

The Signal Sequence of Sporulation-Specific Glucoamylase Directs the Secretion of Bacterial Endo-1,4- β -D-Glucanase in Yeast

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The sporulation-specific glucoamylase (SGA) of *Saccharomyces diastaticus* is known to be produced in the cytoplasm during sporulation. For the purpose of proving that SGA has secretory potential, we constructed a hybrid plasmid, pYESC25, containing the promoter and the putative signal sequence of the SGA fused in frame to the endo-1,4- β -D-glucanase (CMCase) gene of *Bacillus subtilis* without its own signal sequence. The recipient yeast strain of *S. diastaticus* Y1Y345 was transformed with the hybrid plasmid. CMCase secretion from *S. diastaticus* harboring pYESC25 into culture medium was confirmed by the formation of yellowish halos around transformants after staining with Congo red on a CMC agar plate. The transformant culture was fractionated to the extracellular, periplasmic, and intracellular fraction, followed by the measurement of CMCase activity. About 63% and 13% enzyme activity were detected in the culture supernatant (extracellular fraction) and periplasmic fraction, respectively. Furthermore, ConA-Sepharose chromatography, native gel electrophoresis, and activity staining revealed that CMCase produced in yeast was glycosylated and its molecular weight was larger than that of the unglycosylated form from *B. subtilis*. Taking these findings together, SGA has the potential of secretion to culture medium, and the putative signal sequence of SGA can efficiently direct bacterial CMCase to the yeast secretion pathway.

Key words : Endo-1,4- β -D-glucanase, glycosylation, *Saccharomyces diastaticus*, secretion, sporulation-specific glucoamylase

Introduction

Eukaryotic cells synthesize many different proteins, each of which must reach a specific intracellular location or extracellular location to perform its function. Some proteins are transported to specific membrane-bound organelles. The proteins transported to extracellular location have a signal sequence directing proteins to their target sites, which is usually a short stretch of amino acids at the NH-terminus of the protein itself [17].

Signal sequences target nascent polypeptides to lumen of endoplasmic reticulum, a component of the secretion pathway. There are three distinctive structures of signal peptides from various proteins; a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c-region. The (-3,-1)-rule states that small and neutral amino

acid residues should be at -3 and -1 position for correct cleavage [10].

During meiosis in *Saccharomyces cerevisiae* and *Saccharomyces diastaticus*, the polysaccharide, glycogen is first synthesized and then degraded during the period of spore maturation. Sporulation-specific glucoamylase (SGA) is responsible for the glycogen catabolism [1]. This enzyme has been regarded as an intracellular enzyme, because the enzyme is detected in the intracellular region during sporulation. Other groups reported that the SGA is glycosylated and compartmented in cell organelle, vacuole. This result suggested that the SGA may contain the signal sequence for entry into the secretion pathway of the cell [6,12,13].

We have previously cloned the SGA gene from *S. diastaticus* and expressed it in vegetative stage [4]. Moreover, we purified and characterized the SGA from the transformant cell harboring the SGA gene [5]. We found that the major proportion of enzyme activity was detected in extracellular region. This suggests that the SGA has secretion

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potential. To assess the signal sequence of the SGA, we tried several times N-terminal amino acid sequencing with the purified SGA enzyme. But we could not get the data because N-terminal amino acid was blocked.

We used another approach to prove that the SGA has the signal sequence. In this paper, we constructed a hybrid plasmid containing the SGA promoter and nucleotide sequences encoding putative signal peptide fused to *Bacillus subtilis* endo-1,4- β -D-glucanase (CMCase) gene lacking its own promoter and signal sequence, and monitored localization of CMCase activity and the extent of glycosylation.

Materials and Methods

Bacterial strains culture media

Escherichia coli TOP10 [*F mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ *M15* Δ *lacX74* *deoR* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL*(*Str*^R) *endA1* *nupG*] was used for gene cloning and plasmid amplification. *E. coli* was grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl).

The yeast *S. diastaticus* Y1Y345 (*a, ura3-52 leu2-3,112 his4 sta^o infl*) was used as a host strain for the expression of bacterial CMCase gene. 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* Y1Y345 were cultured in YNBD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2.0% dextrose) supplemented with 20 μ g/ml of L-histidine \cdot HCl and 30 μ g/ml of L-leucine.

