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Molecular Cloning and Analysis of Sporulation-Specific Glucoamylase (SGA) Gene of *Saccharomyces diastaticus*

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Sporulation-specific glucoamylase (SGA) gene was isolated from genomic library of *Saccharomyces diastaticus* 5114-9A by using glucoamylase non-producing mutant of *S. diastaticus* as a recipient. When the glucoamylase activities of culture supernatant, periplasmic, and intracellular fraction of cells transformed with hybrid plasmid containing *SGA* gene were measured, the highest activity was detected in culture supernatant. SGA produced by transformant and extracellular glucoamylase produced by *S. diastaticus* 5114-9A differed in enzyme characteristics such as optimum temperature, thermostability, and resistance to SDS and urea. But the characteristics of SGA produced by sporulating yeast cells and vegetatively growing transformants were identical.

Key words: *S. diastaticus*, *SGA* gene cloning, gene disruption, secretory protein

Saccharomyces diastaticus is known to be very similar to *S. cerevisiae* except that *S. diastaticus* has the ability to produce extracellular glucoamylase. *S. diastaticus* is able to mate sexually with *S. cerevisiae*, and genetic and physiological characteristics of the strains are almost identical to each other. The extracellular glucoamylase is encoded by one of the unlinked *STA* genes (*STA1, STA2, STA3*) (1). These *STA* genes were cloned, and their restriction maps were exactly the same (2, 3, 4, 5, 6). Moreover, all nucleotide sequences of *STA1* gene were revealed (7). Extracellular glucoamylase was also purified and shown to be composed of two non-identical glycosylated subunits, H and Y, which are derived from post-translational modification of a single polypeptide encoded by *STA1* gene (8). Contrary to *S. diastaticus, S. cerevisiae* does not have any of these genes and does not produce extracellular glucoamylase. But it has been known that both strains have another glucoamylase gene expressed only when a/α diploid strains sporulate and therefore the glucoamylase activity cannot be detected in vegetatively growing cells. This gene was named *SGA* (8, 9, 10).

Comparison of nucleotide sequences between *STA1* and *SGA* gene has revealed that extensive region of two genes, especially functional domain of glucoamylase, was nearly identical. Based upon these results, it was proposed that extracellular glucoamylase gene (*STA*) of *S. diastaticus* evolved from *SGA* of *S. cerevisiae* by acquisition of different upstream promoter sequence responsible for transcription, and a signal sequence for protein secretion (11). But not much genetic and biochemical studies on SGA were done in comparison with extracellular glucoamylase.

For the purpose of investigating the secretory potential of SGA and estimating the utility of SGA signal sequence for heterologous protein secretion, we cloned *SGA* gene by using screening method which is capable of selecting transformants containing glucoamylase activity by starch-hydrolyzing activity on minimal starch agar medium. Moreover, we partially purified SGA produced by transformant and sporulating cells and extracellular glucoamylase secreted by strain *S. diastaticus* 5114-9A in order to compare enzymatic characteristics of these glucoamylases.

Materials and Methods

Strains and plasmids

Yeast and bacterial strains used in this work are listed in Table 1. *E. coli* HB101 served as a host for gene cloning and gene library amplification. The

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Table 1. Microbial strains used in this study

Strains	Relevant genotype	Source
S. diastaticus YIY345	*a, ura3-52 leu2-3,112 his4 sta*$^{+}$ *inh*$^{+}$	Yamashita
S. diastaticus YIY342	*a, ura3-52 lys7 sta*$^{+}$ *inh*$^{+}$	"
S. cerevisiae SHY3	*a, ste-VC9 ade1-101 ura3-52 trp1-289 leu2-3 leu2-112 his3-Δ1 can1-100*	KCTC
S. diastaticus 5114-9A	*a, arg4 STA1*	Tamaki
S. diastaticus 5209-9A	*a, leu2 STA3*	"
S. diastaticus 5209-11B	α, *his2 STA2*	"
E. coli HB101	F^{-} *Δ(gpt-proA)62 leuB6 SupE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20*(Str^{r}) *xyl-5 mtl-1 recA13*	KCTC

yeast *S. diastaticus* 5114-9A and YIY345 were used as *SGA* gene source and as recipient strain for the expression of *SGA* gene, respectively. A plasmid YEp24, *E. coli*-yeast shuttle vector, was used to construct gene library of *S. diastaticus* 5114-9A, and YIp5, yeast integrating plasmid, was used for the purpose of *SGA* gene disruption.

