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Expression and Secretion of Heterologous Protein in Yeast

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To investigate the expression and the secretion of heterologous proteins in yeast, we constructed an yeast secretion vector and produced a human secretory protein, α 1-antitrypsin (α 1-AT), from yeast cells. The secretion vector pGAT8 was constructed by inserting the signal sequence of yeast acid phosphatase gene (*PHO5*) into the α 1-AT expression vector pGAT6 which contained α 1-AT cDNA fused to GAL10-CYC1 promoter. The α 1-AT was produced efficiently in the yeast cells transformed with plasmid pGAT8, which was confirmed both by the α 1-AT activity assay and by the immunoblot method using α 1-AT antibody. We also showed the secretion of α 1-AT into the culture media and into the periplasmic space by immunoblot.

KEY WORDS □ **Secretion, Heterologous protein, α 1-antitrypsin, Signal sequence, Yeast**

Yeast has been considered as an attractive host organism for the efficient expression of recombinant eucaryotic genes and the protein secretion. A number of human proteins of medical importance (for example, immunoglobulin, transferrin, α1-antitrypsin, growth hormone, trypsin, chymotrypsin, etc.) are secretory proteins and become glycosylated and post translationally processed to be active and mature forms. For the production of these secretory proteins, it is desirable to develop an heterologous gene expression and secretion system in yeast where secretion and glycosylation occur as in other higher eucaryotic cells and the secretion pathway has been thoroughly characterized both genetically and biochemically (7, 8).

To develop a good system for the expression and the secretion of heterologous proteins in yeast, we used human α 1-antitrypsin (α 1-AT) as a model protein. α 1-AT is a 394 amino acid glycoprotein which is primarily synthesized and glycosylated in the liver and is secreted into plasma. α 1-AT is an inhibitor of serine proteases and its major physiological function is to inhibit neutrophil elastase in the lung (1). Genetic deficiencies in α 1-AT concentration in blood cause life-threatening emphysema.

Previously we developed an yeast expression system of α 1-AT by constructing an α 1-AT expression vector and a suitable host yeast strain (4). In our present study, we constructed an α 1-AT secretion vector by employing a signal sequence of yeast acid phosphatase gene (*PHO5*) and succeeded in α 1-AT production and secretion from yeast cells.

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MATERIALS AND METHODS

Strains and plasmids

The yeast strain used in this study is *Saccharomyces cerevisiae* KY8 (*MATa, ura3-52, lys2-801, ade6* or *2, Gal*$^+$) which was described previously (4). *Escherichia coli* JM109 [*recA1, endA 1, gyrA96, thi, hsdR17, supE44, relA1, strA, sbcBΔ (lac, proAB), F' (traD36, proAB, lacI*^{q}*, lacZ M15)*] was used for bacterial transformation and plasmid growth. Plasmids pGAT6 and pGAT7 were described previously (4).

Media and growth conditions

Yeast media and culture conditions were as described by Sherman et al. (9). YEPD contained 1% yeast extract, 2% peptone and 2% dextrose. YNB contained 0.67% yeast nitrogen base w/o amino acid and 2% dextrose. SC-Ura is a synthetic copmlete medium without uracil (YNB with all amino acids added except uracil). SC-Ura was used for the cultivation of strain KY8 harboring plasmid. Galactose (2%) was added instead of glucose in case of induction with galactose.

Transformation and DNA manipulation techniques

Yeast transformation was performed by the lithium acetate method (2) and *E. coli* transformation was by the method of Mandel and Higa (5). Plasmid DNA from *E. coli* was isolated by the modified method of alkaline lysis (6). Restriction endonuclease analysis and agarose gel elctrophoresis were carried out as described in Maniatis *et al.* (6).

Preparation of crude yeast cell extracts

Strain KY8 harboring plasmid pGAT6, pGAT7, or pGAT8 was grown in SC-Ura containing glucose and was subsequently transferred to either galactose or glucose medium. Fifty milliliters of yeast culture ($O.D._{600}=1.0$) was harvested, washed, and resuspended in 200 μl of assay buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl). This cell suspension was treated with 0.45 g of glass beads for the cell-lysis and centrifuged at 15,000 rpm for 20 minutes. The supernatants were saved for the enzyme assay.

Assay for the α 1-AT activity

The activity of α 1-AT was measured as elastase inhibitory activity. The assay was performed with slight modification on the method of Travis and Johnson (12). Fifty microliters of elastase (0.18 mg/m*l*) and the inhibitor (cell extracts or α 1-AT) were mixed in the assay buffer to make a volume of 500 μl and were incubated for 1 min. Twenty microliters of 40 mM substrate, succinyl-(L-alanyl) 3-p-nitroanilide, and 480 μl of assay buffer were added and mixed well. The increase in absorbance at 410 nm was measured for 1 min. The human α 1-AT, porcine elastase, and succinyl-(L-alanyl)$_3$-*p*-nitroanilide were purchased from Sigma Chemical Company.

