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재조합 플라스미드 포함 효모와 염색체 삽입 효모에서의 외래 Glucoamylase의 분비 특성

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Secretion Characteristics of Foreign Glucoamylase from Recombinant Plasmid-Harboring and Chromosome-Integrated *Saccharomyces cerevisiaes*

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ABSTRACT

Secretion efficiency is generally affected by promoter, signal sequence, characteristics of foreign protein and host. Secretion efficiencies of glucoamylase in recombinant plasmid-harboring yeast and chromosome-integrated yeast which had STA signal sequences were 74% and 65% at the 4th day of incubation, respectively. The high secretion efficiencies of the yeasts were obtained due to the fact that the expression levels were not reached at the secretory apparatus capacities of the host yeasts. In both yeasts, most of the intracellular glucoamylase were detected in cytoplasm and small portion (below 10%) of glucoamylase were located in periplasm. The characteristics of secreted heterologous glucoamylase from recombinant *Saccharomyces cerevisiae* were investigated by using Western blot analysis. The secreted mature glucoamylase was heterogeneous and its molecular weight was about 200 to 300 kilodalton. The carbohydrate content of mature glucoamylase was higher than 80%, and several bands of about 55 to 65 kilodalton indicate the endoplasmic reticulum forms of intracellular glucoamylase.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* has been often used as one of recombinant hosts that express foreign genes and secrete proteins because of its advantages(1). Glucoamylase (E. C. 3.2.1. 3) which saccharifys starchy feedstocks in commercial processes for glucose and ethanol productions, is not synthesized by *S. cerevisiae*. Having this in mind, the glucoamylase gene of *Saccharomyces diastaticus STA* was chosen as a glucoamylase gene source(2) for this research. The *STA* gene which was used in this work was cloned from a wild type *S. diastaticus* DS101. In our previous study, the multi-copy recombinant plasmid which contained fused *SUC2-STA* gene was constructed and expressed sucessfully(3).

When working with eucaryotic organism such

as yeast, recovery of desired intracellular product is difficult due to its thick cell wall. In recent years, the researches have been therefore performed to find efficient means to secrete the desired product to the culture broth. The secretion of the target product is highly desirable from a biotechnological point of view for several reasons such as simplicity of recovery and purification of product, capability of post-translational modification, prohibition of cell growth inhibition by toxic product and prerequisition for the development of more efficient immobilized cell bioreactors. Because the secretion mechanism of yeast is similar to that of mammalian cells(4), yeast is a useful microorganism for large-scale production of mammalian proteins which requires post-translational modification such as glycosylation and phosphorylation. Most of the secreted proteins in yeast are glycosylated and have different secretion characteristics depending on the target protein. The factors which affect the localization of secreted proteins are still not understood exactly. However, it is certain that the secretion signal sequence used for secretion of protein plays a very important role in localization of secreted proteins. The signal sequences which are widely used for secretion of protein for a recombinant yeast are such as *SUC2*(5), *PHO5*(6), Killer toxin(7), *MFα1* (8) etc. Secretion efficiency is known to be affected by the promoter used, secretion signal sequence, size, net charge and degree of glycosylation of secreted protein, and a kind of used host strain(8). In the case of a protein of which molecular weight was higher than 50,000 dalton in yeast, secretion efficiency was decreased and most of protein were accumulated in the cell(15). Smaller proteins such as β-endorphin (31 amino acids) and calcitonin (32 amino acids) were secreted efficiently into the culture broth, but larger proteins such as α-interferon (166 amino acids) were secreted into the culture broth in relatively small amounts through the cell wall(8). Vanoni and coworkers(16) reported the expression of *S. diastaticus* glucoamylase fused with the original *STA* promoter and strong, inducible *GAL1-10* (galactose dehydrogenase) promoter which was induced by galactose addition in the culture medium. The secretion efficiency was about 30~40% in the case of the original *STA* promoter (data on secretion efficiency were recalculated). In case of *GAL1-10* promoter, the level of glucoamylase activity was lower and the secretion efficiency was increased and detected at about 50%. The secretion efficiency of invertase was 21.6% in recombinant yeast that contained the *SUC2* structural gene, *MFα1* promoter and *MFα1* signal sequence(5). And in case of recombinant yeast that contained whole *SUC2* gene (*SUC2* signal sequence used), the secretion efficiency was 12.4%. The secretion efficiency of recombinant yeast containing *Bacillus* α-amylase, *MFα1* promoter and *MFα1* signal sequence was detected about 55% at 4th day and about 67% at 8th day of cultivations(17). Also, the human α-antitrypsin fused with *SUC2* signal sequence and yeast *TPI* promoter was secreted only about 20% into the culture broth(18). Most of yeast glycoproteins such as invertase and acid phosphatase and heterogeneous secreted proteins accumulated in periplasm or cell wall. The *STA* signal sequence is a sequence of yeast *S. diastaticus* glucoamylase which secretes the glucoamylase into the culture broth. Recently, the researches of recombinant protein secretion using this *STA* signal sequence have been reported(9, 10). In the case of recombinant *S. cerevisiae* that contained *STA2* signal sequence and *Escherichia coli* β-galactosidase, the secretion did not occur into the culture broth instead 76% of β-galactosidase was secreted into the periplasm(9). Also, in the case of *Bacillus subtilis* endo-glucanase fused with *STA1* signal sequence, the secretion occurred successively at about 93% into the culture broth at 4th day of cultivation(10).

