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The Glucoamylase Signal Sequence Directs the Efficient Secretion of Human α1-Antitrypsin in Yeast Cells

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Five different secretion vectors were constructed by varying the signal sequences and α1-antitrypsin (α1-AT), a human secretory protein, was produced from yeast cells. The signal sequences used are those of acid phosphatase (*PHO5*) and α-factor (*Mfα1*) of *Saccharomyces cerevisiae*, glucoamylase (*STA1*) of *Saccharomyces diastaticus*, and human α1-AT. Four vectors directed the efficient secretion of α1-AT into the culture media. The secretion vector carrying the glucoamylase signal sequence (pGAT11) showed the highest efficiency of secretion. About 70% of α1-AT produced were secreted into the media. The endo H treatment of partially purified α1-AT indicates that the secreted α1-AT appeared to be glycosylated. This glycosylation pattern was altered when amino acid substitution mutations were introduced at the three glycosylation sites of α1-AT.

KEY WORDS □ **secretion, signal sequence, α1-AT, glucoamylase**

The efficient secretion of heterologous protein from yeast cells has been of interest for many years mainly due to the facts that a number of human proteins of medical importance are secretory proteins and that the yeast secretory pathway is very similar to that observed in higher eukaryotic cells (15). The yeast secretory protein is initially translated as a precursor form carrying an amino-terminal signal sequence and become post-translationally processed and glycosylated to be an active and mature form (1, 4). This process occurs through the secretory pathway which involves a series of intracellular organelles such as endoplasmic reticulum, Golgi complex, and secretory vesicles (13). The efficiency of protein secretion in yeast is reported to be governed partly by the nature of the signal sequence which directs the translocation of the precursor across the ER membrane. The hydrophobicity and the charge density of the signal sequence are the main factors for the secretion efficiency (6).

In our previous work, we developed an yeast secretion vector by using the signal sequence of yeast acid phosphatase and succeeded in production and secretion of human α1-antitrypsin (α1-AT) from yeast cells (8). In our present study, various signal sequences such as those of yeast glucoamylase, α-factor, and human α1-AT were employed in order to increase the efficiency of secretion and to get active proteins with proper post-translational modifications. The approaches of varying or mutagenizing secretion signals have been described by other groups (3, 17, 19). Increased secretion of heterologous protein from yeast cells has been reported by identifying oversecreting mutant strains (18, 19). Mutations in *PMR1* encoding *p*-type ATPase was also shown to enhance the secretion of bovine growth factor (18). We report here the highly efficient secretion of heterologous protein directed by the glucoamylase signal sequence of *Saccharomyces diastaticus*.

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MATERIALS AND METHODS

Strains and plasmids

The yeast strains used in this study are *Saccharomyces cerevisiae* KY8 (*MATa, ura3-52, lys2-801, ade6* or *2, Gal*$^+$) (7) and *Saccharomyces diastaticus* YIY345 (*MATα, ura3, leu2-3,112, his4, sta*^{0}, *inh*^{0}) (20). *Escherichia coli* strains HB101 and JM110 were used for bacterial transformation and plasmid propagation.

Plasmid pGAT8 was described previously (8). Plasmid pGAT9 was constructed by isolating the *Eco*RI-partial-*Sal*I restriction fragment containing α1-AT cDNA from plasmid pUC-AT(R) (10) and

inserting it between *Bam*HI and *Sal*I sites of plasmid pCGS109 (gift of G. R. Fink). The *Bam*HI-*Sma*I restriction fragment of pUC-AT(R) was ligated into *Bam*HI and *Sma*I sites of plasmid pYESC25 to generate plasmid pGAT10. Plasmid pGAT10 was digested with *Bam*HI and was treated with mung bean nuclease to construct plasmid pGAT11. Plasmid pGAT12 was constructed by inserting the *Stu*I-*Bst*EII fragment of pGAT11 into *Bam*HI and *Bst*EII sites of pGAT6 (7). Plasmid pGAT13 carries a 50-bp oligonucleotide containing α-factor signal sequence at the *Bam*HI site of pGAT6. Plasmids pGAT11-N46Q, pGAT11-N83Q, pGAT11-N247Q, pGAT11-NtripleQ were constructed by inserting the *Bcl*I-*Sma*I fragment of pEAT8 carrying each mutation into *Bcl*I-*Sma*I sites of pGAT11. The substitution mutations at the three Asn positions were obtained by oligonucleotide-directed mutagenesis. Details of mutagenesis will be described elsewhere (9).

