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Cloning and Expression of *Schwanniomyces castellii* Starch Gene

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Schwanniomyces castellii 전분 유전자의 Cloning과 발현

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The gene encoding glucoamylase from *Schwanniomyces castellii* CBS 2863 was cloned and expressed in *Saccharomyces cerevisiae*. Southern blot analysis confirmed that this glucoamylase gene was derived from the genomic DNA of *Schwanniomyces castellii* and that no DNA fragments corresponding to 5.1 or 1.3 kb of *Sch. castellii* DNA were detected in *S. cerevisiae*. The glucoamylase activity from *S. cerevisiae* transformant was approximately 2,000 times less than that of donor yeast. No expression was found in *E. coli*. The secreted glucoamylase from *S. cerevisiae* transformant was indistinguishable from that of *Sch. castellii* on the basis of molecular weight and enzyme properties.

The amylolytic yeasts, *Schwanniomyces* species, produce extracellular glucoamylase which is capable of degrading α-1.4 and α-1.6 linkage of starch (1-7) or pullulan (5). Sills *et al.*(6) have reported that *Sch. castellii* glucoamylase contains no carbohydrate. Oteng-Gyang *et al.*(3) have isolated two forms of enzymes consisting of 90 and 45 kDa from starch grown cells of *Sch. occidentalis*. Recently, Deibel *et al.* (1) have reported that its glucoamylase contains approximately 12% carbohydrate of total molecular weight and exists as a monomeric polypeptide with approximately 143 kDa. The disparity in molecular weight, subunit and carbohydrate content of enzyme purified from *Sch. castellii* (3, 6) and *Sch. occidentalis* (1, 3) which are of the same species as evidenced by Spencer and Gorin (8), Kurtzman *et al.*(9) and Price *et al.* (10) may be ascribed to either the difference in experimental method between reporters or an erroneous estimation. However, this discrepancy may result from either different proteolysis (2, 11) or the degree of N-and/or O-glycosylation (12). The possibility of polymorphic genes (13, 14) could not be ruled out.

We do not know of the gene structure of *Schwanniomyces* glucoamylase to clearly demonstrate the dissimilarity of some physical properties between the *Schwanniomyces* enzymes reported up to date. By comparing both the nucleotide sequence of glucoamylase gene and the deduced amino acid sequence between *Schwanniomyces* strains, it will be possible to determine whether or not such a discrepancy has really occurred between them. In this respect, we first cloned and expressed glucoamylase gene of *Sch. castellii* in *S. cerevisiae*.

Key words: *Schwanniomyces*, glucoamylase, gene cloning, expression
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Materials and Methods

Strains and plasmids

Sch. castellii CBS 2863 and *S. cerevisiae* SHY 3 (a, ste-VCP, ura 3-52, trp 1-289, leu 2-3, leu 2-112, his - Δ1, ade 1-101 and can 1-100) were used as a donor strain of glucoamylase gene and a recipient for transformation, respectively. *Escherichia coli* HB 101 (F^-, hsdS20 [rB^-, mB^-], recA13, ara-14, proA2, rspL20 [Sm^r], xyl-5, mtl-1, lacY1, galK2, supE44, λ^-) was

used to construct a genomic library of *Sch. castelli*. A cloning vector pYcDE-1 was kindly provided by Dr. B.D. Hall, University of Washington, Seattle, U.S.A.

Culture condition

YPD (1% yeast extract, 2% Bactopeptone and 2% dextrose) was used as the medium for the growth of yeast cells while MSM (minimal starch medium; 0.67% yeast nitrogen base [YNB, Difco Laboratories, Detroit, Michigan, U.S.A.], 2% Bacto agar and 3% soluble starch) was used as the selection medium of yeast containing putative starch gene. When needed, the selection medium was supplemented with appropriate amino acids, the concentration of which was 20 mg/m*l* except leucine (30 mg/m*l*). The media for bacterial growth were LB and LA as described by Maniatis *et al.* (15).