Construction of genomic library and screening of starch-fermenting colonies

Chromosomal DNA purified from *S. diastaticus* 5114-9A was partially digested with *Bam*HI endonuclease, and the digested fragments were fractionated by sucrose density gradient ultracentrifugation (12, 13). Fragments larger than 4 kb were collected and ligated to YEp24 digested with *Bam*HI and dephosphorylated. *E. coli* HB101 was transformed with the ligated mixture to generate a genomic library (14). All hybrid plasmids were isolated from the pool of resulting bacterial colonies (15) and used to transform *S. diastaticus* YIY345 by competent cell method (16).

After transformation, yeast cells were spread on a solid minimal medium containing soluble starch (0.67% yeast nitrogen base w/o amino acids, 2% glucose, 1% soluble starch, 2% agar, 50 μg/ml L-Leucine, and 50 μg/ml L-Histidine-HCl). Plates were incubated for about 3 days at 30°C and then stored at 4°C for 2 days to allow starch to precipitate and turn white. But starch around the starch-fermenting colonies did not precipitate and instead formed clear halos.

Genetic methods of mating and sporulation

Mating, sporulation, and other standard genetic procedures were essentially carried out according to Sherman *et al.* (17). Sporulation medium for meiosis of diploid strains contained 1% potassium acetate and appropriate amount of amino acids for auxotrophic requirments.

Southern blot analysis

Yeast genomic DNA was digested with *Bam*HI and the digested fragments were separated in 0.8% agarose gel electrophoresis and transferred to nitrocellulose filter. The probe was 3.9-kb *Bam*HI fragment in pYES2 labeled with [α-^{32}P]dATP. Hybridization was performed according to the procedures described by Southern (18).

Glucoamylase activity assay

Glucoamylase activitiy was measured by determining the amount of glucose released from starch. Reaction mixture (final volume of 0.5 ml) containing 0.5% soluble starch, 0.1 M sodium acetate buffer (pH 5.0), and 30 μl of enzyme solution was incubated at 50°C for 10 minutes, and the amount of glucose produced was measured with PGO enzyme kit composed of glucose oxidase, peroxidase, and O-dianisidine (Sigma No. 510-A). Protein content was estimated by Bradford method (19). One unit of glucoamylase activity was defined as the amount of enzyme producing 100 μg of glucose from soluble starch under the above condition.

Characterization of SGA

Glucoamylase from culture supernatant of strain YIY 345 transformed with the plasmid containing *SGA* gene, cell extracts of sporulating cells, and culture supernatant of strain 5114-9A were partially purified through sequential steps of ammonium sulfate precipitation and CM-Sephadex C-50 column chromatography [8]. With the resulting enzyme solutions, enzymatic characteristics, such as optimum temperature, thermostability, resistance to SDS, optimum pH, and resistance to urea, were compared (20, 21).