Media and growth conditions

Yeast media and culture conditions were as described by Sherman *et al.* (16). YEPD contained 1% yeast extract, 2% peptone and 2% dextrose. YNB contained 0.67% yeast nitrogen base without amino acids and 2% dextrose. SC-Ura is a synthetic complete 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 (5) and *E. coli* transformation was by the method of Mandel and Higa (12). Plasmid DNA from *E. coli* was isolated by the modified method of alkaline lysis (14). Restriction endonuclease analysis and agarose gel elctrophoresis were carried out as described in Sambrook *et al.* (14).

Secretion assay

Fifty milliliters of yeast culture grown to $O.D._{600}$ =2.0 was centrifuged at 5,000g for 5 min. The supernatant was concentrated to 5 m*l* by ultrafiltration (Amicon YM10 membrane) and saved as media fraction. Cells in the precipitant were resuspended in 0.5 m*l* of solution (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl) and lysed with glass beads. Cell lysates were centrifuged at 12,000g for 20 min and the supernatants were used as the internal fraction. The assay for the α1-AT activity and the immunoblot analysis were performed essentially same as described previously (8).

Partial purification of secreted α1-AT

Yeast strain YIY345 with the secretion vector pGAT11 was grown in 10 m*l* of YNB+His+Leu media for 16~18 hrs to maintain the vector and subsequently transferred to 100 m*l* of YEPD media. Cultures grown for 24 hrs were centrifuged at 4,000g for 10 min and the supernatant was concentrated to 2 m*l* by ultrafiltration (Amicon PM 10 membrane). Ion exchange chromatography (DEAE-celluose) and FPLC (GPC) system were used to purify α1-AT. This partially purified α1-AT was used for Endo H treatment.

RESULTS AND DISCUSSION

Construction of secretion vectors carrying various signal sequences

To improve the efficiency of heterologous protein secretion from yeast cells, we employed four different signal sequences for the construction of secretion vectors (Fig. 1). These secretion signals were from the glucoamylase gene (*STA1*) of *Saccharomyces diastaticus*, acid phosphatase (*PHO 5*) and α-factor (*Mfα1*) genes of *Saccharomyces cerevisiae*, and human α1-antitrypsin (α1-AT) gene. α1-AT is an abundant human serum protein produced and secreted by liver cells (2). α1-AT is a glycoprotein of 394 amino acids and functions as a serine protease inhibitor. We used α1-AT as a heterologous protein for its expression and secretion in yeast. The use of α1-AT's own signal sequence was to see if a human secretion signal functions properly in yeast cells as well.

Secretion vector pGAT8, developed in our previous work (8), carries α1-AT cDNA ligated to the secretion signal of yeast acid phosphatase gene (*PHO5*) whose expression is under the control of yeast GAL10-CYC1 promoter. Vector pGAT9 carries human α1-AT's own signal sequence. This vector was constructed by inserting 1.3 kb *Eco*RI fragment of α1-AT cDNA retaining the translation initiation codon, ATG, and the signal sequence into the yeast expression vector pCGS109 carrying GAL1-10 promoter. Vectors