In this work, we compared the localization characteristics of glucoamylase in the chromosome integrated yeast with those in the plasmid-harboring yeast and investigated the characteristics of secreted recombinant glucoamylase.

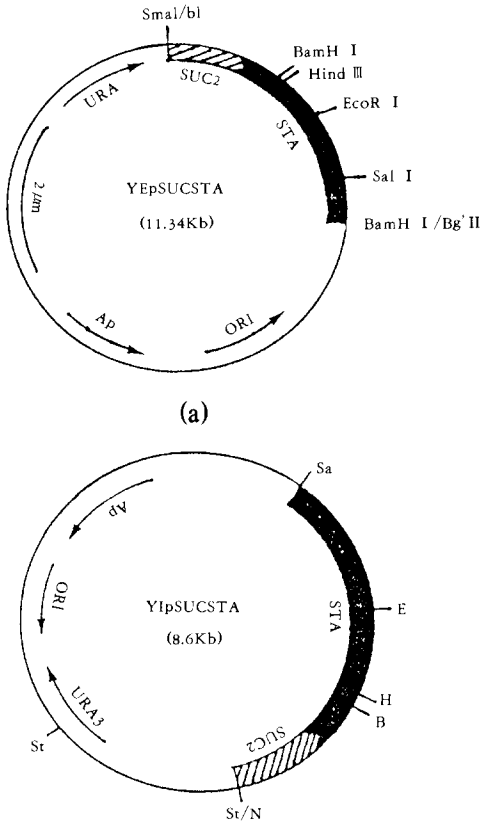


Fig. 1. Gene map for recombinant plasmids YEpSUCSTA and YIpSUCSTA.
(a) YEpSUCSTA (b) YIpSUCSTA.
Abbreviations; ORI: *E. coli* replicating origin, 2μm: yeast replicating origin, URA: URA3 gene, Ap: ampicillin resistance gene, B: BamH I, Bg: Bgl I, E: EcoR I, H: Hind III, Sa: Sal I, Sm: Sma I, bl: blunt end.

MATERIALS AND METHODS

Yeast, Plasmids, and Medium

Saccharomyces cerevisiae MMY2 (a, *ura3-52*, *sta0*, *sta10*) strain was used as a host. Recombinant plasmid YEpSUCSTA (3) containing the glucoamylase coding *STA* gene fused with the *SUC2* promoter and original *STA* signal sequence shown in Fig. 1(a) and chromosomal integrating vector YIpSUCSTA (11) containing the SUC2-STA fragment of YEpSUCSTA shown in Fig. 1 (b). The transformants were named MMY2-SUCSTA and MMY2SUCSTA-I, respectiviely. Yeast was grown at 30℃ in complex(YPDS) medium containing 1% yeast extract (Difco), 2% bacto-peptone (Difco), 1% glucose, and 1% potato starch (Sigma).