Enzymes and reagents

All restriction and modifying enzymes were obtained from Promega Corporation (Fish Hatchery Road, Madison, U.S.A). dNTPs and random primer were purchased from Bethesda Research Laboratories (Gaithersburg. MD, U.S.A). Other chemicals and media were purchased from Sigma Chemical Company (St. Louis, Mo. U.S.A.) and Difco Laboratories (Detroit, Michigan, U.S.A), respectively.

Isolation of total genomic DNA of *Sch. castellii* and plasmid

Total genomic DNA of *Sch. castellii* was prepared by the established protocol A of Rodriquez and Tait (16) and plasmid DNA was isolated by the method of Maniatis *et al.* (15).

Construction of recombinant DNA and genomic library

Total genomic DNA from yeast was partially digested with restriction enzyme *Eco*RI, and the resultant DNA fragments ranging from 5 to 10 kb were ligated to the *Eco*RI site of a cloning vector pYcDE-1 which carries ampicillin resistance (AP^+) for *E. coli* and also the *Saccharomyces* TRP gene for the selection of yeast transformants.

Transformation of *E. coli* and *S. cerevisiae*

The recombinant plasmid DNAs were used to transform *E. coli*. The yeast transformation was performed by the procedure as noted by Gingold (17) except the use of KCl as the osmostabilyzer, and bacterial transformation was carried out with the $CaCl_2$, treated cells.

Southern blot hybridization

The primer extention of inserted foreign DNA to obtain ^{32}P-labelled DNA probe and subsequently direct gel hybridization (18, 19) to genomic DNA isolated from *Sch. castellii* and *S. cerevisiae* were carried out by the previously established procedures (15).

Restriction analysis

The recombinant plasmid carrying a glucoamylase gene was digested with several restriction endonucleases (1-10 U/μg DNA), and the resulting DNA were analized by 0.8-1.0% agarose gel electrophoresis (20).

Isolation and characterization of glucoamylase

S. cerevisiae transformed with the plasmid containing the glucoamylase gene and *Sch. castellii* were separately grown in YNB containing 2% soluble starch or maltose at 30°C to late log phase. The supernatant obtained from maltose grown cells of transformant was 2,000-fold concentrated and dialyzed extensively against 100 mM citrate-phosphate buffer (pH 5.5) by using ultrafiltration system (Vision Scientific Co., Korea) with 10,000 molecular weight cut-off membrane under 60 lb/in^2 of nitrogen. The concentrated and dialyzed supernatants were used as crude enzyme. In case of *Sch. castellii* glucoamylase, supernatant itself was used as a corresponding enzyme. The enzyme reaction mixture contained 10 μl of crude enzyme solution in 990 μl of 100 mM citrate phosphate buffer (pH 5.5) with 0.5% soluble starch or 0.5% isomaltose. The enzyme reaction was carried out for 60 min at 40°C and then stopped by boiling for 5 min. The glucose released from soluble starch or isomaltose was determined by the peroxidase-glucose oxidase assay (Sigma Technical Bulletin No. 510). One unit of enzyme activity is defined by the amount of enzyme which liberates 1 μM of glucose from soluble starch or isomaltose.

Protein electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the procedure of Davis (21). SDS-PAGE was perfomed in vertical slab gel apparatus (2001 vertical system; Ge 2/4,

LKB. Sweden) by the method of Laemmli (22).

Preparation of antiserum to *Sch. castellii* glucoamylase and immunodiffusion

The antiserum against purified glucoamylase from donor strains, *Sch. castellii,* was prepared according to the procedure described by Jurd (23) using New Zealand White rabbit. The immunological activity of rabbit antiserum to purified glucoamylase or other proteins was tested by the double diffusion agarose gel plate (24). The detection of glucoamylase by this serum was shown to be ranging from 10 to 100 nanogram/ml by enzyme-linked immunosorbant assay (25).