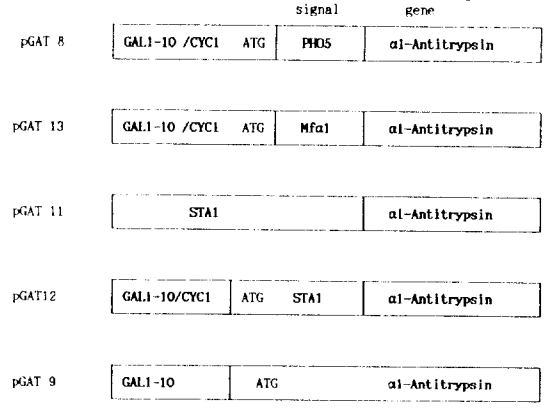

Fig. 1. *Components of the secretion vectors constructed.*

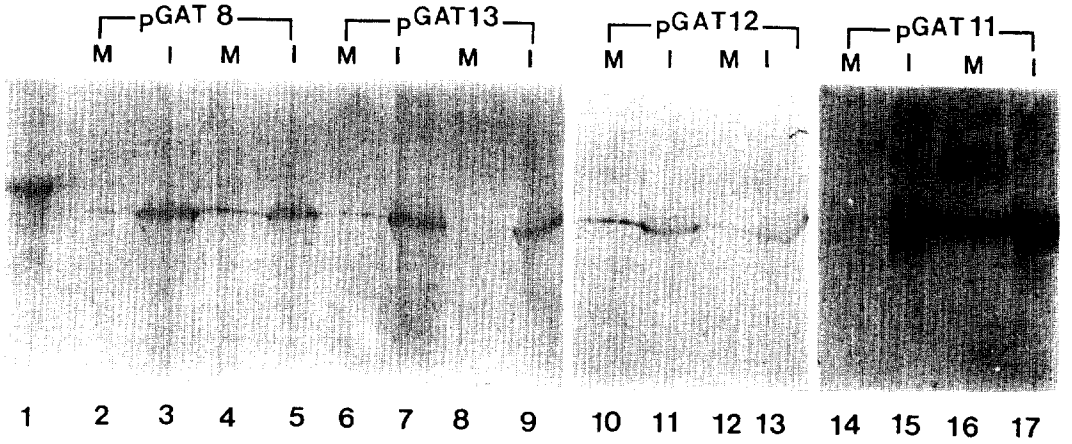

Fig. 2. *Immunoblot analysis of α1-AT secretion.*
Lane 1, authentic human α1-AT; lane 2-5, the media (M) and the internal (I) fractions of yeast culture with vector pGAT8 when O.D.=1.0 (2, 3) or 2.0 (4, 5); lane 6~9, the media (M) and the internal (I) fractions of yeast culture with vector pGAT13 when O.D.=1.0 (6, 7) or 2.0 (8, 9); lane 10~13, the media (M) and the internal (I) fractions of yeast culture with vector pGAT12 when O.D.=1.0 (10, 11) or 2.0 (12, 13); lane 14~17, the media (M) and the internal (I) fractions of yeast culture with vector pGAT11 when O.D.=1.0 (14, 15) or 2.0 (16, 17).

pGAT11 or pGAT12 has the signal sequence of glucoamylase with its own promoter or GAL10-CYC1 promoter, respectively (20). Vector pGAT13 carries the signal sequence of α-factor. A 50-bp oligonucleotide synthesized according to the known amino acid sequence of the α-factor signal sequence was inserted into the *Bam*HI site of plasmid pGAT6 (7).

Secretion of α1-AT directed by secretion vectors

Five secretion vectors constructed were introduced into host yeast strains by transformation in order to examine the efficiency of the expression and the secretion of α1-AT in yeast cells. *S. cerevisiae* strain KY8 was used for all vectors but except pGAT11 carrying *STA1* promoter which was transformed into *S. diastaticus* strain YIY345. The yeast transformants were grown in galactose media until $OD_{600}=2.0$ for the induction of GAL promoter (for *S. diastaticus* with vector pGAT11 YEPD media was used). The cultures were fractionated into the media and the internal fractions by centrifugation. The α1-AT produced and secreted successfully from yeast cells would be detected in the media fraction.

Total proteins from each fraction were subjected to the immunoblot analysis using α1-AT antibody. As shown in Fig. 2, secretion vectors pGAT8, pGAT11, pGAT12, and pGAT13 showed polypeptide bands interacting specifically with α1-AT antibody in the media fractions as well as in the internal fractions, indicating that α1-AT was secreted efficiently into the media. Vector pGAT9 showed extremely low level of expression and secretion (data not shown). Vector pGAT11 carrying the signal sequence of glucoamylase gene (*STA1*) appeared to show the highest efficiency of the secretion. Approximately two thirds of α1-AT polypeptides were detected in the media fraction (Fig. 2, lane 16~17). The size of the secreted polypeptides seems to be larger than that of the internal [illegible]es and almost same as that of authentic human α1-AT, suggesting that the secreted polypeptides were glycosylated.

The secretion of α1-AT into the media was also examined by [illegible]T activity assay. As summarized in Table 1, the media fraction of pGAT11 and pGAT12 showed drastic inhibition of elastase activity (100% and 50% inhibition, respectively), which is greater than the internal fraction. These results are in good agreement with those from immunoblot method and indicate that the newly constructed secretion vectors carrying the glucoamylase signal sequence are highly efficient in secretion of heterologous protein from yeast cells.

Glycosylation of secreted protein

Human α1-AT is known to be glycosylated at three sites, Asn46, 83, and 247. We asked if α1-AT secreted from yeast cells carrying vector pGAT11 was glycosylated. α1-AT polypeptides in the media were partially purified by utilizing ion exchange chromatography (DEAE-cellulose) and FPLC (GPC) and were treated with endo-β-N-acetylglucosaminidase H (endo H) which catalyzes the cleavage of N-linked oligosaccharides. As a result, a protein band of smaller size appeared after the endo H reaction (Fig. 3). This result suggests that the secreted protein of the

Table 1. *Secretion of α1-AT analyzed by α-AT activity assay.*

Internal (I) or media (M)		OD	% of Elastase activity remained
Control		0.040	100
pGAT6	(I)	0.040	100
	(M)	0.040	100
pGAT8	(I)	0.026	66
	(M)	0.040	100
pGAT13	(I)	0.027	67.5
	(M)	0.040	100
pGAT12	(I)	0.035	87.5
	(M)	0.020	50
pGAT11	(I)	0.036	90
	(M)	0.000	0

50 μl of cell extracts was added to the reaction mixture. OD value indicates the elastase activity assayed by measuring the increase in absorbance of the reaction mixture at 410 nm for 1 min. Absorbance was measured when quantity of protein is 1.