Immunoblot

SDS-polyacrylamide gel electrophoresis was carried out as the method of Laemmli (10) and immunoblot procedure was essentially same as that of Towbin *et al.* (11). Total yeast proteins for electrophoresis were isolated by precipitation with trichloric acid (TCA) after lysis with glass beads. Anti-α 1-AT rabbit antibody and peroxidase-conjugated anti-rabbit IgG goat antibody were purchased from Sigma Chemical Company.

Cell fractionation for secretion assay

Fifty milliliters of yeast culture grown to log phase ($O.D._{600}=1.0$) was centrifuged at 5,000 rpm for 5 min. The supernatant was concentrated to 4 m*l* by ultrafiltration and saved as media fraction. Cells in the precipitant were resuspended in 4 m*l* of solution (12.5 mM Tris-HCl, 5 mM NaN_3, 1.4 M sorbitol, 25 mM phosphate buffer) and 8 μl of β-mercaptoethanol and 20 μl of zymolyase (100T, Kirin Company) solution (2 mg/m*l*) were added subsequently. After incubation at 30°C for 1 hr, this cell mixture was centrifuged at 2,000 rpm for 5 min. The supernatant was saved as periplasmic fraction and the precipitant was used as internal fraction after being resuspended in cell lysis buffer (1% Triton X100, 25 mM Tris-HCl, pH 7.5) or electrophoresis sample buffer.

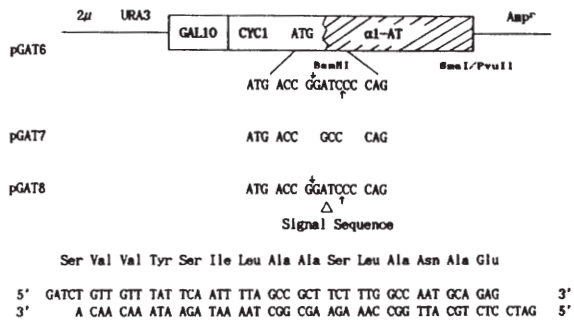

Fig. 1. *Construction of a secretion vector, pGAT8.* Plasmid pGAT6 contained 1.3 kb BamHI-SmaI restriction fragment of α 1-AT cDNA fused to the GAL10-CYC1 hybrid promoter (4). A secretion vector pGAT8 was constructed by inserting a synthetic oligonucleotide encoding the signal sequence of yeast acid phosphatase (*PHO5*) at a unique BamHI site of plasmid pGAT6. Plasmid pGAT7 is α 1-AT expression vector derived from plasmid pGAT6 by removing the BamHI site with SI nuclease to adjust the reading frame.

RESULTS AND DISCUSSION

Construction of α 1-AT secretion vector

The efficiency of protein secretion in yeast is known to be governed primarily by the amino-terminal signal sequences found in precursors of secreted proteins (3). Therefore, to direct a secretion of heterologous protein from yeast cells, we constructed a secretion vector in such a way that a signal sequence of a known yeast secretory protein could be located at the amino-terminal of the heterologous protein. Plasmid pGAT6 constructed in a previous work (4) carries 1.3 kb BamHI-SmaI restriction fragment of α 1-AT cDNA under the regulation of the GAL10-CYC1 hybrid promoter and has a unique BamHI site between the translation initiation codon and the first amino acid codon of the mature form of α 1-AT (Figure 1). The coding region of α 1-AT in pGAT6 is out of frame with the initiation methionine codon. Plasmid pGAT7 is the α 1-AT expression vector in which the reading frame was adjusted by using S1 nuclease (4). To construct α 1-AT secretion vector, we inserted the signal sequence of yeast acid phosphatase gene (*PHO5*) at BamHI site of pGAT6, producing GAL/PHO5/α 1-AT fusion gene. The *PHO5* signal sequence used in this study was a DNA oligonucleotide

Table 1. *Synthesis of α 1-AT assayed by the inhibition of elastase activity*

Cell extract (100 μl)	OD*	% of Elastase activity remained
Control	0.375	100.0
pGAT6(glu)	0.305	81.3
(gal)	0.325	86.6
pGAT7(glu)	0.356	94.9
(gal)	0.205	54.6
pGAT8(glu)	0.290	77.3
(gal)	0.167	44.5

*OD value indicates the elastase activity assayed by measuring the increase in absorbance of the reaction mixture at 410 nm for 1 min.

of 47 bp which was synthesized according to the known amino acid sequence. The secretion vector with GAL/PHO5/α 1-AT fusion gene was named as pGAT8.

