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Characterization of Sporulation-Specific Glucoamylase of *Saccharomyces diastaticus*

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The yeast strains of *Saccharomyces diastaticus* produce one of three isozymes of an extracellular glucoamylase I, II or III, a type of exo-enzyme which can hydrolyse starch to generate glucose molecules from non-reducing ends. These enzymes are encoded by the *STA1*, *STA2* and *STA3* genes. Another gene, sporulation-specific glucoamylase (*SGA*), also exists in the genus *Saccharomyces* which is very homologous to the *STA* genes. The SGA has been known to be produced in the cytosol during sporulation. However, we hypothesized that the SGA is capable of being secreted to the extracellular region because of about 20 hydrophobic amino acid residues at the N-terminus which can function as a signal peptide. We expressed the cloned *SGA* gene in *S. diastaticus* YIY345. In order to compare the biochemical properties of the extracellular glucoamylase and the SGA, the SGA was purified from the culture supernatant through ammonium sulfate precipitation, DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-200 chromatography. The molecular weight of the intact SGA was estimated to be about 130 kDa by gel filtration chromatography with high performance liquid chromatography (HPLC) column. Sodium dedecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed it was composed of two heterogeneous subunits, 63 kDa and 68 kDa. The deglycosylation of the SGA generated a new 59 kDa band on the SDS-PAGE analysis, indicating that two subunits are glycosylated but the extent of glycosylation is different between them. The optimum pH and temperature of the SGA were 5.5 and 45°C, respectively, whereas those for the extracellular glucoamylase were 5.0 and 50°C. The SGA were more sensitive to heat and SDS than the extracellular glucoamylase.

Key words : Extracellular glucoamylase, purification, *Saccharomyces diastaticus*, secretion, sporulation-specific glucoamylase

Introduction

Many microorganisms generally secrete hydrolytic enzymes and the role of these secreted enzymes is to degrade macromolecules in the environment into forms which can be utilized by organisms. Fungal cells secrete the starch-hydrolytic enzymes, of which glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) are glycoslyated and the most active at the relatively low pH characteristic of fungal growth habitats [11].

Glucoamylase is industrially an important biocatalyst that decomposes starch into glucose by tearing-off α-1,4-linked glucose residue from the non-reduced end of the polysaccharide chain. Glucoamylase is mostly produced by many molds, mainly *Aspergillus* sp. *Rhizomucor pusilus* and *Endomyces* sp. [4,5,16]

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In the yeast, *Sacchoromyces diastaticus*, three isoenzymes of the extracellular glucoamylase have been reported; glucoamylase I, glucoamylase II and glucoamylase III. These isozymes are encoded by the *STA1* (*DEX2*), *STA2* (*DEX1*) and *STA3* (*DEX3*) gene, respectively [14]. Three types of the *STA* genes from *S. diastaticus* have been cloned and their nucleotide sequences were analyzed [16,20]. It has been suggested that the extracellular glucoamylase I is translated as a polypeptide of 778 amino acids containing two domains; the one comprising the amino terminal 338 amino acid residues of a signal peptide and prosequence, and the other corresponding to the mature extracellular glucoamylase. This mature glucoamylase was purified and proved to consist of two subunits, H and Y [17,19].

In contrast to *S. diastaticus*, *S. cerevisiae* does not produce an extracellular glucoamylase and also can not utilize starch as a carbon source. Nevertheless, both *S. diastaticus* and *S. cerevisiae* contain common DNA sequences highly homologous to the *STA 1* gene [20]. This common DNA sequences

encode the sporulation-specific glucoamylase and is designated as the *SGA* gene. Although *S. cerevisiae* has no glucoamylase activity in the vegetative stage of life cycle, it has a glucoamylase activity in intracellular region during sporulation process [1]. This sporulation-specific glucoamylase is unusual in its intracellular location.

