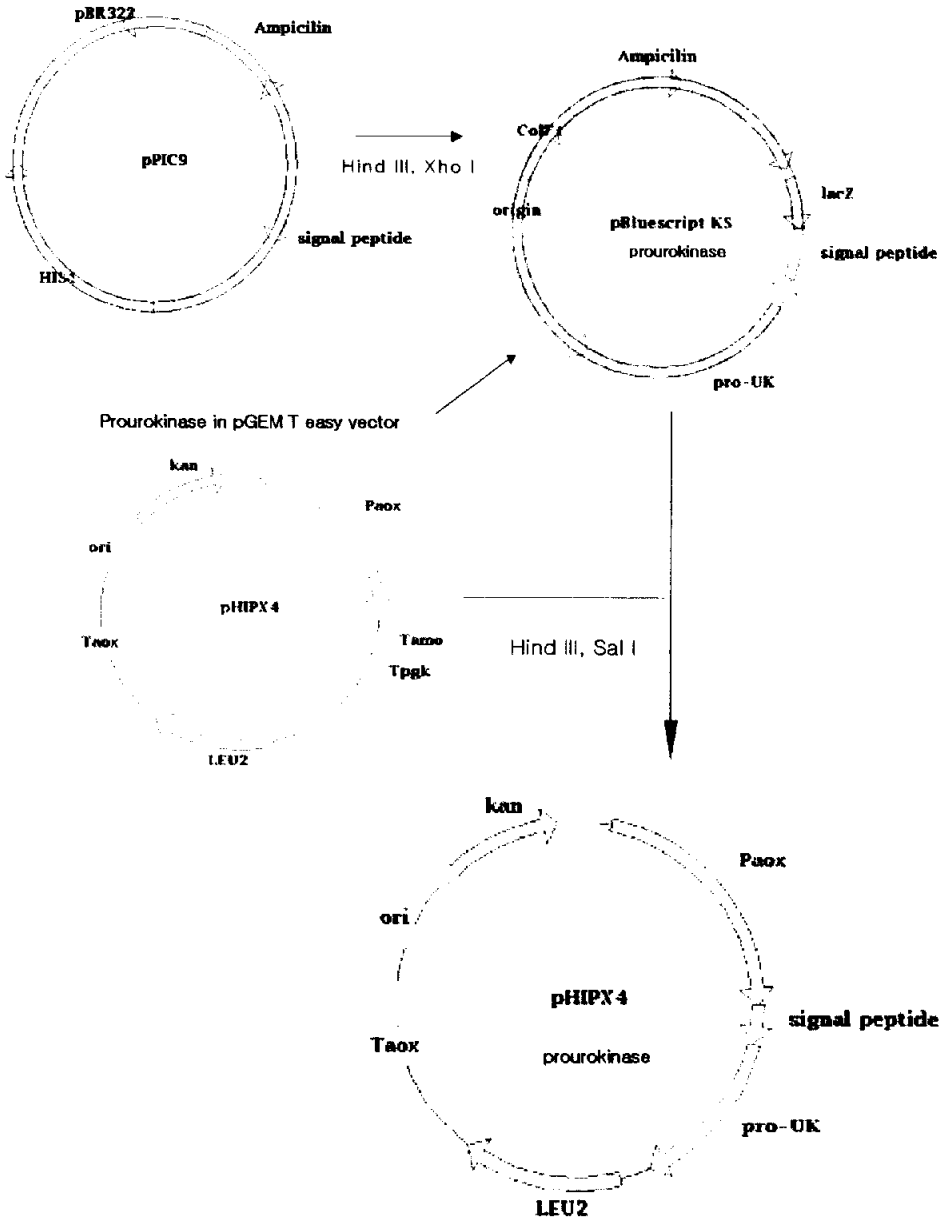

Fig. 2. Construction of expression vector for expression of prourokinase in *Hansenula polymorpha*.