Cell Mass Measurement

The measurements of cell mass were carried out with spectrophotometer(Kontron; UVICON-930) at 600 nm wavelength.

Glucoamylase Activity

The 0.7ml culture supernatants were incubated in 0.1 ml 1M sodium acetate buffer (pH 5.0) and 0.2 ml 8% soluble starch (Junsei) at 50℃ for 30 minutes and boiled at 100℃ for 5 minutes for inactivating glucoamylase. Glucose produced by the action of glucoamylase on soluble starch was assayed by using a glucose-diagnostic kit (Sigma, No. 510). Glucoamylase activity was expressed as unit and one unit is one mole of glucose released per one minutes at the above conditions.

Cell Fractionation

Cells were harvested and washed with 10mM sodium azide. The cell pellet was resuspended in spheroplast buffer (0.1 M sodium acetate(pH 5.0), 1M manitol, 10 mM sodium azide, 1mM EDTA and 0.1 M β-mercaptoethanol). After addition of 15mg/ml Zymolase 20T (Seikagaku Corp.) in TEN buffer (1 mM EDTA, 10 mM Nacl and 10 mM Tris-Cl(pH 7.6)), the mixture was incubated at 45 ℃ for one hour. Spheroplasts were harvested by centrifugation with 12,000 rpm at 4℃ for 1 minute. The supernatant was operationally defined as periplasmic glucoamylase source. The spheroplast was resuspended in lysis buffer (0.1 M sodium acetate(pH 5.0), 10 mM sodium azide, 1mM EDTA and 0.1%(v/v) Triton X-100) and mechanically lysed by vortexing with glass bead (Sigma, 425-600 microns). After intermittent vortexing and cooling on ice, the suspension was

harvested by centrifugation with 12,000 rpm at 4℃ for 5 minutes. This supernatant was operationally defined as cytoplasmic glucoamylase source.

Production of Primary Anti-Glucoamylase Antibody

For producing the primary polyclonal anti-glucoamylase antibody, multiple intradermal injection method(12) was used. The purified glucoamylase sample was mixed with complete adjuvant (Sigma) (1:1) and injected into 1.5Kg New Zealand female rabbit. After that, the purified glucoamylase was mixed with incomplete adjuvant and boosted two times into the rabbit per three weeks. The serum was separated from rabbit blood by 1 hour incubation at 37℃ and centrifuged at 4℃, 10000g for 10 minutes. We identified the construction of anti-glucoamylase antibody using a double diffusion precipitation method. Immune serum was stored at −20℃.