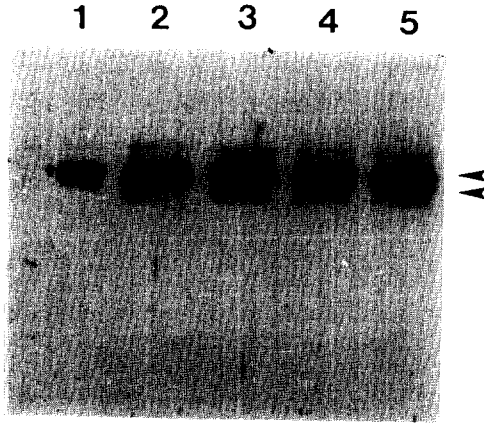

Fig. 3. *Deglycosylation of partially purified α1-AT by a treatment with endo-H.*
Lane 1, authentic human α1-AT; lane 2, 0 mU; lane 3, 1.25 mU; lane 4, 2.5 mU; lane 5, 5 mU.

same size as authentic human α1-AT was a glycosylated form. With the increasing amount of endo H treated, the band of the smaller size (deglycosylated form) became thicker.

Mutational analysis of glycosylation of secreted α1-AT

To investigate the proper glycosylation of α1-AT produced and secreted by our secretion vector, we cloned into the secretion vector pGAT11 the mutant forms of α1-AT gene in which amino acid residues at the known N-linked glycosylation sites,

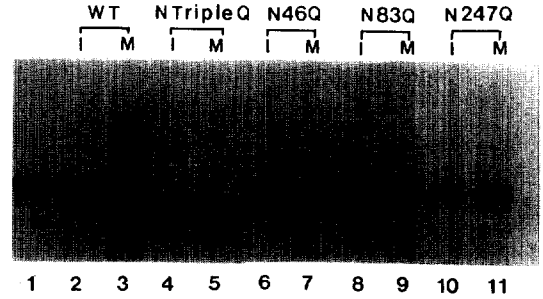

Fig. 4. *Immunoblot analysis of mutant α1-AT with amino acid substitutions at the glycosylation sites.*
Lane 1, authentic human α1-AT; lane 2,3, the internal (I) and the media (M) fractions of yeast culture with vector pGAT11 containing the wild type α1-AT; lane 4, 5, yeast culture with mutant vector pGAT11-NtripleQ; lane 6, 7, yeast culture with mutant vector pGAT 11-N46Q; lane 8, 9, yeast culture with mutant vector pGAT11-N83Q; lane 10, 11, yeast culture with mutant vector pGAT11-N247Q.

Asn46, Asn83, or Asn247, were substituted with Gln residues. These substitution mutations were generated such that each mutation could be identified by the presence of the unique restriction enzyme site. Three single-mutation vectors, pGAT 11-N46Q, pGAT11-N83Q, and pGAT11-N247Q, and one triple-mutation vector pGAT11-NtripleQ were constructed and were transformed into the host yeast strain.

α1-AT polypeptides secreted from yeast cells carrying these mutant vectors were analyzed by the immunoblot method. As shown in Fig. 4, the polypeptide bands of the mutant α1-AT in the media fraction were smaller in size than the wild type and were almost same as the internal nonglycosylated form. The reduction in the band size was the most apparent with the triple mutant. These results indicate that α1-AT secreted from yeast cells by our secretion vector pGAT11 was glycosylated at the putative sites which have been reported to be the natural N-linked glycosylation sites of human α1-AT (11).

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초 록: 효모에서 Glucoamylase 신호서열에 의한 인체 α1-Antitrypsin의 분비 효율 향상

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*Saccharomyces cerevisiae*의 acid phosphatase (*PHO5*)와 α-factor (*Mfα1*), *Saccharomyces diastaticus*의 glucoamylase (*STA1*), 그리고 인체 알파원앤티트립신 등의 분비 신호서열을 이용한 다섯가지의 분비벡터를 제조하여, 인체 분비단백질인 알파원앤티트립신을 효모에서 효과적으로 분비시킬 수 있었다. 네 가지 벡터가 알파원앤티트립신을 세포밖의 배양액으로 분비시켰으며, 이중 glucoamylase 분비 신호서열을 갖는 pGAT11 벡터의 분비효율이 가장 높아 생성된 단백질의 약 70%가 분비됨을 확인할 수 있었다. Endoglycosidase H 효소를 처리함으로써 분비된 알파원앤티트립신이 N-linked glycosylation되었음을 알 수 있었고, 알파원앤티트립신의 glycosylation되는 위치로 알려진 Asn 잔기를 Gln 잔기로 치환한 돌연변이들을 도입하였을 때 glycosylation되지 않음을 보여주었다.