Results and Discussion

The cloning of glucoamylase gene

Recombinant plasmid DNA from *Sch. castellii* genomic library was used to transform *S. cerevisiae* SHY 3 to tryptophan prototrophy (TRP^+). Transformants harboring the plasmids containing putative glucoamylase gene were then selected by their ability to form turbid precipitates around colonies on TRP^+ selection plates (26). Of the 5×10^4 TRP^+ transformants obtained, 7 were selected which had small turbid halo after incubation of the plates at 30°C for 7 to 9 days. Plasmid DNA was recovered from seven transformants, and they were identical. When these transformations were separately subcultured into a selective medium, one of them exhibited large halo aroud its colonies in 3 days (Fig. 1). The plasmid preparation from this transformant was used to retransform *E. coli.* The recloned plasmid was isolated and used to retransform *S. cerevisiae.* Retransformed yeast cells showed glucoamylase activity. However, no expression of glucoamylase activity was found in *E. coli.* We named this recombinant plasmid pScGlu.

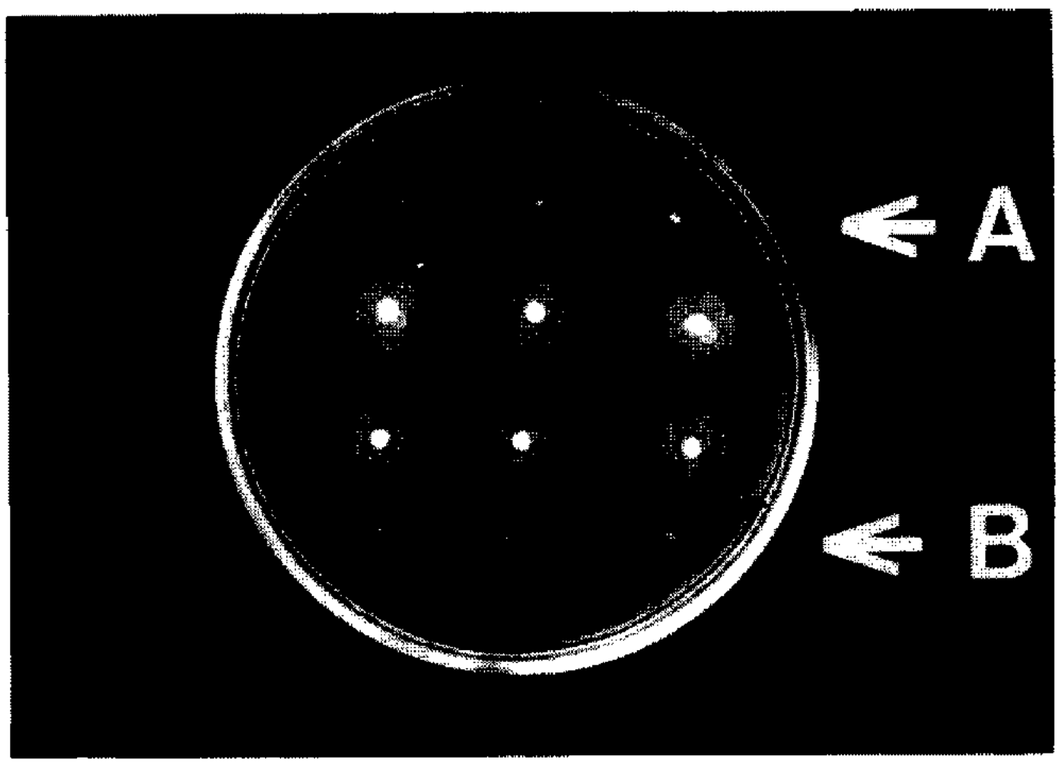

Fig. 1. Secretion of glucoamylase from *S. cerevisiae* transformed with plasmid pScGlu.
Yeast was grown for 3 days at 30°C on MSM without tryptophan. A halo formed without staining with iodine shows that the colony secrets glucoamylase. Arrows indicate *S. cerevisiae* transformant containing plasmid pYcDE-1 only (A) and *S. cerevisiae* itself (B).