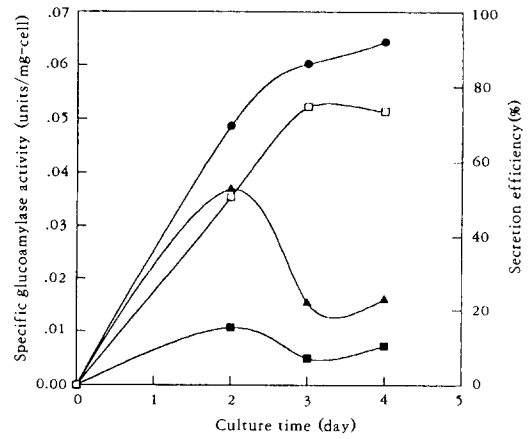
Western Blot Analysis

After gel electrophoresis, the transfer of proteins in gel to nitrocellulose membrane was done using semi-dry transfer unit(Hoefer Scientific Ins., TE70 Semiphor). Non-specific binding sites of membrane was blocked by immersing the membrane in 5% non-fat dried milk (Sigma) in PBS-T buffer (pH 7.5, 80 mM $Na2HPO_2 \cdot 12H_2O$, 20 mM $NaH_2PO_4 \cdot 2H_2O$, 100 mM NaCl and 0.1% Tween-20) for one hour at room temperature on an orbital shaker. This primary antiglucoamylase antibody was mixed with the culture broth of host MMY2 which had no glucoamylase cross-reacting materials for removal of impurities in serum. This mixture was let at 4℃ for overnight. After centrifuged at 4℃, 12000 rpm for 20 minutes, the supernatent was taken and used as a primary antibody. Then, the membrane was washed and incubated in the diluted anti-rabbit secondary antibody (Amersham Life Science) for one hour at room temperature. The detection of glucoamylase bands on nitrocellulose membrane was performed using ECL (Amersham Life Science, RPN 2108; Enhanced Chemi-Luminescence) detection reagents.

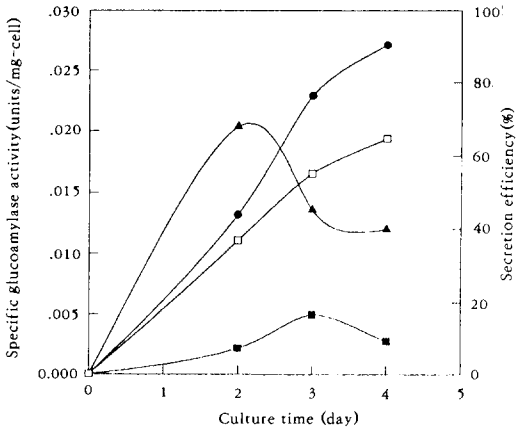
RESULTS AND DISCUSSION

Localization and Secretion Efficiency of Recombinant Glucoamylase

The localizations of glucoamylase in recombinant plasmid-harboring yeast MMY2SUCSTA and chromosome-integrated yeast MMY2SUCSTA-I were investigated. Protein localization can be divided into cytoplasm, periplasm and culture broth. Secretion efficiency was defined as the ratio of glucoamylase activity in the culture broth per total glucoamylase activity in the fermentor. The secretion efficiencies of both yeast strains were increased proportional to the culture time as shown in Fig. 2. In the case of MMY2SUCSTA, the increase of secretion efficiency was stopped at 3rd day of cultivation and maintained about 74 % as shown in Fig. 2(a). But in the case of MMY2SUCSTA-I, the secretion efficiency was increased linearly and about 65% at 4th day of cultivation as shown in Fig. 2(b). These results are summarized in Table 1. Generally, adverse effect which is caused by the accumulation of product in secretory apparatus may occur when expression rate of product is faster than secretion rate in the case of recombinant yeast having high plasmid copy number(6, 13). When this adverse effect occurs, secretion efficiency becomes decreased. Therefore, researches towards the increase of secretion efficiency have been performed by integrating desired genes into the host chromosome in order to reduce the adverse effect (6, 14). But in our research, the plasmid-harboring yeast MMY2SUCSTA and the chromosome-integrated yeast MMY2SUCSTA-I had high secretion efficiency. These were thought to be a result that MMY2SUCSTA expression level did not reach the secretory apparatus capacity of the host yeast MMY2. In other words, the adverse effect did not occur in this case because the amounts of glucoamylase which was expressed in MMY2SUCSTA were smaller than those of glucoamylase which could be secreted into the



(a)



(b)

Fig. 2. Time courses of glucoamylase localization in recombinant yeasts.
(a) plasmid-harboring yeast MMY2SUCSTA
(b) chromosome-integrated yeast MMY2-SUCSTA-I.
Yeast was grown in YPDS medium containing 1% glucose and 1% starch for 4 days at 30℃. Symbols; ●: culture broth glucoamylase (units/mg-cell), ■: periplasmic glucoamylase (units/mg-cell), ▲: cytoplasmic glucoamylase (units/mg-cell), □: secretion efficiency (%).

culture broth by yeast. Chromosome-integrated

Table 1. Localization of glucoamylase during batch culture of plasmid-harboring yeast MMY2SUCSTA and chromosome-integrated yeast MMY2SUCSTA-I. Yeasts were grown in YPDS medium containing 1% glucose and 1% starch for 4 days at 30℃.