The comparison of the nucleotide sequences between the *STA1* and the *SGA* gene has revealed that the carboxy terminal region is almost identical and both contain potential sites for N-linked glycosylation [17,19]. The *STA1* gene contains, in addition, a serine- and threonine-rich (TS) region in the amino terminal domain which presumably provides sites for secretion information. It has been suggested that the *STA1* gene evolved from the *SGA* gene by the acquisition of the amino terminal domain consisting of the signal peptide and the TS region [18].

The SGA is suggested to be glycosylated and located in the vacuole, the hydrolytic organelle of yeast which is analogous to mammalian lysosome. Thus, although it is detected in the intracellular region, the SGA contains the information for entry into the early stage of the secretory pathway of the cell [12].

As an attempt for biochemical study of the SGA, we have cloned the *SGA* gene and constructed recombinant expression system of the *SGA* gene in *S. diastaticus*, producing the SGA at vegetative stage [5].

In this paper, we describe the purification of the SGA from the yeast cells of *S. diastaticus* transformed with a hybrid plasmid harboring the *SGA* gene and the comparison of enzyme characteristics between the extracellular glucoamylase and the SGA.

Materials and Methods

Microbial strains and culture media

The yeast *S. diastaticus* 5114-9A (*a, arg4 STA1*) and YIY 345 (*a, ura3-52 leu2-3,112 his4 sta^o inh^o*) were used as extracellular glucoamylase source and a recipient strain for the expression of the *SGA* gene, respectively. Yeast cells were grown in YPD broth (1.0% yeast extract, 2.0% peptone, 2.0% dextrose) at 30°C. The transformants of *S. diastaticus* YIY345 were cultured in YNBD medium (0.67% Bacto-yeast nitrogen base w/o amino acids, 2.0% dextrose) supplemented with histidine and leucine.

Glucoamylase activity assay

Half milliliter of reaction mixtures containing 1% soluble starch, 0.1 M sodium acetate buffer, pH 5.0 and 50 ㎕ of enzyme solution were incubated at 45°C for 30 min and the amount of glucose released was measured by PGO enzyme kit (Sigma-Aldrich Chemical Co., St. Louis, MO) [4]. One unit of the enzyme activity was defined at the amount of enzyme which releases 100 μg of glucose from soluble starch for 1 hr under the above condition.

Protein quantitation

Protein concentration was determined by Lowry method with bovine serum albumin as the standard [9]. The protein elution patterns on column chromatography were estimated by the absorbance at 280 nm.

Cellular localization of sporulation-specific glucoamylase

Yeast cells were grown in 50 ml of YPD medium at 30°C for 2 days. The fraction of extracellular region, culture supernatant, was recovered by centrifugation. Precipitated cells were resuspended in 5 ml of protoplasting buffer (0.1 M K_2HPO_4, 0.5 M KCl, 60 mM EDTA, pH 7.0) with protease inhibitors. The mixtures were incubated at 30°C for 40 min with shaking and centrifuged. The cell pellets were resuspended in 2.5 ml of protoplasting buffer containing 25 μl of 5 mg/ml of zymolyase 6,000 (Seikaguchi, Japan). The mixture were incubated at 30°C for 30 min with gentle shaking. Protoplasts were harvested by centrifugation and supernatant was regarded as the fraction of periplasmic space region. The protoplasts were washed with potassium phosphate buffer twice and resuspended in 2.5 ml of distilled water. The mixtures were vortexed with glass-bead. The lysed cells were centrifuged and supernatant was recovered as the fraction of intracellular region [12].