Synthesis of α 1-AT by the secretion vector

Yeast strain KY8 was transformed with the secretion vector pGAT8 and was induced in galactose medium in order to examine the expression of α 1-AT in yeast cells. The crude yeast cell extracts were prepared for the α 1-AT activity assay. As summarized in Table 1, cell extracts prepared from yeast strain carrying plasmid pGAT8 grown under the induced condition (galactose medium) inhibited about 55% of the elastase activity. Cell extracts from the same strain grown under the uninduced condition (glucose medium) showed about 80% of elastase activity, indicating that the inhibition was negligible. Cell extracts from the strain carrying the control plasmid pGAT6 grown under either the induced or the uninduced condition also showed 80-90% of elastase activity. These results suggest that the newly constructed secretion plasmid pGAT8 were efficient in production of human α 1-AT in yeast cells. According to the α 1-AT activities in Table 1 and the results from our repeated experiments, the α 1-AT expression by plasmid pGAT8 seemed to be at the almost same level as or at a slightly higher level than that of the previously reported expression plasmid pGAT7.

To examine the production of α 1-AT polypeptides by the secretion vector, total proteins isolated from the cell extracts were separated on SDS-polyacrylamide gel electrophoresis and analyzed by the immunoblot method using antibodies to human α 1-AT (Figure 2). Plasmid pGAT8 expressed polypeptides of 44 kDa under the induced condition but not under the uninduced condition. The 44 kDa protein produced by pGAT8 was similar in size to that produced by pGAT7. However, the amount of

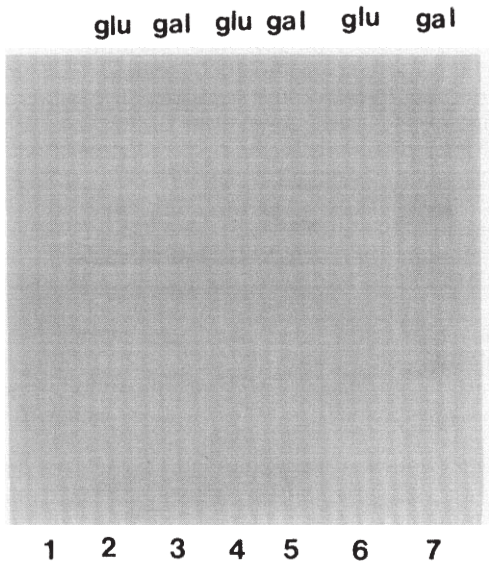

Fig. 2. *Immunoblot showing the synthesis of α 1-AT by the secretion vector.*
Lanes contain total proteins prepared from yeast culture grown either in glucose medium (Glu, uninduced condition) or in galactose medium (Gal, induced condition). Lane 1, authentic human α 1-AT ; lanes 2 and 3, proteins from a strain carrying the control plasmid pGAT6; lanes 4 and 5, proteins from a strain carrying the expression plasmid pGAT7; lanes 6 and 7, proteins from a strain carrying the secretion plasmid pGAT8.

polypeptides produced by pGAT8 seemed to be greater than that by pGAT7. These results are in good agreement with the results of the activity assay. We assume that the α 1-AT expression by plasmid pGAT8 was more efficient than that by plasmid pGAT7. The control vector pGAT6 showed no protein band under both induced and uninduced conditions.

Secretion of α 1-AT from yeast cells

We asked if α 1-AT produced by the secretion vector pGAT8 was secreted from yeast cells to the culture media or the periplasmic space. The yeast culture grown under the induced condition was fractionated to the culture media, the periplasmic space, and the internal fraction. When the presence of α 1-AT in each fraction was examined by the immunoblot using α 1-AT antibody, we could observe in the medial and the periplasmic space fractions a protein band of the same molecular weight as that in the internal fraction (Figure 3). However, when the α 1-AT activity was measured in each fraction, the activity was detected only in the internal fraction. We assume that a small portion of α 1-AT synthesized

