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Secretion of Active Urokinase-type Plasminogen Activator from the Yeast *Yarrowia lipolytica*

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Abstract In order to study the secretion of the human urokinase-type plasminogen activator, u-PA, from the yeast *Yarrowia lipolytica*, three kinds of integrative expression vector were constructed. These vectors differed only in their secretion control regions, pre-, pre-dip- (dipeptide stretch) or pre-dip-pro sequences of the alkaline extracellular protease, which were joined in-frame to the human u-PA cDNA. The recombinant *Y. lipolytica* strains, transformed with the expression vectors, secreted the hyperglycosylated u-PA. A fibrin plate assay of the culture supernatants showed that the hyperglycosylated u-PA proteins could catalyze fibrinolysis, and that the pre-dip sequence was the most efficient secretory signal for the secretion of the u-PA from *Y. lipolytica*. This result suggests that *Y. lipolytica* can be developed as a potential host for the production of recombinant human u-PA.

Keywords: *Yarrowia lipolytica*, urokinase-type plasminogen activator, secretion

Yeasts have been exploited for their secretion of a variety of biologically active proteins. However, these levels of secretion can range from tens of micrograms to over a gram per liter of culture broth, dependent on the kinds of protein and host organism. That is, the amount and quality of an expressed heterologous protein suffers from organism-specific limitations [1]. Despite similarities in the protein secretion mechanisms of different organisms, certain parts of the secretory pathways seem to be quite species-specific. Therefore, extensive development is required to attain high levels of secretion of important heterologous proteins from a specific organism [2,3].

Yarrowia lipolytica, a heterothallic yeast, is considered a good potential host for heterologous gene expression due to its ability to secrete large amounts of extracellular proteins [4-7] and its ease of cultivation to a high cell density [8-10]. In addition, *Y. lipolytica* may also be useful in the bioconversion of hydrophobic substrates because of its ability to degrade hydrophobic compounds, such as alkanes, fatty acids and oils [5,11,12]. Therefore, *Y. lipolytica* is a highly attractive yeast in the field of biotechnology.

Urokinase-type plasminogen activator (u-PA) is a serine protease that activates plasminogen to plasmin and causes fibrinolysis [13]. Human u-PA is initially synthesized as a single-chain u-PA (scu-PA, pro-urokinase), which is a glycoprotein containing 12 disulfide bonds, and the scu-PA is cleaved by plasmin into a two-chain u-PA (high molecular weight or low molecular weight tcu-PA), in which the two chains are held together by one disulfide bond [14]. Both scu-PA and tcu-PA can be used in thrombolytic therapy for patients with acute myocardial infarction. Therefore, u-PA has been expressed in *Escherichia coli* [15], *Saccharomyces cerevisiae* [16,17], *Pichia pastoris* [18,19] and mammalian cells [20]. In this report, we describe the secretory expression of human u-PA from *Y. lipolytica*.

In an attempt to secrete u-PA from *Y. lipolytica* we constructed three kinds of expression vector containing the human u-PA gene fused to three kinds of secretion sequence: the pre, pre-dipeptide stretches (pre-dip), and pre-dip-pro sequences of the alkaline extracellular protease (AEP), which is the most efficiently secreted protein from *Y. lipolytica*. These secretion sequences have frequently been used to direct the secretion of recombinant proteins from *Y. lipolytica* [6]. The pre, pre-dip, and pre-dip-pro sequences were amplified by PCR, using the primer sets Sig-For and Sig-Pre, Sig-For and Sig-Dip, and Sig-For and Sig-Pro, respectively (Table 1). The human u-PA gene was amplified by PCR from the plasmid pSM1 [19], using the primer sets Pre-PA2 and Ter-PA, Dip-PA2 and Ter-PA, and Pro-PA2 and Ter-PA (Table 1). Since 18 nucleotides at the N-termini of the amplified u-PA genes were complementary to those at the C-termini of the three secretion signal sequences, the pre, pre-dip, and pre-dip-pro sequences could be fused to the u-PA cDNA in-frame by PCR, using the primers Sig-For and Ter-PA. The three PCR products were inserted into a *Y. lipolytica* integration vector, pAU-Ar, thus generating three types of u-PA expression vector: pUpaX-Ar, pUpaY-Ar and pUpaZ-Ar, with the pre, pre-dip and pre-dip-pro regions, respectively (Fig. 1).