Purification of sporulation-specific glucoamylase

The SGA was purified by the modified method of Yamashita *et al.* [19], Pugh *et al.* [12] and Kleinmann *et al.* [6]. *S. diastaticus* YIY 345 transformants harboring the *SGA* gene were vigorously cultured in 1 liter of YPD medium at 30°C for 4 days. Culture supernatant was concentrated with 80% solid ammonium sulfate precipitation. The precipitate formed was collected by centrifugation and dissolved in 50 ml of 10 mM sodium phosphate buffer (pH 6.8). The enzyme solution was dialysed against 5 liters of phosphate buffer. The dialysed enzyme solution was precipitated with acetone at -20°C. The precipitate was collected by cen-

trifugation, dissolved in 50 ml of phosphate buffer and dialysed against the same buffer. The dialysed solution was loaded to a DEAE-Sephadex A-50 column (1.6×20 cm) equilibrated with phosphate buffer. After the column was washed with 400 ml, the enzyme was eluted with a linear gradient of NaCl (0 to 0.5 M) in phosphate buffer.

Active fractions were collected and concentrated by ultrafiltration using PM 10 membrane (Amicon, Houston, TX). The pH of ultrafiltrated solution was adjusted to 4.6 by the addition of 1.0 M sodium acetate buffer (pH 4.6) and applied to a CM-Sephadex C-50 column (1.6×20 cm) equilibrated with 10 mM sodium acetate buffer (pH 4.6). Active fractions were detected in flow-through of washing step and concentrated by ultrafiltration (PM 10 membrane). The enzyme solution was loaded to Sephadex G-200 column (2.6×94 cm) equilibrated with phosphate buffer. Active fractions were collected and concentrated with ultrafiltration (PM 10 membrane).

Determination of molecular weight

The molecular weight was determined by gel filtration chromatography using HPLC column (Protein Pak 300 SW, Waters) equilibrated with 0.1 M sodium phosphate buffer (pH 6.8) [12]. A calibration curve was made with standard proteins (Sigma-Aldrich Chemical Co.); aldolase (M.W. 158 kDa), bovine serum albumin (68 kDa), carbonic anhydrase (29 kDa) and lysozyme (14 kDa). The molecular weights of subunits were estimated by 9% SDS-PAGE according to the method of Laemmli [15]. A calibration curve was made with standard proteins; myosin (200 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase (97 kDa), bovine serum albumin (66 kDa) and hen egg ovalbumin (43 kDa).

Con-A Sepharose chromatography

Half milliliter of 5.0 M NaCl solution was added to 5 ml of enzyme solution in phosphate buffer containing 100 units of the purified SGA. The purified enzyme solution was applied to the column of Con A-Sepharose (1.2×2 cm) equilibrated with phosphate buffer containing 0.5 M NaCl. After the column was washed with same buffer, the enzyme was eluted with 0.25 M methyl-α-D-mannoside in the same buffer.

Enzymatic deglycosylation of the sporulation-specific glucoamylase

About 2.5 μg of the purified SGA dissolved in phosphate buffer was lyophilized with speed Vac concentrator and redissolved in 20 μl of DW. SDS was added to enzyme solution to the final concentration of 0.2% and heated at 100°C for 5 min. The reaction mixture was dissolved in 50 μl sodium phosphate buffer (pH 5.5). Fifty mUnit of Endoglycosidase H (Genzyme, Cambridge, MA) was added to the reaction mixture and incubated at 37°C for 24 hr followed by 9% SDS-PAGE analysis [2].

Effect of pH on enzyme activity

Two units of the purified SGA were incubated at 45°C with 1% soluble starch in 0.1 M citrate/phosphate buffer at pH values ranging from 3.0 to 7.5. The extracellular glucoamylase purified from *S. diastaticus* 5114-9A were incubated at 50°C under the same conditions to compare optimum pH with that of sporulation-specific glucoamylase.

Effect of temperature on enzyme activity

The enzyme activities of the purified extracellular glucoamylase or SGA were assayed at various temperature to compare the optimum temperature of two enzymes. The thermostabilities of them were also compared by preincubation of two units of the purified glucoamylases at different temperature without soluble starch. After preincubation at tested temperatures for 10 min, soluble starch was added to the final concentration of 1% and the residual enzyme activity was measured with PGO enzyme kit [11].