Results and Discussion

Cloning of SGA gene

The genomic library of *S. diastaticus* 5114-9A was used to transform starch non-fermenting strain, *S. diastaticus* YIY345. Of about 10,000 uracil(+) transformants, two clones showing starch-hydrolyzing activity were selected. The plasmids isolated from each of two

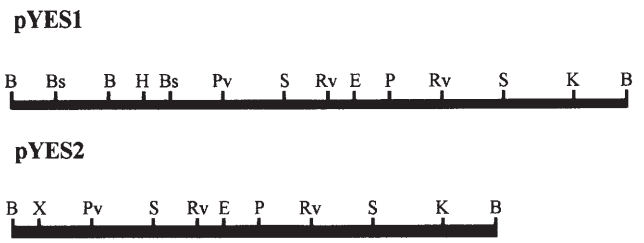

Fig. 1. Restriction map of cloned DNA fragments of pYES1 and pYES2. Recombinant plasmid pYES1 and pYES2 were digested with several restriction endonucleases, and their restriction maps were deduced from electrophoretic analysis of each digest in agarose gel. Abbreviation: B, *Bam*HI; Bs, *Bst*EII; *E, Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; X, *Xho*I.

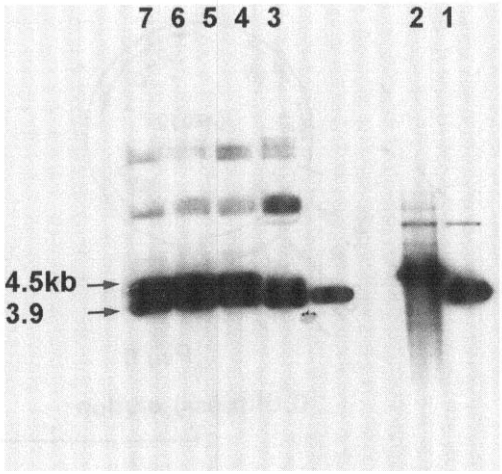

Fig. 2. Southern blot analysis of cloned DNA fragments and yeast genomic DNAs. Chromosomal DNAs were isolated and digested with *Bam*HI. About 10 μg of each sample were subjected to 0.8% agarose gel electrophoresis, transferred to nitrocellulose membrane, and then probed with [α-^{32}P]dATP-labeled 3.9-kb *Bam*HI fragment of pYES2. Lane 1, pYES2-*Bam*HI; 2, pYES1-*Bam*HI; 3, *S. cerevisiae* SHY3; 4, *S. diastaticus* YIY345 (*sta*^{o}); 5, *S. diastaticus* 5114-9A (*STA1*); 6, *S. diastaticus* 5209-11B (*STA2*); 7, *S. diastaticus* 5209-9A (*STA3*).

clones were named pYES1 (5.8-kb *Bam*HI fragment) and pYES2 (3.9-kb *Bam*HI fragment), respectively, and analyzed with several restriction endonucleases (Fig. 1). The restriction map of inserted DNA fragment in pYES1 was identical to those of *STA* genes previously reported by several groups (2, 3, 22). Cloned DNA fragment in pYES2 also showed almost the same restriction map as that of *SGA* gene (3, 11) except for some deletion of 5' flanking region which is equivalent to regulatory region of *SGA* gene.

Southern blot analysis of *SGA* and *STA* gene

Southern blot analysis of genomic DNAs purified from *S. cerevisiae* SHY3, *S. diastaticus* YIY345, *S. diastaticus* 5114-9A, 5209-11B, and 5209-9A were carried out. As shown in Fig. 2, when 3.9-kb *Bam*HI fragment of pYES2 was used as a probe, 3.9-kb fragment of putative *SGA* gene hybridized in all strains, and 4.5-kb fragment of *STA1* gene also hybridized only in strains containing extracellular glucoamylase gene (*STA*). *SGA* gene existed in all tested strains of *S. cerevisiae* and *S. diastaticus* and also had high homology to *STA* genes.