2.2 Transformation into *H. polymorpha* A16

Transformation was performed by lithium chloride method (Sohn *et al.*, 1993). One colony of *H. polymorpha* A16 was inoculated into 5.0 ㎖ of YPD medium and cultivated to reach the cell density of OD600nm 0.3-0.4. Cells were harvested and washed once with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.4). Cell pellets resuspended in 10 ㎖ of TE buffer containing 0.1 M LiCl and 40 ㎕ of 2-mercaptoethanol were incubated at 30℃ for 1 hr, pelleted by centrifugation at 6,000×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/㎖. About 100-200 ㎕ of competent cells were added to 10-20 ㎍ of transforming DNA which was digested with Stu I in order to integrate into chromosomal DNA 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 1 hr. The preparations were heat-pulsed at 46℃ for 5-10 min and placed at 4℃ for 10-15 hrs. The cell suspensions were plated onto selective medium without washing and incubated at 37℃ for 4-7 days, and several colonies were selected for PCR confirmation. Cell PCR was carried out using two primers; MF-F and UK-R.

2.3 Genomic DNA extraction

The transformants verified by PCR were streaked on selective YNB agar. To confirm integration of plasmid into chromosomal DNA, the transformants were cultured in YPD medium several times. Then, genomic DNA was extracted by following methods. The cell was centrifuged and resuspended in 500 $\mu\ell$ lysis buffer. Cells were mixed for 2 min with glass beads, centrifuged and the liquid phase was transferred into another tube. After 275 $\mu\ell$ of 7.0 M ammonium acetate (pH 7.0) was added into liquid followed by incubating at 65℃ for 5 min and kept on ice 5 min. Five hundreds $\mu\ell$ of chloroform was added into this mixture and centrifuged. Thereafter, the supernatant was precipitated with isopropanol at room temperature. Precipitate was washed with 70% ethanol, dried out and dissoved in 50 $\mu\ell$ H_2O. The extracted genomic DNA was proceeded PCR using MF-F and UK-R primers (Table 2).

2.4 Induction of prourokinase

Prourokinase induction with and without methanol was inoculated in YPD broth culture. The cells were initially grown in complex medium containing glycerol (2.0% bacto peptone, 1.0% yeast extract, 2.0% glycerol). Then, 1% methanol was added into culture media intermittently. To compare an influence of methanol, the other was used without methanol.

2.5 SDS-PAGE analysis of human prourokinase expression

The SDS-PAGE analysis of human prourokinase was carried out in both cultures with and without methanol.

Twenty five ㎖ of the culture was centrifuged 1,620×g for 5 min at room temperature to separate culture supernatant and cells. Collected cells were resuspended in 0.5 ㎖ of ice cold sample buffer (10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% bromophenol blue) with freshly PMSF (0.5 mM) and benzimidine (0.5 mM). After adding glass beads, mixture was mixed by vortex 4 times for 45 sec with standing on ice for 30 sec between each mixing. Next, it was centrifuged at 4℃ for 5 min. Supernatant was transferred to a new tube, boiled for 10 min. The samples were centrifuged 11,600×g for 1 min at room temperature. The supernatant was loaded on a 12% SDS-PAGE. Until bromophenol blue reaches the bottom of the gel, the gel was run at 150 voltage for 2 hrs. The gel was stained with coomasie brilliant blue and destained with destaining solution (10% acetic acid, 45% methanol).

To collect secreted proteins, 900 ㎖ of culture was centrifuged and the supernatant was taken and dried using deep freezer. Then, the dried supernatant was melted in 50 ㎖ distilled water. To decrease viscosity, it was filtrated by Microcon (Amicon co, USA) and 20 ㎕ of the filtrate sample including sample buffer was boiled and loaded on 12% SDS PAGE.

2.6 Western blot analysis

After total proteins containing prourokinase were separated on a 12% SDS-PAGE, the gel was transferred to nitrocellulose membrane at 100 mA for 16 hrs in transfer buffer (25 mM Tris, 120 mM glycine, 20% methanol). The nitrocellulose membrane was washed with TTBS buffer (20 mM Tris-Cl, 0.5 M NaCl, 2.5 mM KCl, pH 7.4) containing 0.1% Tween 20 and blocked with TBS buffer containing 5% skim milk for 1 hr. The membrane was washed for 5 min in TTBS buffer and treated for 90 min with urokinase primary antibody which diluted 1:5,000 in TTBS buffer containing 5% skim milk. After washing three times for 5 min with TTBS buffer, the membrane was treated for 1 hr with alkaline phosphatase conjugated anti-rabbit IgG in TTBS buffer containing 5% skim milk. The membrane was washed three times for 5 min in TTBS buffer and developed by NBT and BCIP solution in alkaline phosphate buffer (0.1 M Tris-Cl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5)