Effect of SDS on enzyme activity

Two units of the purified SGA were preincubated at different concentration of SDS without soluble starch. After preincubation at 45°C for 10 min, soluble starch was added to the final concentration of 1%. The remaining enzyme activity was estimated with PGO enzyme kit.

Results

Cellular localization of glucoamylases

We carried out enzyme activity assay of the fractions of different cellular regions of various yeast strains. As shown in Table 1, the strain, *S. diastaticus* YIY 345, which is *sta*^{o}, had no enzyme activity in any cellular region. The strain, *S. cerevisiae*, which is also *sta*^{o}, did not show an enzyme activity, either. In the previous cloning experiment, we used *S. diastaticus* YIY 345 as a recipient cell and *S. diastaticus* YIY 5114-9A as a donor cell. The strain, *S. diastaticus* 5114-9A, which harbors the *STA1* gene, had the glucoamylase activity

Table 1. Cellular localization of glucoamylase in yeast cells

Strain	Enzyme activity (unit)		
	Extracellular region	Periplasmic space region	Intracellular region
S. cerevisiae (*sta*[a])	-	-	-
S. diastaticus YIY345 (*sta*[a])	-	-	-
*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)

Table 2. Purification of the SGA from *S. diastaticus*[a] transformant

Purification step	Total activity[b]	Protein (mg)	Specific activity	Yield (%)
Culture fluid	2814	350	8.0	100
$AmSO_4$ precipitation	2260	90	25.1	80.3
Ultrafiltration	1088	20	49.4	38.6
DEAE-Sephadex	1266	5	253.2	44.9
CM-Sephadex	896	1	396	31.8
Sephadex G-200	306	0.22	1390.9	10.8

[a]*S. diastaticus* YIY345 transformed with pYES 2 (*SGA* gene).
[b]One unit is defined as 100 μg glucose released from starch per 1 hr at 45°C.

and the major proportion (about 80%) was detected in the extracellular region. The transformant cell of *S. diastaticus* YIY 345 (*pYES1*) containing the STA1 gene, also had the same enzyme activity as *S. diastaticus* YIY 5114-9A. The transformant cell of *S. diastaticus* YIY 345 (*pYES2*) containing the SGA gene, had the same pattern of enzyme activity as that of the extracellular glucoamylase. This is an interesting observation, for the SGA has been known to be an intracellular enzyme. So, we have purified the SGA from transformant cell of *S. diastaticus* YIY 345 (*SGA*).

Purification of sporulation-specific glucoamylase

We followed the method described by Yamashita *et al*. Culture fluid was precipitated by 80% ammonium sulfate and washed using ultrafiltration kit (PM10). Column chromatography was carried out using DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-200. The purification step was summarized in Table 2. The enzyme (0.22 mg) was purified to 1391 units of specific activity and 10.8% yield.

Molecular weight estimation

To estimate the molecular weight of the SGA, the pure enzyme was loaded to gel filtration chromatography column (Protein pak 300 SW. Waters). The profile of protein peak was shown in Fig. 1. The molecular weight. of the SGA was estimated to be about 130 kDa. For the analysis of subunit structure, 2 μg of pure enzyme was analysed by 9% SDS-PAGE (Fig. 2). The Fig. 2 shows that the subunit of en-