Restriction analysis

The pScGlu was subjected to restriction analysis (Fig. 2). The plasmid contained ca. 6.4 kb insert. This insert of pScGlu plasmid had one *Eco*RI site, one *Sal*I site, two *Hap*I sites, two *Kpn*I sites, three *Cla*I sites, and three *Hind*III sites. To obtain the subclone positive for the glucoamylase activity, pScGlu containing 6.4 kb insert was digested with *Eco*RI. *Eco*RI restriction fragment (5.1 kb) obtained therein was ligated to *Eco*RI site of pYcDE-1 plasmid with cohesive ends. The resulting recombinant plasmid, pScGlu 1, had glucoamylase activity. To further reduce the size of this insert, pScGlu 1 was doubly digested with *Hpa*I and *Kpn*I. Two fragments, *Hpa*I fragment (3.4 kb) and *Kpn*I-*Eco*RI fragment (2.7 kb), were isolated and blunt-end ligated to the corresponding *Eco*RI sites of pYcDE-1. The resulting plasmids, pScGlu 2 (3.4 kb) and pScGlu 3 (2.7 kb), did not show any enzyme activity. Since glucoamylase activity was not detected either in pScGlu 2 which lacked both 0.8 kb *Eco*RI-*Hpa*I and 2.2 kb *Hpa*I-*Eco*RI fragments or in pScGlu 3 which lacked 3.7 kb *Kpn*I-*Eco*RI fragment, subcloning was accomplished by inserting 5.1 kb *Eco*RI fragment in *Eco*RI site of pBR322. pBR322 containing 5.1 kb fragment was digested with *Ava*I. The resulting linear fragment (9.43 kb) was deleted with *Bal* 31. The fragment deleted from both end of 9.43 kb was digested with *Eco*RI and the resulting 3.6 kb was obtained. This fragment was ligated to *Eco*RI site of pYcDE-1 using *Eco*RI lingker (12 mer). We found the resulting plasmid, pScGlu 4, to have glucoamylase activity. Therefore, it was found that glucoamylase gene was located in 3.6 kb fragment and that the 2.2 kb *Hpa*I-*Eco*RI fragment deleted from 5.1 kb insert was essential for gene expression. The fact that 5.1 or 3.6 kb inserts was cloned in the same orientation as original 6.4 kb was ascertained by obtaining 3.8 kb *Hind*III fragment for 5.1 kb and 2.8 kb *Kpn*I fragment for 3.6 kb insert, respectively.