2.7 Activity test of prourokinase on fibrin plates

Bovine fibrin plate for assay the activity of the secreted prourokinase as method of Moir and Davidow, 1991 included three reagent solutions as follows. (1) The fibrinogen reagent contains 1% bovine fibrinogen (80% clottable) dissolved in sterile water plus 1/4 volume sterile 10× phosphate buffer (26.9 g $Na_2H_2PO_4 \cdot H_2O$, 43.3 g

Na_2HPO_4 and 58.5 g NaCl per liter, pH 7.0). Extensive stirring was needed to dissolve the fibrinogen. Ampicillin (25 ㎍/㎖) was added to avoid bacterial contamination. (2) The thrombin reagent contained 1,000 units of thrombin plus 10 ㎖ of sterile 1×phosphate buffer. (3) Agar concentration was 2.0%.

Fibrin plates were prepared as follows. Two portions of 5 ㎖ fibrinogen reagent and 0.1 ㎖ thrombin reagent were pipetted into two separated spots on petri dish. To the other place, 5 ㎖ agar was added, followed by shaking the plate for approximately 5 sec on table surface to mix three compositions for polymerizing the fibrinogen and solidifying agar uniformly. The plates were inoculated with 10 ㎕ samples and were incubated at 37℃ for 12 hrs. Subsequently, clear zone forming certified prourokinase activity.

Ⅲ. RESULTS

1. Construction of expression vector

In order to express human prourokinase in *H. polymorpha*, pHIPX4-prourokinase expression system was constructed (Fig. 2). Prourokinase mature gene, which was located in pGEM T easy vector, was ligated in pBluescript KS Ⅱ (+), and 1.25kb band was confirmed by PCR (Fig. 3). Then, signal sequence from pPIC9 was introduced into N-terminus of prourokinase mature gene, and certified the insertion by PCR (Fig. 4). Next, prourokinase containing signal sequence was introduced into pHIPX4, expression vector of *H. polymorpha* and then, confirmed by PCR using MF-F primer and UK-R primer (Fig. 5).

To know the exact sequence, amplified PCR product by above primers was sequenced and analyzed. The sequencing result was shown in Fig. 6.

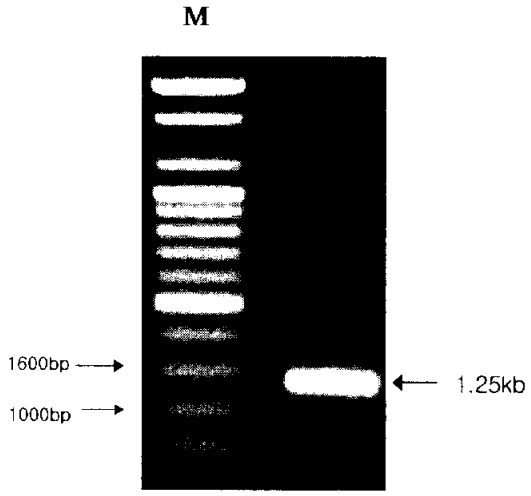

Fig. 3. Confirmation of prourokinase in pBluescript KS II (+) by PCR.

M: 1Kb of DNA standard marker.

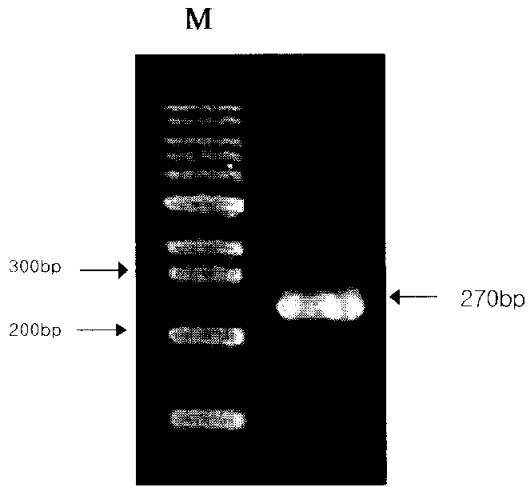

Fig. 4. Confirmation of signal sequence in pBluescript KS II (+) by PCR.