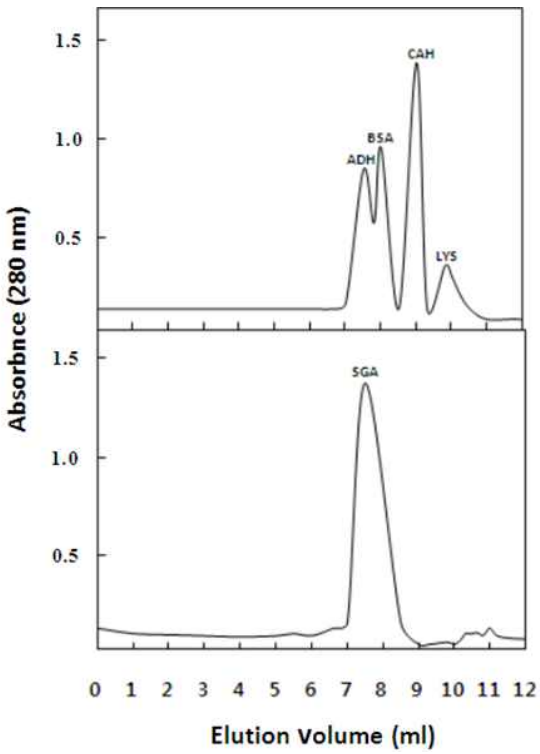

Fig. 1. Molecular weight estimation of the purified SGA by gel permeation HPLC.
The molecular weight of the intact SGA was estimated by gel filtration chromatography using HPLC column (Protein Pak 300 sw, Waters) equilibrated with 0.1 M sodium phosphate buffer (pH 6.8). Standard proteins; ADH, aldolase (158 kDa); BSA, bovine serum albumin (68 kDa); CAH, carbonic anhydrase (29 kDa); LYS, lysozyme (14 kDa).

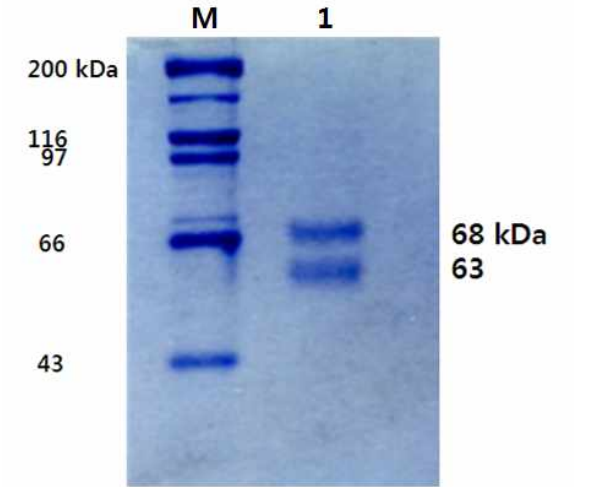

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of the purified SGA.
The purified SGA was analyzed on 9% SDS-PAGE to estimate molecular weight. Lane M, protein standards: myosin (200 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase (97 kDa), bovine serum albumin (66 kDa) and hen egg ovalbumin (43 kDa). Lane 1, the purified SGA.

zyme is heterogeneous in molecular weight and their sizes are 68 kDa and 63 kDa, respectively. From the above result, the SGA was proved to be a dimeric enzyme.

Con A-Sepharose affinity column chromatography and deglycosylation

It is well known that secretory enzymes are usually N- or O-glycosylated and the sizes of enzymes are heterogeneous because of the diverse extent of glycosylation. The SGA was loaded to the column of Con A-Sepharose to examine the glycosylation (Fig. 3A). There was no glucoamyalse activity in the fractions of washing steps. On the other hand, the 13th and 14th fractions eluted with methyl-α-D-mannoside had enzyme activity. The SDS-PAGE analysis of two active fractions showed two bands, 68 kDa and 63 kDa, which are the same pattern as the purified SGA (Fig. 3B). These results imply two bands are all glycosylated and are subunits of the SGA. To estimate the size of deglycosylated subunit, the SGA was denatured with SDS and was incubated with Endoglycosidase H. The data shows deglycosylation of the SGA produced a new smaller-sized band, 59 kDa (Fig. 3C). This result denotes that the SGA consists of two polypeptides although the sizes of subunit are heterogeneous. When glycosylation patern is compared with other secreted glucoamylases, the SGA was relatively less