Strains	Compartments	Fraction of glucoamylase activity		
		2nd day	3rd day	4th day
MMY2SUCSTA	Culture broth	0.506	0.747	0.735
	Periplasm	0.111	0.062	0.083
	Cytoplasm	0.383	0.191	0.182
MMY2SUCSTA-1	Culture broth	0.369	0.552	0.647
	Periplasm	0.061	0.121	0.068
	Cytoplasm	0.570	0.327	0.285

recombinant yeast MMY2SUCSTA-I which had relatively slower expression of glucoamylase than recombinant plasmid-harboring yeast MMY2-SUCSTA, is required longer culture time in order to reach to maximal glucoamylase secretion efficiency.

In both yeasts, most of the intracellular glucoamylase were detected in cytoplasm and small portion of glucoamylase were located in periplasm as shown in Fig. 2(a) and Fig. 2(b). The large amounts of cytoplasmic glucoamylase was detected until 2nd day of cultivation. After that time the cytoplamic glucoamylase was decreased with the culture time and its portion was about 18% in MMY2SUCSTA and about 28% in MMY2SUCSTA-I. The percentage of glucoamylase in the periplasm of both yeasts remained below 10% during the entire culture period. From these glucoamylase localization, we knew that the transport of glucoamylase through the cell wall was not rate-limiting step in recombinant glucoamylase secretion.

In both the recombinant yeast strains used in our research, glucoamylase was secreted mainly into the culture broth (more than about 75%) although glucoamylase was very large protein (420 amino acids and glycosylated). Therefore, the STA signal sequence which was used in this research, was effective in secreting the recombi-

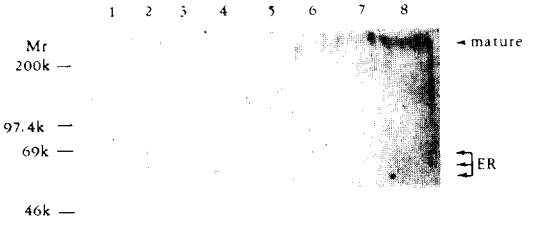


Fig. 3. Glucoamylase expression profiles from the culture of the recombinant plasmid-harboring yeast MMY2SUCSTA.
Yeast was grown in YPDS medium containing 1% glucose and 1% starch at 30℃. lane 1: Cell extract at 1st day of cultivation, lane 2: Cell extract at 2nd day of cultivation, lane 3: Cell extract at 3rd day of cultivation, lane 4: Cell extract at 4th day of cultivation, lane 5: Culture broth at 1st day of cultivation, lane 6: Culture broth at 2nd day of cultivation, lane 7: Culture broth at 3rd day of cultivation, lane 8: Culture broth at 4th day of cultivation.

nant glucoamylase.

Characteristics of Recombinant Glucoamylase

The expression profiles of glucoamylase of the recombinant plasmid-harboring yeast MMY2SUCSTA with the culture time were investigated by using Western blot as shown in Fig. 3. Three bands appeared between about 55 kilodalton and 65 kilodalton without large differences of band densities in the case of intracellular glucoamylase of 1 to 4 lanes. And one band was detected above about 200 kilodalton in the case of the culture broth glucoamylase of 5 to 8 lanes. The density of this band was increased proportional to the culture time. Therefore this band of above 200 kilodalton can be considered as mature glucoamylase.