M: 100 bp of DNA standard marker.

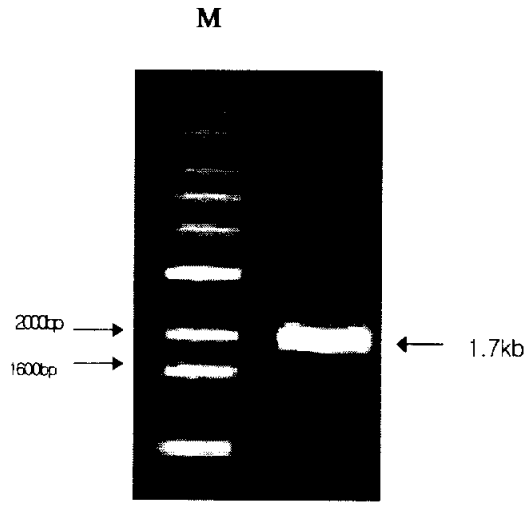

Fig. 5. Confirmation of prourokinase containing signal sequence in pHIPX4 by PCR.

M: 1Kb of DNA standard marker.

```
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    P  V  N  T  T  T  E  D  E  T  A  Q  I  P  A  E  A  V  I  G
 61 CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT
    Y  S  D  L  E  G  D  F  D  V  A  V  L  P  F  S  N  S  T  N
121 TAC TCA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT
    N  G  L  L  F  I  N  T  T  I  A  S  I  A  A  K  E  E  G  V
181 AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA
    S  L  E  K  R  S  N  E  L  H  Q  V  P  S  N  C  D  C  L  N
241 TCT CTC GAG AAA AGA AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA AAT
    G  G  T  C  V  S  N  K  Y  F  S  N  I  H  W  C  N  C  P  K
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1381 TGG GGC CGT GGA TGT GCC CTG AAG GAC AAG CCA GGC GTC TAC ACG AGA GTC TCA CAC TTC
    L  P  W  I  R  S  H  T  K  E  E  N  G  L  A  L
1441 TTA CCC TGG ATC CGC AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC TGA
```

Fig. 6. Sequence of signal sequence and prourokinase in pHIPX4 prourokinase.

2. Transformation

After transformation procedure, the transformants were selected on the selective YND agar and one colony was inoculated in YPD broth. Transformants were confirmed by PCR using oligonucleotide primers; 1) MF-F and MF-R, 2) UK-F and UK-R, 3) MF-F and UK-R. Three bands resulted from cell PCR were shown in Fig 7.

3. Confirmation of pHIPX4-prourokinase integration into genomic DNA

After genomic DNA extraction, PCR of genomic DNA was proceeded with MF-F primer and UK-R primer, the band with size between 2000bp and 1600bp was obtained (Fig. 8).

4. Western blotting of expressed prourokinase

When subjected to western blotting, the cell pellet preparations caused the bands with sizes about 64kDA. The supernatant, however, resulted the bands with the sizes larger than 64kDA (Fig. 10).

5. Prourokinase activity test on fibrin plate

Prourokinase activity test on fibrin plate were carried out with supernatant and cell pellet samples. In both samples, clear zones formed as shown in Fig. 11.