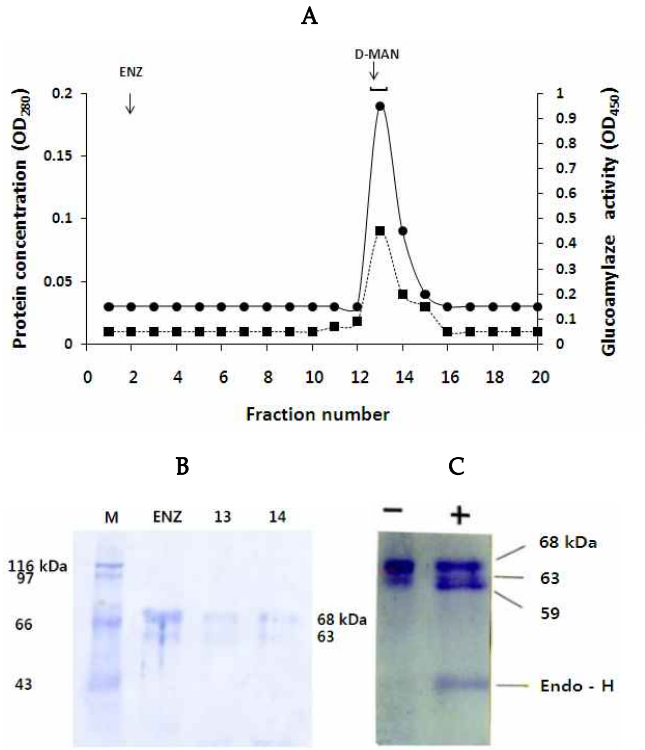

Fig. 3. Affinity chromatography of the purified SGA on Con A-Sepharose and SDS-PAGE analysis of active fractions and the deglycosylated SGA.
The purified SGA was applied to a column of Con A-Sepharose (1.2×2 cm) equilibrated with phosphate buffer containing 0.5 M NaCl. The enzyme was eluted with 0.25 M methyl-α-D-mannoside in the same buffer (A). Active fractions of #13 and #14 were analyzed by 9% SDS-PAGE, transferred to nitrocellulose membrane and stained with Coomassie brilliant blue R-250 (B). The denatured SGA was incubated with 50 mUnit of Endoglycosidase H (Genzyme) in 50 μl sodium phosphate buffer (pH 5.5) at 37°C for 24 hr followed by 9% SDS-PAGE analysis. The lane: —, no Endoglycosidase H treatment; +, Endoglycosidase H treatment (C). The symbols: —●—, optical density at 280 nm ; —■—, glucoamylase activity.

glycosylated.

Characteristics of enzymes

The Fig. 4 represents the optimum pH of the SGA was 5.5 and the enzyme activity gradually decreased at pH values above and below 5.5. But the extracellular glucoamylase had about 5.0 of pH optimum [10] and retained almost maximal activity at pH values from 4.0 to 5.5. The optimum temperatures of the SGA and the extracellular glucoamylase were 45°C and 50°C, respectively (Fig. 5).

According to the result of thermostability, the SGA was

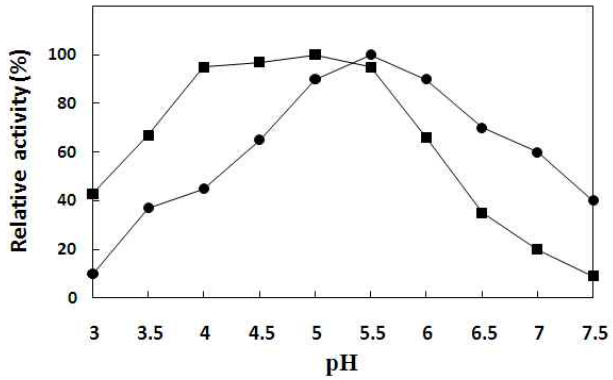

Fig. 4. Effect of pH on enzyme activities of the extracellular glucoamylse and the SGA.
The symbols: —■—, extracellular glucoamylase; —●—, the SGA.