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Table 1. Strains, plasmids and primers used in this study

Strains	Characteristics	Source
SMS397A	*MATA ade1 ura3 xpr2*	[6]
YLUXIn	SMS397A harboring pUpaX-Ar	This study
YLUYIn	SMS397A harboring pUpaY-Ar	This study
YLUZIn	SMS397A harboring pUpaZ-Ar	This study
Plasmids		
pSM1	A plasmid carrying the human uPA gene	[19]
pAU-Ar	*Y. lipolytica* integration vector	Unpublished
pUpaX-Ar	pAU-Ar with pXPR2::pre::uPA::tXPR2	This study
pUpaY-Ar	pAU-Ar with pXPR2::pre-dip::uPA::tXPR2	This study
pUpaZ-Ar	pAU-Ar with pXPR2::pre-dip-pro::uPA::tXPR2	This study
Primers		
Sig-For	5'CCCTGTCAGATATCGTGAG3'	
Sig-Pre	5'GGCCAGAACGGCAGTGAG3'	
Sig-Dip	5'AGGCACAGCAGCAGGGGC3'	
Sig-Pro	5'TCGCTTGGCATTAGAAGAAG3'	
Pre-PA	5'CTCACTGCCGTTCTGGCCCCGAACGAACTGCATCAGGTTC3'	
Dip-PA	5'GCCCCTGCTGCTGTGCCTCCGAACGAACTGCATCAGGTTC3'	
Pro-PA	5'TCTTCTAATGCCAAGCGACCGAACGAACTGCATCAGGTTC3'	
Ter-PA	5'CCCTAAGCTTCATCAGAGGGCCAGGCCATT3'	

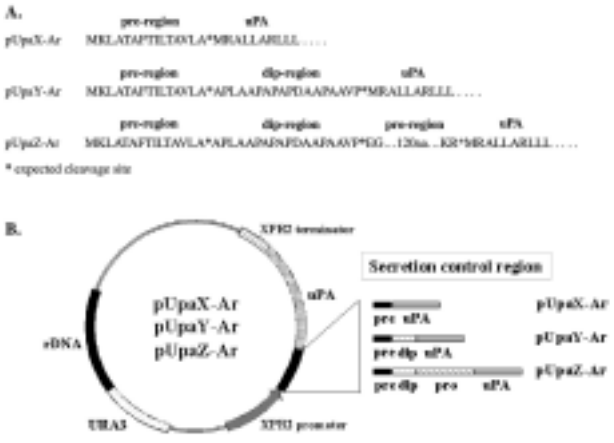

Fig. 1. (A) Amino acid sequences and expected cleavage sites for the pre-, dip-, and pro-regions of the *Y. lipolytica* AEP protein. (B) Schematic representation of the u-PA expression vectors, pUPaX-Ar, pUPaY-Ar and pUPaZ-Ar. The plasmid map is not drawn to exact scale.

Y. lipolytica SMS397A (*MATA ade1 ura3 xpr2*) was transformed with the circular expression vectors, and the colonies that had appeared on a synthetic complete medium without uracil (SC-Ura) were selected for further tests. The SC-Ura medium was composed of 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and drop-out amino acid mixture, including all the amino acids required [21]. Since the expression vectors were integration vectors targeting ribosomal DNA, we checked for the stable integration of these expression vectors into the ribosomal DNA of host chromosome using Southern blotting (data not shown). The strains transformed with pUpaX-Ar, pUpaY-Ar or pUpaZ-Ar, were designated YLUXIn, YLUYIn and YLUZIn, respectively.

To compare the secretion efficiency of each signal sequence, we selected six transformants (two strains from each recombinant strain), and investigated the amounts of u-PA secreted from the six recombinant strains. Since the band intensities of the u-PA gene in the Southern blotting of the selected transformants were similar (data not shown), they were considered to have the same copy number of the u-PA gene. The recombinant *Y. lipolytica* strains were cultivated for 48 h at 28°C in an induction medium, YPDm (0.2% yeast extract, 0.1% glucose and 5% proteose peptone in 50 mM sodium phosphate buffer at pH 6.8), and the culture supernatants were subject to SDS-PAGE analysis under non-reducing condition and immunoblot analysis. From the molecular weights of the processed or unprocessed u-PA from the SDS-PAGE analysis, we could not detect the discrete protein bands we expected. However, western blotting using goat anti-uPA immunoglobulin revealed that *Y. lipolytica* had secreted hyperglycosylated u-PA proteins of which the molecular weights were much higher than those of the non-glycosylated single chain u-PA (47 kDa) or the glycosylated one expressed in mammalian cells (53 kDa) (Fig. 2). The result also showed that the YLUXIn strain secreted much less u-PA than the YLUYIn and YLUZIn strains, indicating that the pre sequence was not suitable for the secretion of u-PA from *Y. lipolytica*.