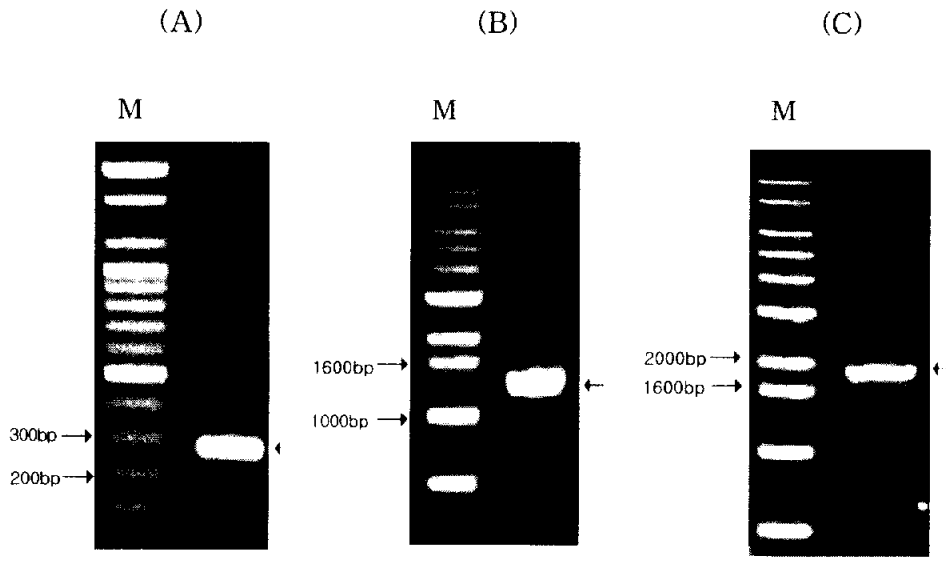

Fig. 7. Transformants confirmation by cell PCR.

M: DNA size marker, A: signal sequence,

B: prourokinase, C: prourokinase including signal sequence.

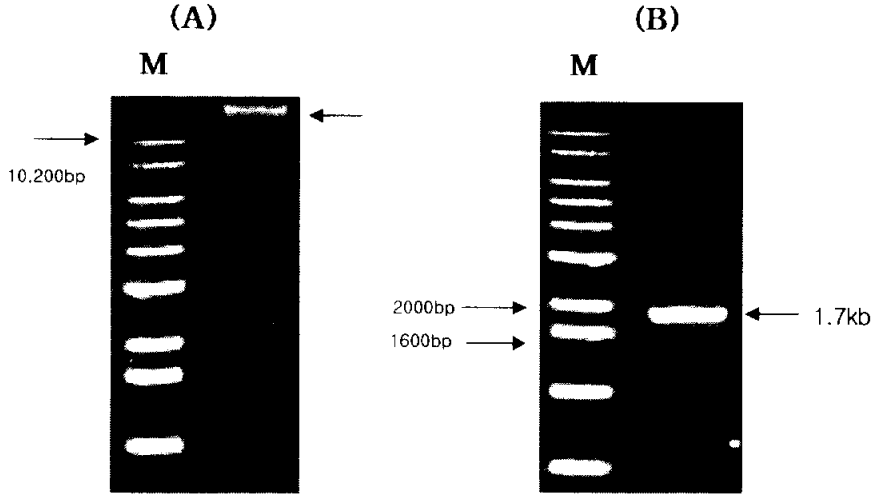

Fig. 8. Genomic extraction of transformants.

(A) Genomic DNA extraction,

(B) Confirmation by cell PCR.

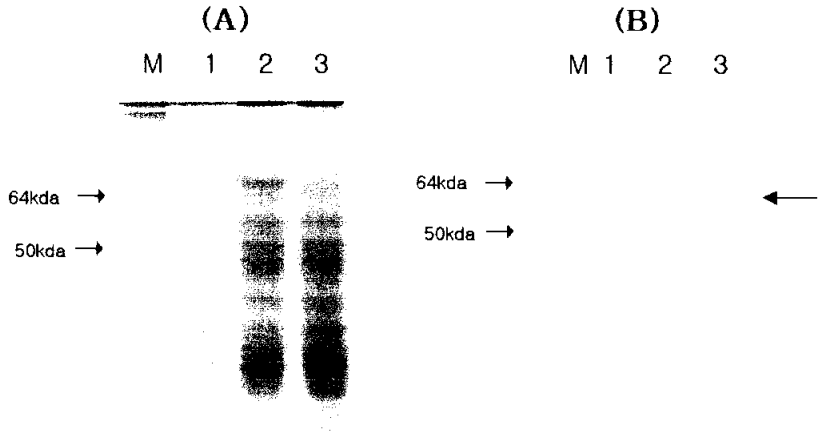

Fig. 9. Analysis of prourokinase expression in cell pellet.

(A) The patterns of SDS-PAGE of the expressed proteins,

(B) Western blot analysis on transferfed membrane,

M ; Protein standard marker,

lane 1; Sample without transformation,

lane 2; Sample with methanol addition,

lane 3; Sample without methanol addition.