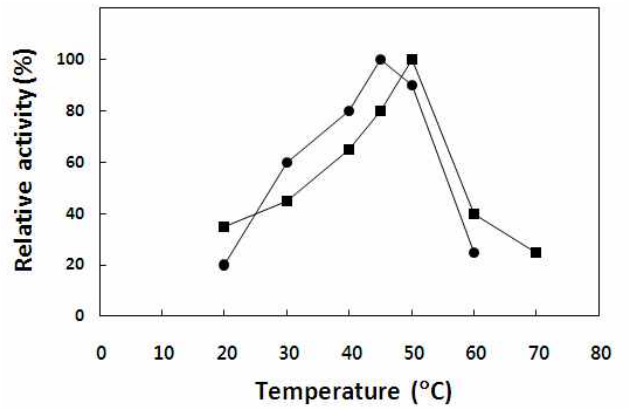

Fig. 5. Effect of temperature on enzyme activities of the extracellular glucoamylse and the SGA.
The symbols: —■—, extracellular glucoamylase; —●—, the SGA.

known to be more labile than the extracellular glucoamylase (Fig. 6). Preheating of the SGA at 40°C for 10 min had no effect on enzyme activity. However, higher temperature dramatically decreased the SGA activity and it reduced to 20% at 55°C. The extracellular glucoamylase retained enzyme activity up to 45°C and more than 20% even at 65°C. Thermostability test represents the extracellular glucoamylase is more thermostable than the SGA at tested temperatures. The SGA was inactivated at lower concentration of SDS and more sensitive to SDS than the extracellular glucoamylase (Fig. 7).

Discussion

The SGA is expressed for a short time during the sporulation stage, which made it difficult to study it. Moreover,

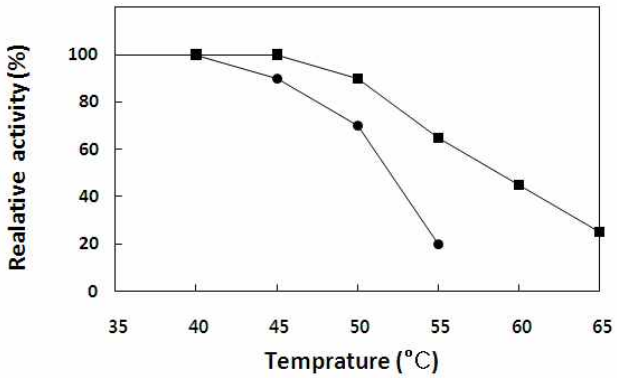

Fig. 6. Comparison of thermostability between the extracellular glucoamylse and the SGA.
The purified extracellular glucoamylase and SGA were preincubated at indicated temperatures for 10 min. After adding starch solution to final concentration of 1%, residual activity was measured with PGO enzyme kit. The symbols: —■—, extracellular glucoamylase; —●—, the SGA.

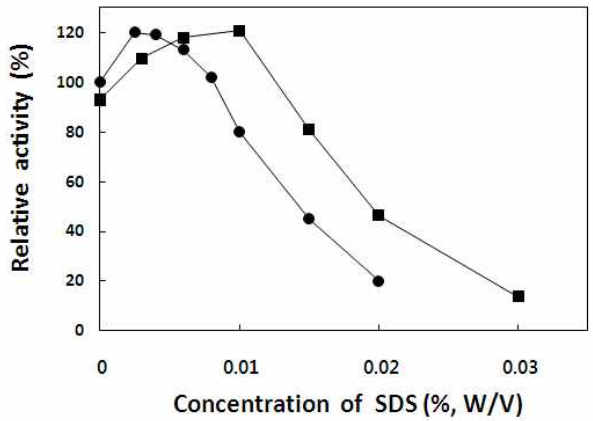

Fig. 7. Effect of SDS on enzyme activities of the extracellular glucoamylse and the SGA.
Two units of the purified SGA and extracellular glucoamylase were preincubated at different concentrations of SDS at 45°C for 10 min. Soluble starch was added to the final concentration of 1%. Residual activity was measured with PGO enzyme kit after the addition of starch solution. The symbols: —■—, extracellular glucoamylase; —●—, the SGA.

the modification of cell wall made the study of enzyme difficult, too. The SGA has been thought to be non-secreted enzyme because of its intracellular localization.