To test the u-PA proteins secreted from the recombinant *Y. lipolytica* strains for biological activity, we performed a fibrin plate assay. Since active u-PA converts plasminogen into plasmin, which lyses fibrin clots, the appearance of a clear zone on a turbid fibrin plate con-

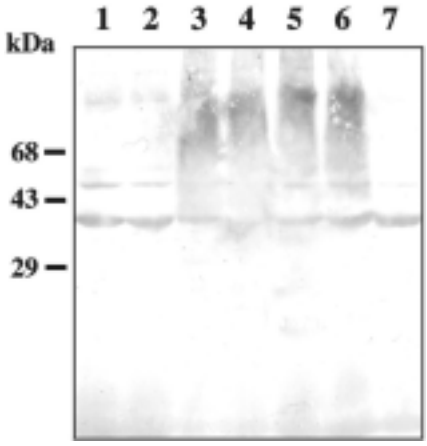

Fig. 2. Western blot analysis of the human u-PA proteins secreted from the recombinant *Y. lipolytica* strains. Culture supernatants were subject to SDS-PAGE (10%) under non-reducing conditions. The primary antibody used was goat IgG raised against human u-PA. Lanes 1 and 2, culture supernatant of the YLUXIn strain; lanes 3 and 4, culture supernatant of the YLUYIn strain; lanes 5 and 6, culture supernatant of the YLUZIn strain; lane 7, culture supernatant of the host strain (control).

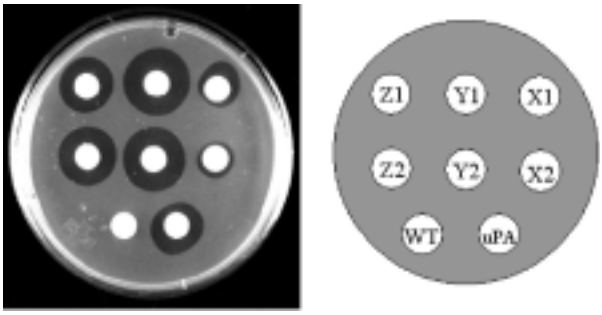

Fig. 3. A fibrin plate assay of the u-PA secreted from recombinant *Yarrowia lipolytica* strains. Culture supernatants (20 μL) of recombinant *Y. lipolytica* were dropped onto each paper disk. The fibrin plate was incubated at 37°C for 17 hrs. X1 and X2, culture supernatant of YLUXIn; Yl and Y2, culture supernatant of YLUYIn; Z1 and Z2, culture supernatant of YLUZIn; W, culture supernatant of the control host strain; uPA, about 60 IU of authentic u-PA (high molecular weight urokinase isolated from human urine).

taining plasminogen and fibrin is an indication of the presence of active u-PA in a test sample. As shown in Fig. 3, clear zones were formed around disk papers onto which the culture supernatants of the recombinant *Y. lipolytica* strains were loaded. The result indicated that the hyperglycosylated u-PA proteins secreted from the recombinant *Y. lipolytica* strains were active in catalyzing fibrinolysis. The sizes of these clear zones suggested that the pre sequence was the least efficient secretion leader for u-PA, which was in agreement with the result of the western blot analysis. When we simply compared the size of the halos, the pre-dip sequence seemed to be a better signal sequence than the pre-dip-pro sequence, for the secretion of the human u-PA. However, we do not currently know if the dipeptide stretches in the pre-dip sequences, which are cleaved by dipeptidyl aminopeptidase, were correctly processed in the secreted u-PA.

In this study, we tested three kinds of secretion signal sequence for their usefulness in the secretory production of human u-PA from *Y. lipolytica* and found that the pre-dip and pre-dip-pro sequences of AEP could efficiently direct the secretion of active u-PA proteins although they were hyperglycosylated. Since it has been reported that a non-glycosylated u-PA lacking an N-glycosylation site (u-PA-Q302), secreted from a yeast closely resembled natural u-PA with respect to its enzymatic activity and fibrinolytic properties [18], it will be interesting to investigate the secretion of u-PA-Q302 from *Y. lipolytica* using the pre-dip and pre-dip-pro sequences.

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