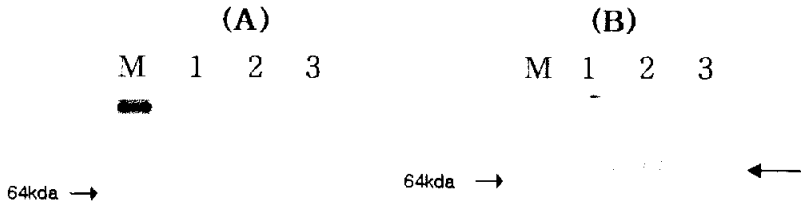

Fig. 10. Analysis of prourokinase expression in supernatants.

(A) The patterns of SDS-PAGE of the expressed proteins,

(B) Western blot analysis on transfered membrane,

M; Protein standard marker,

lane 1; Sample without transformation,

lane 2; Sample with methanol addition,

lane 3; Sample without methanol addition.

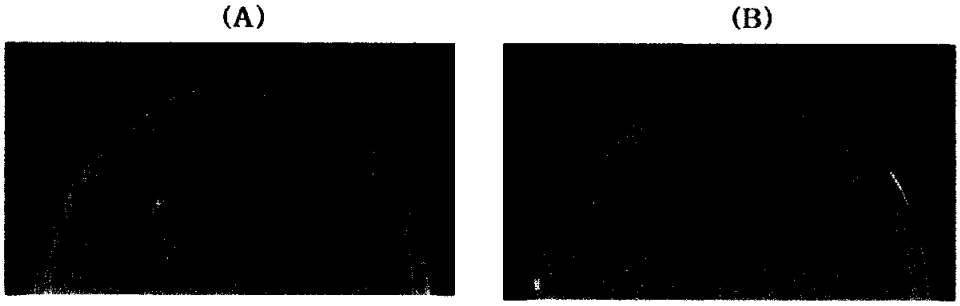

Fig. 11. Prourokinase activity test on fibrin plate.

(A) Supernatant, (B) Cell pellet

1; Wild type of *H. polymorpha* A16,

2; Transformants with prourokinase gene under the presence of methanol.

Ⅳ. DISCUSSION

In this study, human prourokinase was expressed in MOX promoter system of *H. polymorph.* Both samples with and without methanol were expressed human prourokinase. This result is considered due to characteristic of MOX promoter and glycerol. MOX promoter which is the element of methanol utilization pathway, was reported that its regulation follows repression/derepression mechanism, rather than an introduction/repression one in *H. polymorpha* (Mayer *et al.*, 1999). Therefore, it can be expressed at reasonable levels in the absence of their natural substrate, methanol (Hollenberg, 1989). Also, glycerol is a depressing carbon source of the MOX promoter, therefore it allows the expression of the methanol pathway at satisfactory levels when it present as the only carbon source in the medium (Gellissen and Melber, 1996).

One major attraction in choosing *H. polymorpha* as a system for heterologous gene expression is potential of this microorganism to secrete large amounts of proteineaceous products into the medium (Gellissen and Marten, 2001). Secretion provides an efficient mechanism for product recovery without breaking cells, especially since the methylotrophic host secretes genuine polypeptides in low abundance only (Weydemann *et al.*, 1995). A range of secretion leaders are available to direct translation products into the secretory compartment of the yeasts. In this study, α mating signal

sequence from *S. cerevisiae* was introduced into N-terminus of human prourokinase mature gene and facilitated secretion of human prourokinase.

On the other hand, in western blot analysis, the band's size obtained about 64kDa was larger than expected ones. It is considered due to influence of glycosylation. It is reported that human prourokinase normally has an N-glycosylation site. The N-linked oligosaccharides attached to the recombinant glycoprotein in yeast (Wang *et al.*, 2000(b)). Therefore, to know an accurate size of human prourokinase, it is necessary to remove oligosaccharides by elimination of glycosylation process.

Also, the bands sizes of prourokinase were different in the cell pellet and the supernatant. Therefore, further study is needed to explain this problem.

Prourokinase activity on fibrin plate test (Moir and Davidow, 1991) certified that both supernatant and cell pellet samples have prourokinase activity.

In this study, we successfully expressed and secreted human prourokinase under *H. polymorpha* expression system using MOX promoter and signal sequence from *S. cerevisiae*.

We will try to find out the growth condition for optimal production of human prourokinase and the effect of glycosylation against prourokinase secretion.