As shown in Fig. 4, the molecular weight of secreted mature glucoamylase was about 200 to 300 kilodalton and the band of about 130 kilodalton was thought as underglycosylated form. Also three bands of about 55 to 65 kilodalton was thought as ER(endoplasmic reticulum) forms. Be-

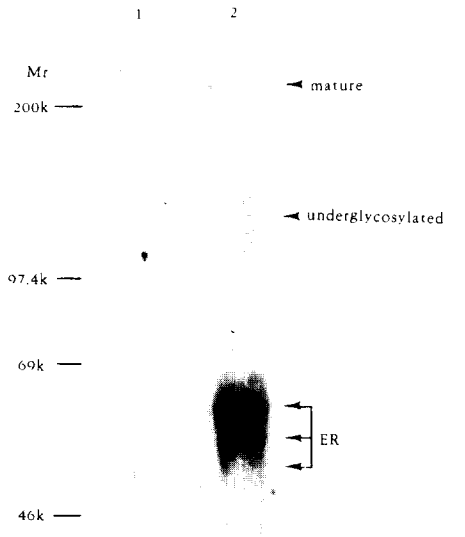


Fig. 4. Western blots of culture broth and cell extract from the recombinant plasmid-harboring yeast MMY2SUCSTA.
Yeast was grown in YPDS medium containing 1% glucose and 1% starch for 3 days at 30℃. lane 1: Culture broth, lane 2: Cell extract

cause functional domain of glucoamylase is composed of about 420 amino acids from glucoamylase nucleotide sequence(2) and the average molecular weight of one amino acid is about 120 dalton, the molecular weight of glucoamylase without carbohydrate can be roughly calculated as 45 kilodalton. The molecular weight of one core carbohydrate is about 3-5 kilodalton. Therefore, three bands of 55 to 65 kilodalton which detected in cell extract, must be ER forms which were core glycosylated at the glucoamylase of 45 kilodalton. Many researches have reported the molecular weight of *S. diastaticus* glucoamylase. In the case of *S. diastaticus* strain 5206-1B which contained *STA2* gene, the molecular weight of glucoamylase was 300 kilodalton from SDS-PAGE method and 670 kilodalton from fast protein liquid chromatography(19). From these results they thought that glucoamylase existed in dimer form. In the case of yeast SPX-101-1C

which contained *STA2* gene, the molecular weight of glucoamylase was 186 kilodalton from SDS-PAGE and 306 kilodalton from high-pressure size-exclusion chromatography(20). The molecular weight of glucoamylase was 84 kilodalton from ultracentrifugation in the case of yeast 5205-10A strain which had STA2 gene(21). In the case of yeast J132b strain containing *DEX1* gene (equal to *STA2*), the molecular weight of glucoamylase was about 107 to 315 kilodalton of heterogeneous form from SDS-PAGE(22). In the case of yeast 5108-7A which had *STA1*, the molecular weight was about 68 kilodalton from ultracentrifugation(21). Yamashita and coworkers reported that the molecular weight of glucoamylase of yeast 5106-9A strain which contained *STA1* gene was 66 kilodalton from gel filtration method(23). From the literature mentioned above, the *STA1* glucoamylase was thought to be composed of two non-equal subunits. In the case of yeast YIY2-12D strain which had *STA1*, the molecular weight of glucoamylase was 250 kilodalton from SDS-PAGE(24). In the case of yeast which had *DEX2* gene, the molecular weight was 98.5 to 305 kilodalton from SDS-PAGE method (22). In the case of yeast 5301-14D which had *STA3*, the molecular weight was 79 kilodalton from ultracentrifugation(21). Also for the *S. diastaticus* which had unknown *STA* gene, the molecular weight of glucoamylase was 70 kilodalton and about 250 kilodalton in native condition(25). From these reports the molecular weight of glucoamylase was very heterogeneously distributed (from 60 to 300 kilodalton) depending on the purification procedures, assay methods of glucoamylase molecular weight and a kind of strain.