In this study, we discovered new facts on the SGA. First, the enzyme was purified in culture fluid, this means that the enzyme has the potential of secretion. This also means that the *SGA* gene encoding the enzyme has the signal peptide region. Second, the SGA was dimer-form enzyme and two subunits were identical each other except for the extent

of glycosylation. The extracellular glucoamylase has been reported to consist of H and Y subunit, which are originated from one premature polypeptide. The *SGA* gene is very homologous to the *STA1* gene of structural region. But the *STA* gene has more region than the *SGA* gene, this region was seemed to be involved in secretion of the extracellular glucoamylase. From our study, however, the region is seemed to be more involved in processing than secretion because the SGA, by itself, could be secreted. Third, the optimum pH and temperature of the SGA was more adapted to the intracellular region than extracellular region. Thermostability and SDS inhibition effect of the enzyme revealed that the SGA is not as stable as other secreted glucoamylases. These intracellular region-adapted characteristics are not consistent with the fact that the enzyme could be secreted. So, we presume that the enzyme must be compartmented in membrane-bound cell organelle during the sporulation stage although it has the signal peptide. The modification of cell wall must make the enzyme intracellular localization. If this suggestion is true, the SGA could be secreted during the vegetative stage. But we need to consider the possibility that the SGA is originally directed to the cell organelle and could not be detected in culture fluid during the vegetative stage. Considering all the results about the SGA, the enzyme is different from the extracellular glucoamylase although structural region of two genes, the *SGA* and *STA1*, are very homologous. The SGA appears to consist of two non-processed subunits, whereas the extracellular glucoamylase consists of H and Y subunit, processed fragment from one transcript of the *STA1* gene. This difference could be originated from different secretion pathway. The information which makes the extracellular enzyme enter into the secretion pathway accompanying processing is contained in the 5′ region of the *STA* gene (S2 region).

Based on our data, we conclude the SGA has secretion potential, suggesting there is a signal peptide in the amino terminal region.

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초록 : *Saccharomyces diastaticus*의 포자형성 특이 글루코아밀라제의 특성

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효모 *Saccharomyces diastaticus* 는 세포 외로 분비되는 glucoamylase I, II, III 동위효소 중 하나를 생산하여 전분을 가수분해하여 포도당을 생성할 수 있다. Glucoamylase I, II, III는 *STA1, STA2, STA3* 유전자에 의해 각각 암호화된다. 효모 *Saccharomyces* 속이 포자가 형성되는 시기에 세포 내에서 특이적으로 발현된다고 알려진 glucoamylase (SGA)의 분자생물학적 및 생화학적 연구를 수행하기 위한 일환으로 *S. diastaticus* YIY 345 형질전환체의 배양 상등액으로부터 SGA 정제를 시도하였다. 황산암모늄 침전, DEAE-Sephadex A-50, CM-Sephadex C-50, Sephadex G-200 chromatography 등의 정제과정을 거쳐서 비특이 활성이 174배 증가된 0.22 mg의 순수한 SGA를 얻었다. HPLC와 SDS-PAGE 분석을 통해 이 효소는 63, 68 kDa의 단위체로 구성된 이합체임을 확인할 수 있었다. Con-A Sepharose 친화성 크로마토그피와 탈당쇄 효소를 처리한 결과로부터 SGA는 N-연결형 당쇄로 수식되었으며 단백질 부분은 59 kDa이었다. 정제한 SGA와 세포 외 분비성 glucoamylase의 효소학적 특성을 조사하고 비교한 결과 SGA의 최적 pH와 온도는 각각 5.5와 45°C로 나타났으며 세포 외 분비성 glucoamylase는 5.0과 50°C로 나타났다. SGA는 세포 외로 분비되는 glucoamylase에 비해 열처리 및 SDS에 대해 더 민감한 반응성을 나타내었다.