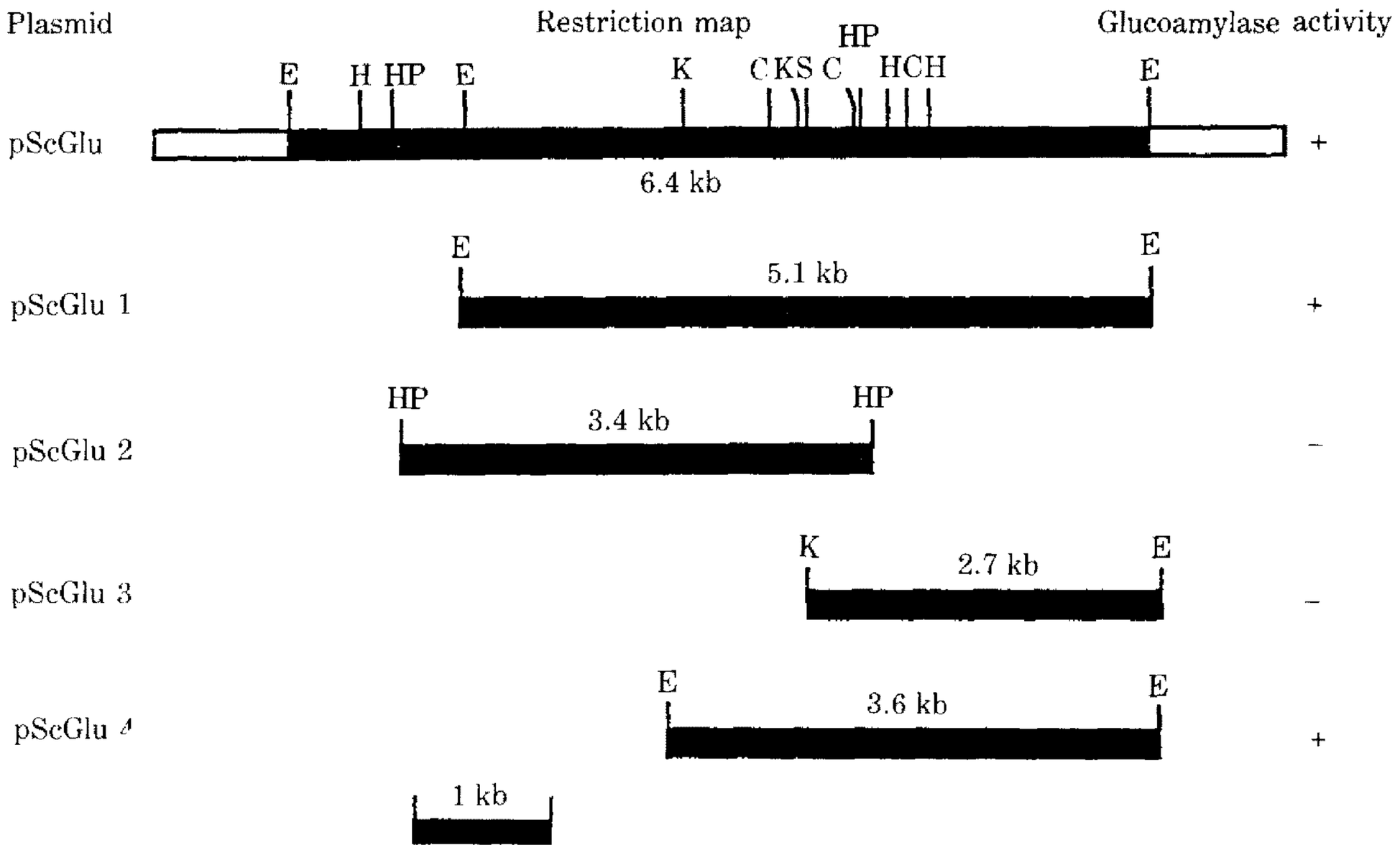

Fig. 2. Restriction maps of the inserted DNA segments.
Plasmid pScGlu and the subcloned plasmids (pScGlu 1, pScGlu 2, pScGlu 3 and pScGlu 4) were subjected to restriction analysis. The restriction sites for *Cla*I(C), *Eco*RI(E), *Hind*III(H), *Hpa*I(Hp), *Kpn*I(K), and *Sal*I(S) are indicated. Open bars of pScGlu represent parts of plasmid pYcDE-1.

Since ADH1 promoter was contained in pScGlu 4, it remains to be determined whether transcription was initiated outside 3.6 kb insert or within the 2.2 kb *Hpa*I-*Eco*RI fragment. Yamashita *et al* (11) reported that 6.4 kb *Bam*HI insert of glucoamylase gene of *Saccharomycopsis fibuligera* and had *Kpn*I site, one *Pst*I site, three *Hpa*I sites, three *Eco*RI sites and thre *Hind*III sites. In contrast, 5.3 kb *Bam*HI insetrt of glucoamylase of *Saccharomyces diastaticus* had two *Bam*HI sites, one *Kpn*I site, two *Pvu*II sites, one *Hind*III site, two *Sal*I sites and one *Eco*RI site (26). On the other hand, Innis *et al.*(27) reported that 3.4 kb *Eco*RI insert of glucoamylase gene of *Aspergillus awamori* cloned in *S. cerevisiae* had one *Sal*I site, one *Sac*I site, three *Nru*I sites, one *Ava*II site, one *Nde*I site, one *Bgl*II site, one *Pst*I site, one *Bam*HI site, one *Pvu*II site, two *Eco*RI sites and one *Hco*I site. Therefore, the restriction enzyme sites of *Sch. castellii* glucoamylase gene were similar to those of *S. diastaticus* or *S. fibuligera* in that they all had *Kpn*I, *Hind*III and *Eco*RI sites.