국문초록

Human prourokinase는 비활성의 plasminogen을 활성형의 plasmin으로 전환시키는 serine protease이다. Plasmin은 혈관내 응고된 혈전(fibrin clot)을 용해시키는 매우 중요한 효소로서 현재 혈전용해제로 사용되는 plasminogen activator로는 streptokinase와 재조합 tissue type plasminogen activator (t-PA) 등이 있으나, streptokinase의 경우 비인체 유래의 것으로 발열의 위험이 있으며 t-PA는 뇌출혈의 부작용을 가지고 있다. 현재, 인체 유래의 prourokinase가 혈전용해제로서 주목받고 있는데 국내에서는 녹십자 (주) 에서 인체의 뇨로부터 직접 정제하여 생산하고 있으나 생산과정이 복잡하고 비용이 많이 든다는 단점을 가지고 있다. 따라서 유전공학적 기법을 이용하여 진핵세포인 yeast, 특히 강력한 promoter를 가진 가지며 외래 단백질 발현시 수거가 용이한 메탄올 자화효모인 *H. polymorpha*에서 human prourokinase를 발현하여 대량 생산을 위한 발판을 마련하고자 한다.

Secretion 효율을 높이기 위하여 *S. cerevisiae* 유래의 Mating α signal sequence를 human prourokinase mature gene N-terminal에 도입하였다. Signal sequence가 포함된 prourokinase를 *H. polymorpha* expression vector인 pHIPX4에 ligation하여 vector를 구축하였다. Transformation은 lithium chloride 방법에 따라 시행하였고 SDS PAGE와 western blotting을 통해 expression을 확인하였다. 또한, fibrin plate를 이용하여 prourokinase activity를 확인하였다.

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Prourokinase gene을 흔쾌히 분양해 주신 정광회 교수님, *Hansenula polymorpha*를 분양해 주신 강현아 박사님 그리고 항상 바쁘실텐데도 질문에 친절히 대답해주시고 벡터를 분양해 주신 네덜란드에 계시는 Jan Kiel 박사님께 감사의 말씀을 전해 드립니다.

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REFERENCES

Gellissen G. and K. Melber. 1996. Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmacculicals. Drug Research. **46**, 943-948.

Gellissen G. and C. P. Hollenberg. 1997. Application of yeast in gene expression studies:comparison of *Saccharomyces cerevisiae, Hansenula polymorpha* and *Kluyveromyces lactics*. *Gene*. **90,** 87-97.

Gellissen G. 2000. Heterologous protein in production in methylotrophic yeasts. *Applied Microbiology and Biotechnology*. **54**, 741-750.

Gellissen G. and Marten V. 2001. The methylotrophic yeast *Hansenula polymorpha*: its use in fundamental research and as a cell factory. *Yeast*. **18,** 1-3.

Hiramatsu R., S. Horinouchi and Y. Beppu. 1991. Isolation and characterization of human prourokinase and its mutants accumulated within the yeast secretory pathway. *Gene*. **99,** 235-241.

Hollenberg C. P.. 1989. DNA molecules coding for FMDH control regions and structural gene for a protein having FMDH-activity and their uses. *European Patent EP*. 0299108.

Holmes W. E., D. Pennica, M. Blaber, M. W. Rey, W. A. Guenzler, G. J. Steffens and H. L. Heyneker. October 1985. Cloning and expression of the gene for pro-urokinase in *Escherichia coli*. *Biotechnolgy*. **3**, 923-929.

Kang H. A., J. H. Sohn, E. S. Choi, B. H. Chung, M. H. Yu and S. K. Rhee. 1989. Glycosylation of human α_1-antitrypsin in *Saccharomyces cerevisiae* and methylotrophic yeasts. *Yeast*. **14,** 371-381.

Kang H. A. and S. K. Rhee. 2001(a). Methylotrophic yeast as a new host for heterologous protein expression. *Korean Journal of Biotechnology and Bioengineering*. **16**, 15-23.

Kang H. A., W. K. Kang, W. K. Hong, M. W. Kim, J. Y. Kim, J. H. Sohn, E. S. Choi, K. B. Choe and S. K. Lee. 2001(b). Development of expression system for the production of recombinant human serum albumin using MOX the promoter. *Journal of Microbiology and Biotechnology*. **29,** 248-252.