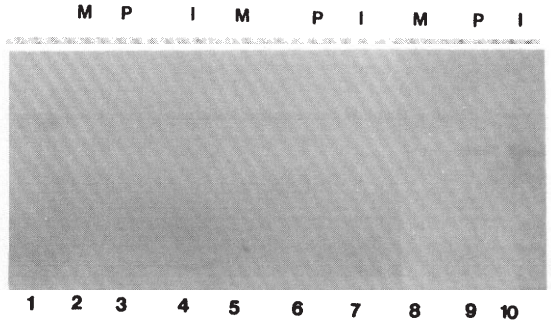

Fig. 3. *Immunoblot showing the secretion of α 1-AT by the secretion plasmid.*
Yeast cultures grown under the induced condition were fractionated to the culture media (M), the periplasmic space (P), and the internal fraction (I). Lanes 1, authentic human α 1-AT; lanes 2-4, cell fractions from a strain carrying the expression plasmid pGAT7; lanes 5-10, cell fractions from a strain carrying the secretion plasmid pGAT8, lanes 5-7 and lanes 8-10 are results from two independent experiments.

was secreted into the culure media and into the periplasmic space, which can be shown by the immunoblot. For the activity assay, the procedure itself requires large a amount of α 1-AT in the reaction mixture and the amount of secreted protein was not enough to see the presence of the enzyme activity as compared to that of the internal fraction. Cell fractions prepared from yeast strain carrying the expression plasmid pGAT7 did not show the α 1-AT protein band either in the media nor in the periplasmic space, indicating the specificity of the secretion signal.

Since the yeast acid phosphatase is normally targeted to the plasma membrane, the α 1-AT fused to the *PHO5* secretion signal was expected to be secreted mainly to the periplasmic space. However, we achieved secretion of α 1-AT from yeast cells into the media as well as in the periplasmic space. The migration of secreted α 1-AT in the SDS-PAGE gel was almost same as that of internal α 1-AT and was faster than purified authentic human α 1-AT. This result is probably due to the lack of glycosylation. The polypeptides went through the yeast secretion pathway but may have not been subjected to the glycosylation process. We are currently in the process of constructing secretion vectors using various other secretion signals in order to increase the efficiency of secretion and to get active proteins with proper postranslational modifications.

REFERENCES

1. **Marrell, R. W., J.-O. Jeppsson, C.-B. Laurell, S. O. Brennan, M. C. Owen, I. Vaughan, and D. R. Boswell,** 1982. Structure and variation of human α-1-antitrypsin. *Nature* **298**, 329-334.
2. **Ito, H., Y. Fukada, K. Murata, and A. Kimura,** 1983. Transformation of intact yeast cells with alkali cation. *J. Bacteriol.* **153**, 163-168.
3. **Kaiser, C.A., D. Preuss, P. Grisafi, and D. Botstein,** 1987. Many random sequences funtionally replace the secretion signal sequence of yeast invertase. *Science* **235**, 312-317.
4. **Kim, J., K. N. Lee, and M.- H. Yu,** 1990. Cloning and expression of human α 1-antitrypsin cDNA in yeast *Saccharomyces cerevisiae. Kor. J. Biochem.* **23**, 263-268.
5. **Maniatis, T., J. Sambrook, and E. F. Fritsch,** 1989. Molecular cloning. 2nd ed. Cold Spring Harbor Lab. Cold Spring Harbor, N. Y.
6. **Mandel, M. and A. Higa,** 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**, 159-162.
7. **Novick, P., S. Ferro, and R. Schekman,** 1981. Order of events in the yeast secretory pathway. *Cell* **25**, 461-467.
8. **Schekman, R. and P. Novick,** 1981. The secretory process and yeast cell-surface assembly. *In* The Molecular Biology of the Yeast *Saccchromyces.* Vol. 2 Cold Spring Harbor Lab. Cold Spring Harbor, N. Y.
9. **Cherman, F., G. R. Fink, and C. W. Lawrence,** 1979. Methods in yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
10. **Laemmli, U. K.,** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**, 680-685.
11. **Towbin, H., T. Staehelin and J. Gordon,** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
12. **Travis, J. and Jonson,** 1982. Human α 1-protease inhibitor. Methods in Enzymology **80**, 754-765.

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초 록: 효모에서 이종단백질의 발현과 분비

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이종단백질을 효모에서 발현시키고 분비시키기위한 목적으로 효모 분비벡터를 제조하여 인체 분비단백질인 알파원앤티트립신을 효모에서 생성하고 그의 분비를 확인하였다. 분비벡터 pGAT8은 효모의 acid phosphatase (*PHO5*) 유전자의 분비 신호서열을 알파원앤티트립신 발현벡터 pGAT6에 삽입하여 GAL/PHO5/α1/-AT 융합유전자가 되도록 제조되었다. 이 분비벡터로 형질전환시킨 효모균주에서 알파원앤티트립신이 생성됨을 활성도 측정및 면역블럿 방법으로 확인하였으며 생성된 알파원앤티트립신의 일부가 배양액및 peripliasmic space로 분비됨을 면역블럿 방법으로 보일 수 있었다.