SGA gene disruption

Chromosomal *SGA* of *S. diastaticus* 5114-9A was disrupted to prove whether the inserted DNA fragment in pYES2 really encoded *SGA*. About 0.6-kb *Pvu*II-*Eco*RI fragment corresponding to N-terminal half of mature putative SGA was subcloned into *Eco*RI and *Nru*I site of a yeast integrating plasmid YIp5, resulting in YIp-ΔSGA. According to the model of integrative transformation suggested by Orr-Weaver *et al.* (23), the possible mechanism for *SGA* gene disruption is shown in Fig. 3. After transforming yeast strains, *S. diastaticus* YIY345 and YIY342, to uracil prototrophy, the integration of the plasmid was proved by Southern blot analysis (Fig. 4). Two transformants with different mating types (lane 4 and 7) were cultured in YPD broth (1% yeast extract, 2% peptone, 2% dextrose), and their glucoamylase activities in supernatant and cell lysates were measured. Two transformants had no glucoamylase activity. Furthermore, no glucoamylase activity was detected in supernatant and cell lysates prepared from diploid cells crossed with transformants having different mating type during sporulation. But when diploid cells crossed with transformants of YIp-SGA containing 3.9-kb fragment of entire putative *SGA* gene were used, glucoamylase activity was much higher than that of transformants of pYES2 (data not shown). This result could be explained by the increase in gene copies resulting from integration of putative *SGA* gene into the chromosomal *SGA* gene. These results revealed that the cloned gene in pYES 2 was an authentic *SGA* gene.

Cellular localization of *SGA*

In general, SGA is known to be an intracellular enzyme which is expressed only when a/α diploid cells of *S. cerevisiae* or *S. diastaticus* sporulate. But we could isolate the *SGA* gene by screening for the transformants with halo formation (starch-hydrolyzing activity) as described in Materials and Methods. It was also unexpected that the *SGA* gene could be expressed in the vegetatively growing *S. diastaticus* cells to produce an active enzyme which is able to hydrolyze the starch in the medium, strongly sugge-