Southern blot analysis

Both 5.1 and 1.3 kb *Eco*RI DNA fragments were used as probes for hybridization with *Eco*RI-restricted total DNA of *Sch. castellii* and *S. cerevisiae* as well as pScGlu and pYcDE-1 plasmid. Hybridization signal was obtained at 5.1 or 1.3 kb fragment for *Sch. castellii* DNA only (Fig. 3). This indicated that the

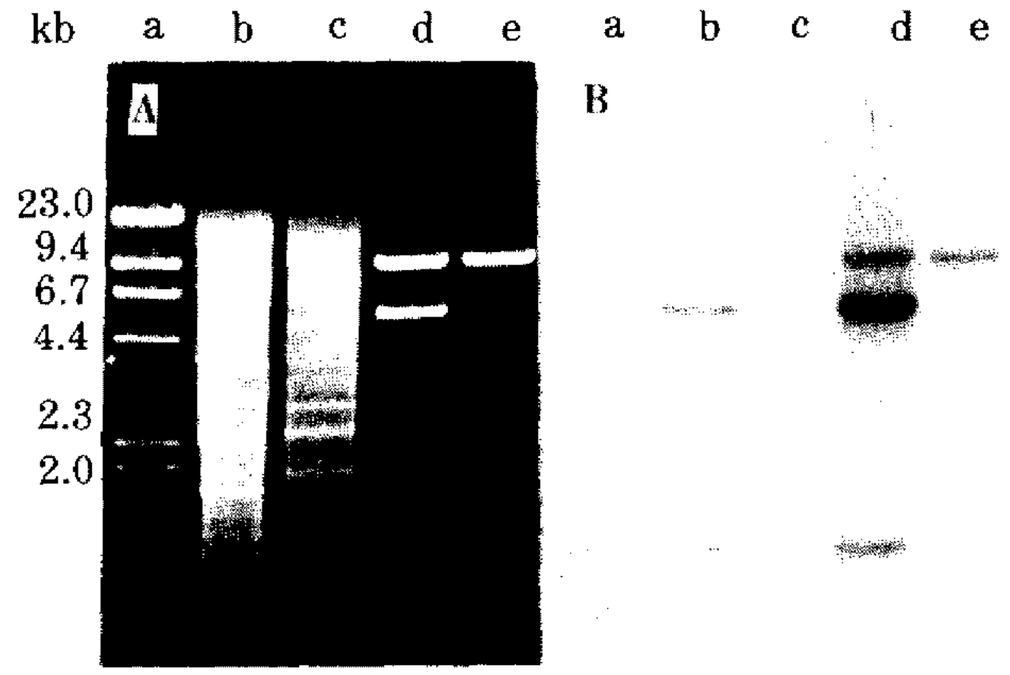

Fig. 3. Southern blot analysis of restriction digests of pScGlu plasmid DNAs.
Restriction fragments of *Eco*RI-digested 5.1 and 1.3 kb were separated on a 0.8% agarose gel (plane A) and used as probe^{32}p-labelled pScGlu DNA. The sizes (in kb) of *Hind*III-digested λ phage DNA restriction fragment (lane a) are shown on the left margin; (lane b) *Sch. castellii* genomic DNA; (lane c) *S. cerevisiae* genomic DNA digested with*Eco*RI; (lane d) pScGlu and (lane e) pYcDE-1 plasmid DNA digested with *Eco*RI.