Kim H. Y., J. H. Sohn, C. H. Ki, K. J. Rao, E. S. Choi, M. K. Kim and S. K. Rhee. 2000. Rapid Selection of Multiple Gene Intergrant for the Procuction of Recombinant Hirudin in *Hansenula polymorpha*. *Biothchnology and Bioprocess Engineer.* **5**, 1-6.

Lahtchev. K. L., V. D. Semenova, I. I. Tolstorukov, K. V. Klei and M. Veenhuis. 2002. Isolation and properties of genetically defined strains of the methylotrophic yeast *Hasenula polymorpha* CBS4732. *Archives of Microbiology.* **177**, 150-158.

Mayer A. F., K. Hellmuth, H. Schlieker, R. Lopez-Ulibarri, S. Oerter, U. Dahlem, A. W. M. Strasser and A. P. G. Van Loon. 1999. An expression system matures:a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. *Biotechnology and Bioengineering.* **63,** 373-381.

Moir D. T. and L. S. Davidow. 1991. Production of Proteins by Secretion from Yeast. *Methods in enzymology.* **194**, 491-507.

Nagamine Y, D. Pearson, M. S. Altus and E. Reich. 1984. cDNA and gene nucleotide sequence of porcine plasminogen activator.

Nuleic Acid Research. **12**, 9525-9541.

Narciandi R. E., L. Rodriguez, E. Rodriguez, R. Diaz, J. Delgado and L. Hrrera. 1995. High level production of recombinant invertase in *Hansenula polymorpha. Biotechnology Letter.* **17**, 949-952.

Okabayashi K., M. Tsujikawa, M. Morita, K. Einaga, K. Tanaka, T. tanabe, K. Yamanouchi, M. Hirama and J. F. Tait. 1996. Secretory production of recombinant urokinase-type plasminogen activator-annexin V chimeras in *Pichia pastoris. Gene.* **177**, 69-76.

Oh Y. I., J. H. Sohn, E. S. Choi, H. C. Kim and S. K. Rhee. 1994. Gene Expression and Secretion of Human Epidermal Growth Factor in a Methylotrophic Yeast *Hansenula polymorpha. Korean Micorobiology and Biotechnology.* **2**, 477-484.

Sohn J. H., M. Y. Beburov, E. S. Choi and S. K. Rhee. 1993. Heterologous Gene Expression and Secretion of the Anticoagulant Hirudin in a Methylotrophic Yeast *Hansenula polymorpha. Journal of Microbiology and Biotechnology.* **3**, 65-72.

Tang Wei, Z. Y. Sun, Ralph P., Victor G. and J. N. Liu. 1997. An

efficient system for production of recombinant urokinase-type plasminogen activator. *Protein Exression and Purification.* **11**, 279-283.

Wang P., J. Zhang, Z. Sun, Y. Chen, V. Gurewich and J. N. Liu. 2000(a). Catalytic and Fibrinolytic Proterties of Recombinant Urokinase Plasminogen Activator from *E. coli*, Mammalian, and Yeast Cells. *Thrombosis Research.* **100**, 461-467.

Wang P., J. Zhang, Z. Sun, Y. Chen and J. N. Liu. 2000(b). Glycosylation of Prourokinase produced by *Pichia pastoris* Impairs Enzymatic Activity but Not Secretion. *Protein Exression and Purification.* **20**, 179-185.

Weydemann. U., P. Keup, M. Piontek, A. W. M. Strasser, J. Schweden, G. Gellissen and Z. A. Janowicz. 1995. High-level secretion of hirudin by *Hansenula polymorpha*-authentic processing of three different preprohirudins. *Applied Microbiology and Biotechnology.* **44**, 377-385.

Yebra M. J., A. Blasco and P. Sanz. 1999. Expression and secretion of *Bacillus polymyxa* neopullulanase in *Saccharomyces cerevisiae*. *Microbiology Letters.* **170**, 41-49

Zaworski P. G., K. R. Marotti, V. MacKay, C. Yip and G. S. Gill. 1989. Production and secretion of porcine urokinase in *Saccharomyces cerevisiae*; characterization of the secreted gene product. *Gene*. **85**, 545-551.