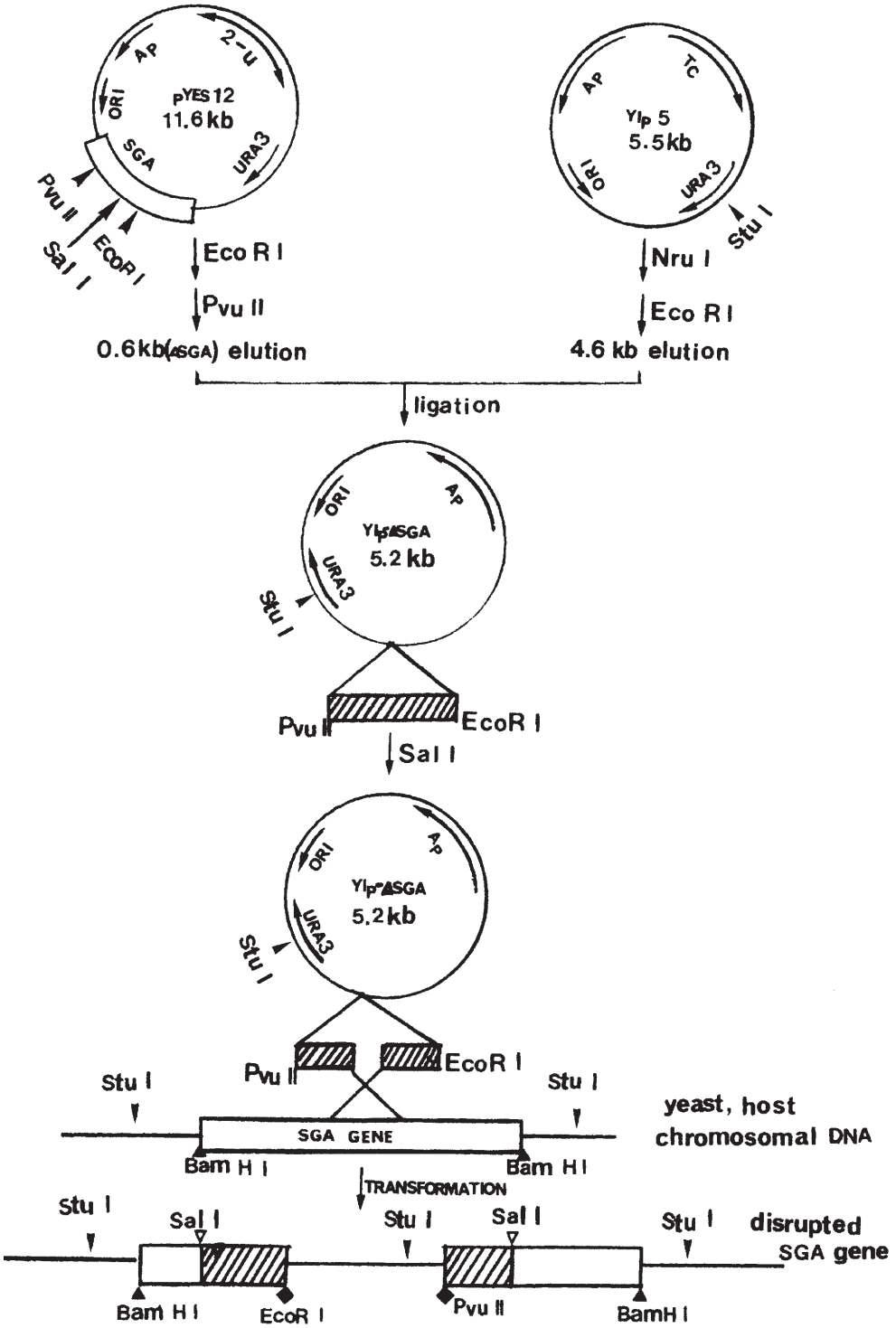

Fig. 3. Overall scheme for the disruption of *SGA* gene. Constructed YIp-ΔSGA was linearized with *Sal*I and targeted into the chromosomal *SGA* locus of *S. diastaticus* YIY345 and YIY342, respectively, by integrative transformation.

sting secretion of SGA to the medium. We presumed that the expression of *SGA* gene in vegetatively growing tranformants was due to the deletion of 5' flanking region of cloned *SGA* gene which might be essential for repressing *SGA* gene expression during vegetative growth phase. So cloned *SGA* gene could have been constitutively expressed through the mechanism independent of sporulation process.

To determine the cellular localization of the SGA produced by vegetatively growing cells transformed

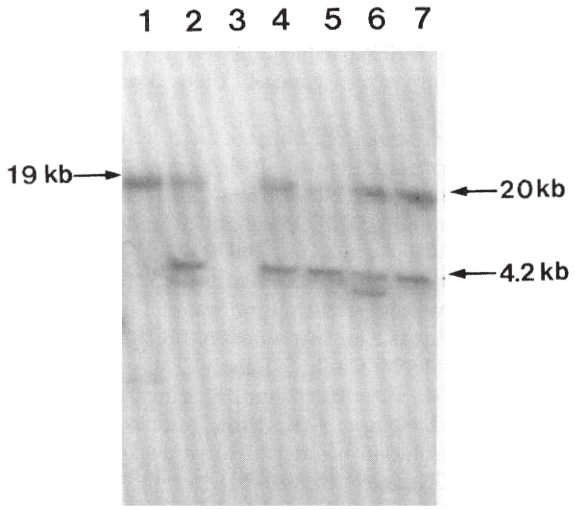

Fig. 4. Southern blot analysis of *SGA* gene-disrupted yeast strain. Chromosomal DNAs were isolated from *S. diastaticus* YIY345 and YIY342 strains transformed with YIp-ΔSGA and followed by the digestion with *Stu*I, transferring to membrane and probing with [α-^{32}P]dATP-labeled 3.9-kb *Bam*HI fragment of pYES2. Lane 1, *S. diastaticus* YIY345; lanes 2~4, YIY345 (YIp-ΔSGA); lanes 5~7, YIY342 (YIp-ΔSGA).