Table 1. The properties of glucoamylase produced by the transformant with plasmid pScGlu 1.

Organisms	Optimal conditions[a]		Thermostability[b]	Glucoamylase activity[c] (U/ml/min)	
	pH	Temp (°C)	(°C)	Starch	Isomaltose
S. cerevisiae transformant	5.5	40	50	0.44	1.22
Sch. castellii	5.5	40	50	0.79	1.13

[a]The enzyme activity was measured over the range of 20 to 80°C and the pH range of 3.0 to 8.0 as described in the text.
[b]The enzyme solution was preincubated for 30 min over the range of 40 to 60°C. A portion of 10 μl was taken and assayed for the residual enzyme activity as described in the text.
[c]The crude enzyme for the assay of glucoamylase activity from *S. cerevisiae* transformant was twenty thousand-fold concentrated.

cloned DNA was derived from *Sch. castellii* but not from *S. cerevisiae* DNA, and that any signals corresponding to 5.1 or 1.3 kb DNA fragment were not detected from *S. cerevisiae* DNA. This result was quite unlike *S. diastaticus* glucoamylase gene which shared three DNA fragments with *S. cerevisiae* (28). In addition to signals described above, another one was observed only in the hybridization between pScGlu and pYcDE-1 plasmid DNA (Fig. 3, lane d and e). The presence of this signal may be ascribed to the hybridization between *E. coli* plasmid DNA fragments which was introduced into the 5.1 or 1.3 kb DNA fractions during fragment preparation from agarose gel.

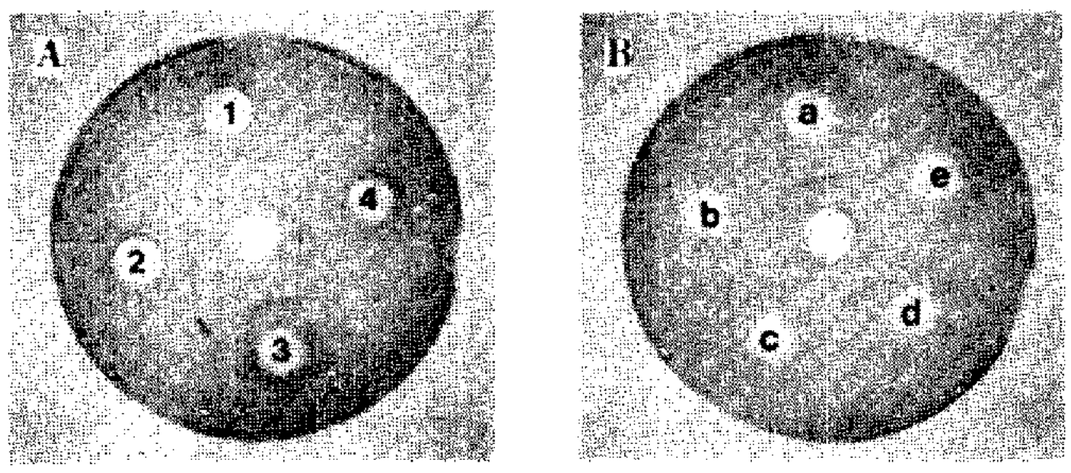

Fig. 4. Ouchterlony double immunodiffusion of *Sch. castellii* glucoamylase directed antiserum to crude enzyme of *S. cerevisiae* transformant.
A. Center well contains 10 μg protein of crude enzyme of *S. cerevisiae* transformant. The outer well 1 to 4 contain 50, 25, 10 and 0 μl of antiserum
B. Center well contains 20 μl of antiserum: well a, 10 μg of crude enzyme of *S. cerevisiae* transformant; well b, 10 μg of α-amylase of *Sch. castellii*; well c, 10 μg of glucoamylase of *Lipomyces kononenkoae;* well d, 10 μg of bovine serum albumin; well e, 10 μg of culture filtrate protein of *S. cerevisiae* SHY 3.