The secretion pathway of proteins in yeast is generally described as follows(4): The protein which is produced in ribosome, is transported in the lumen of endoplasmic reticulum(ER). After that, core-glycosylation and folding of protein occurs in the ER and the protein is transported in the Golgi body. The protein is transferred to secretory vesicle, and then secreted into the culture broth or periplasmic space by plasma membrane

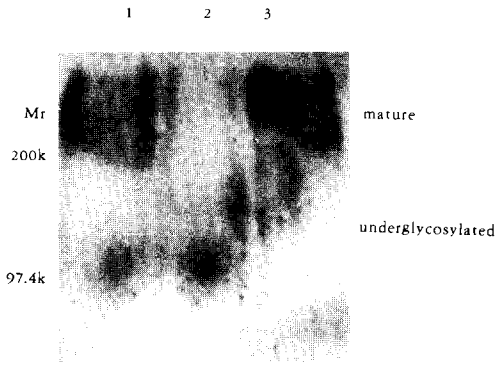


Fig. 5. Western blots of culture broth from the recombinant plasmid-harboring yeast MMY-2SUCSTA, the chromosome-integrated yeast MMY2SUCSTA-I-5 and the recombinant plasmid-harboring yeast MMY2-STAF which contains whole STA gene.
Yeasts were grown in YPDS medium containing 1% glucose and 1% starch for 4 days at 30℃. lane 1: Culture broth of MMY2SUCSTA, lane 2: Culture broth of MMY2SUCSTA-I-5, lane 3: Culture broth of MMY2STAF

fusion. As shown in lane 2 of Fig 4, ER forms had heavy band densities. This was thought to be that the glucoamylase was accumulated in the ER because the translocation of glucoamylase from the ER to the Golgi body was rate-limiting in glucoamylase secretion pathway. The secreted glucoamylase patterns of recombinant plasmid-harboring yeast MMY2SUCSTA, the chromosome-integrated yeast MMY2SUCSTA-I and recombinant plasmid-harboring yeast MMY2STAF which had the whole *STA* gene were compared by Western blot. As shown in Fig. 5, mature glucoamylases in all recombinant yeasts, had heterogeneous glycosylated forms ranging between 200 to 300 kilodalton. The broad pattern of these mature glucoamylase band shows the general characteristics of yeast glycoprotein. In this case, the percentage of carbohydrate of mature glucoamy-

lase was thought larger than 80% because the molecular weight of glucoamylase without carbohydrate was about 45 kilodalton. In many researches, the percentage of carbohydrate of secreted glucoamylase was 67~87%(22) and 28% (24) in the case of *STA1* gene and 80%(19) and 80~89%(22) in the case of *STA2* gene. From these reports, the degree of glycosylation of *S. diastaticus* glucoamylase was very high and similar to the value from this research. Because the sizes and glycosylation patterns of secreted glucoamylases from the three recombinant yeasts were very similar as shown in Fig. 5, it can be said that the localization in plasmid or in chromosome of desired gene did not affect the secretion pattern of the recombinant glucoamylase used in this research.

요 약

재조합 플라스미드 함유 효모 MMY2SUCSTA의 glucoamylase의 분비효율은 배양 4일째에 약 74%이었으며 염색체 삽입 재조합효모 MMY2SUCSTA-I의 경우에는 4일째에 약 65%로 나타났다. 높은 분비효율을 나타낸 것은 glucoamylase의 발현수준이 숙주세포 분비기관의 능력에 미치지 못하기 때문으로 추정된다. 두 재조합 균주에서 모두 대부분의 세포내 glucoamylase는 cytoplasm에 존재하고 약간의 부분만이 (10% 이내) 전 배양기간을 통하여 periplasm에 존재하였다. 재조합 효모로부터 분비되는 glucoamylase의 특성을 Western blot 분석을 통하여 조사하였다. 배양액으로 분비된 glucoamylase는 매우 heterogeneous하였고 그 분자량은 약 200에서 300 kilodalton이었다. 분비된 glucoamylase의 당 잔기량은 약 80% 이상이었고 세포내 glucoamylase의 endoplasmic reticulum 형태는 약 55에서 65kilodalton 사이의 여러 가지의 밴드로 나타났다.

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