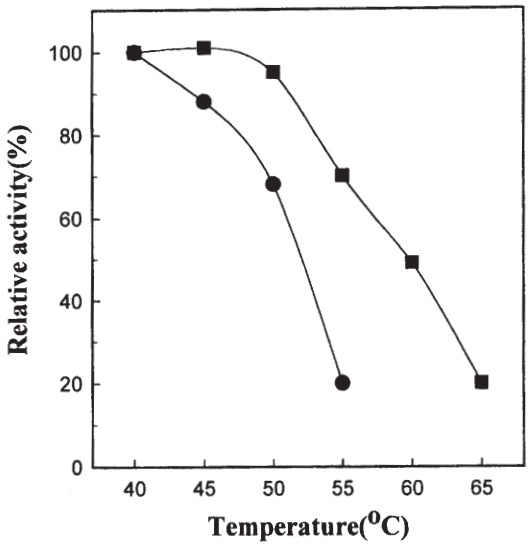

Fig. 5. Comparison of thermostability between extracellular glucoamylase and sporulation-specific glucoamylase. About 2 units of partially purified extracellular glucoamylase and SGA were incubated at indicated temperatures for 10 min, and the residual activity was measured. Filled circle and square represent SGA and extracellular glucoamylase, respectively.

with pYES2, the transformant cultures were fractionated into extracellular (supernatant), periplasmic, and intracellular fractions (24). Glucoamylase activity of each fraction was measured. As shown in Table 2, almost all activities were detected in supernatant. This result implies that SGA is likely to be a secretory protein during vegetative growth phase. But the modification of cell wall in the process of sporulation probably prevented SGA from being secreted to the exterior of cell, which made it an intracellular protein.

Charaterization of *SGA*

It would be interesting to compare the enzymatic properties of extracelluar glucoamylase encoded by *STA1* gene with those of sporulation-specific glucoamylase encoded by *SGA* gene. Two enzymes may have similar protein structures because DNA sequences encoding the functional domain of two enzymes were known to be very similar (7, 11). Moreover, the characteristics of SGA produced by vegetatively growing transformants harboring pYES2 and by sporulating cells of untransformed *S. diastaticus* were compared to one another. But there were no significant differences in enzyme characteristics between them. Extracellular glucoamylase was more thermostable than SGA (Fig. 5) and more resistant to SDS and urea. It also had higher optimum temperature (data not shown).

From the results we thought that despite of the similarity of the DNA sequences encoding functional domains of the extracellular glucoamylase and SGA, some differences must exist in protein structures resulting from different pattern of post-translational modification. And we assumed that the difference in

Table 2. Cellular localization of glucoamylase in yeast cells

Strains	Glucoamylase activity (U/ml)		
	Extracellular	Periplasmic	Intracellular
S. cerevisiae SHY3 (*sta*^{o})	–	–	–
S. diastaticus YIY345 (*sta*^{o})	–	–	–
S. diastaticus YIY345 (pYES1)	448.5(89.6)	32.5(6.5)	19.7(3.9)
S. diastaticus YIY345 (pYES2)	127.1(83.9)	1.1(0.7)	23.3(15.4)
S. diastaticus 5114-9A (*STA1*)	278.5(88.0)	29.3(9.2)	8.5(2.7)

Each yeast strain was grown in YPD broth at 30°C for 3 days, and cultures were fractionated into extracellular (culture supernatant), periplasmic, and intracellular fractions. Their enzyme activities were measured. The numbers in parenthesis denote percentage of each fraction to total activity.

post-translational modification might result from the difference in the DNA sequences encoding secretion signal and the other domains removed from functional domains in the course of post-translational modifications. And finally these differences might lead to differences in enzymatic characteristics between extracellular glucoamylase and SGA.

In conclusion, we cloned *SGA* gene by selecting for glucoamylase non-producing *S. diastaticus* showing starch-hydrolyzing activity. We concluded that SGA was a secretory protein. From the amino acid sequence deduced from the nucleotide sequence of *SGA* gene, we could find a plausible signal sequence of about 20 amino acid residues. The secretion of bacterial endo-glucanase directed by SGA signal sequence is now in progress to evaluate its secretion efficiency.

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