Characterization of glucoamylase from *S. cerevisiae* transformant

The yeast cells transformed with pScGlu 1 plasmid excreted glucoamylase that had the same enzymatic properties as did *Sch. castellii* (Table 1). This excretion was ascertained with the formation of precipitin band only in the culture filtrate of transformant by immunodiffusion (Fig. 4). When this excreted enzyme was subjected to SDS-PAGE, a single protein band was observed in parallel to that of purified glucoamylase from *Sch. castellii* (Fig. 5). This indicated that transformant glucoamylase had the same molecular size as that of donor strains. However, the glucoamylase activity of transformant was approximately 2,000 fold less than that of *Sch. castellii* itself. The glucoamylase activity level exhibited by this transformant was 20 fold less than by *S. cerevisiae* transformed with *S. diataticus* glucoamylase gene (29). This lowered level of gene expression obtained here could be explained by the fact that host yeast, *S. cerevisiae,* is more distantly related to *Sch. castellii* than to *S. diastaticus.* This is supported by the fact that *S. cerevisiae* shared several DNA fragments with *S. diastaticus* (28) but not with *Sch. castellii.* The other factors including the nature of expression vector or promoter may be involved in such lower expression of *Schwanniomyces* glucoamylase gene. This problem could be partially solved by the elucidation of gene structure. We are now in progress towards nucleotide sequencing of the cloned gene and amino acid analysis of the enzyme secreted from the *Saccharomyces* transforment. The result will be published elsewhere.

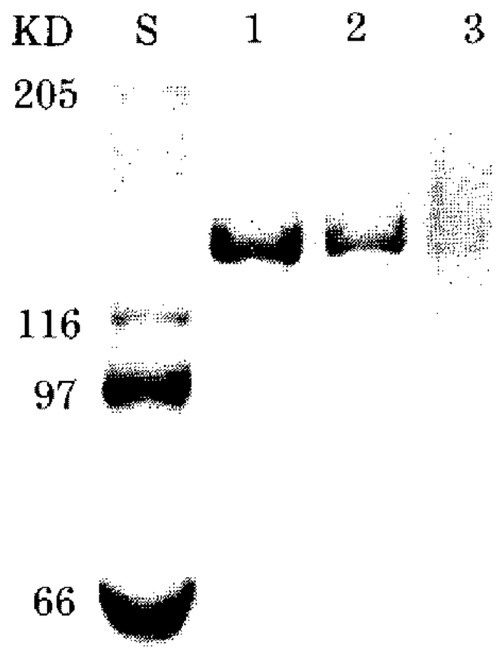

Fig. 5. SDS-PAGE of glucoamylase from *Sch. castellii* and *S. cerevisiae* transformant.
Lanes: 1, culture filtrate from *Sch. castellii* (40 μg protein); 2, purified glucoamylase from *Sch. castellii* (20 μg protein); 3, paratially purified glucoamylase from *S. cerevisiae* transformant (20 μg protein); S, standard protein

요 약

Schwanniomyces castelli CBS 2863의 glucoamylase 유전자를 *Saccharomyces cerevisiae*에 cloning하고 발현시켰다. Southern blot 분석결과, 형질전환체의 glucoamylase 유전자는 *Sch. castellii* genomic DNA 로부터 나온 것임을 확인하였고 5.1 혹은 1.3kb의 *Sch. castellii* 유전자에 해당되는 DNA 절편이 *S. cerevisiae* 에서는 관찰되지 않았다. *S. cerevisiae* 형질전환체의 glucoamylase 활성은 *Sch. castellii*의 그것에 비해 2,000배 정도 낮았고 *E. coli*에서는 발현되지 않았다. *S. cerevisiae* 형질전환체가 생산한 glucoamylase는 *Sch. castellii*의 glucoamylase와 동일한 특성과 분자량을 가지고 있음을 알 수 있었다.

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