Prediction of SGA signal sequence

Thirty amino acid residues of N-terminal SGA were analyzed to identify a signal sequence in the SGA using TatP 1.0 server <http://www.cbs.dtu.dk/services/TatP-1.0/>. The S-score (value), C-score and Y-score of each amino acid of 30 N-terminal residues were obtained from analysis and Y-score was plotted. The cleavage site of signal sequence is generally located in amino side peptide bond of amino acid with the maximal C- and Y-score [10].

Construction of hybrid plasmid

Two plasmids, pYES12 [4] and pUEC19, were used for the construction of the hybrid plasmid, pYSC25. The plasmid pYES12 contained a 3.9-kb *Ban*HI fragment encoding the SGA at the *Ban*HI site of YEp24 vector. The plasmid pUEC19 contains structural gene of *B. subtilis* CMCase. The *Hae*III site is located at the 40th codon from the initiation codon.

The *Hind*III site is located immediately after stop codon of the CMCase. After cutting CMCase gene with *Hae*III and *Hind*III, both termini were converted to blunt ends by Klenow enzyme. The blunt ended 1.4-kb CMCase gene was inserted in *Sma*I site of pUC19 and designated as pUEC19. Thus, the CMCase in pUEC19 consists of only structural region lacking 29-amino acid signal peptide and ten amino acid residues in the N-terminal region of mature CMCase.

The pYES12 was treated with *Xba*I and *Sma*I to remove the structural region of the SGA. About 8.0-kb DNA fragment containing the promoter and 24-amino acid residues of the putative signal sequence of the SGA, was purified from agarose gel. The plasmid, pUEC19 was partially digested with *Eco*RI and its termini were converted to blunt ends with Klenow enzyme and then cut with *Sal*I. The resulting 1.4-kb DNA fragment of the CMCase was recovered and ligated with purified 8.0-kb fragment, generating a recombinant plasmid, pYSC25 (Fig. 2).

Transformation

E. coli transformation was carried out by CaCl₂ method [15]. Ttransformants were selected on LB plates supplemented with 100 μ g/ml of ampicillin. Yeast transformation was carried out by Dohmen method [3]. Yeast transformants were screened on YNBD agar plate containing L-leucine and L-histidine \cdot HCl. Yeast transformants secreting CMCase was selected on YNBD plate containing 1% carboxymethylcellulose (CMC) by Congo red staining for 30 min and then washing with 1.0 M NaCl solution [16].

Subcellular fractionation of yeast cell cultures

Yeast cells were grown in 200 ml of YPD medium at 30°C for 2 days. Culture supernatant was recovered by centrifugation and then concentrated to 5 times by ultrafiltration (PM10). This concentrated culture broth was regarded as extracellular fraction, secreted enzyme. The cell pellets were resuspended in 20 ml of protoplasting buffer (0.1 M K₂HPO₄, 0.5 M KCl, pH 7.0) and mixed with 2 ml of 0.6 M EDTA. The mixture was incubated for 40 min at 30°C with gentle shaking and centrifuged. The cell pellet was resuspended in 20 ml of protoplasting buffer and 200 μ l (5 mg/ml) of zymolyase 100T was added. The mixture was incubated for 40 min at 30°C with gentle shaking. Protoplasts were harvested with centrifugation and supernatant was regarded as periplasmic fraction. The protoplast was washed twice with potassium phosphate buffer and resuspended in 10 ml of

DW. The mixture were sonicated and centrifuged. The supernatant was recovered as intracellular fraction [2].

CMCase activity assay

The CMCase activity was determined by measuring the release of reducing ends from CMC substrate by dinitrosalicylic acid (DNS) method [9]. The reaction mixture contained 0.5 ml of appropriately diluted enzyme solution and 0.5 ml of 1% CMC in 0.05 M citrate buffer, pH 5.5. After the incubation for 30 min at 60°C, 3 ml DNS solution was added, and boiled for 5 min. The absorbance was measured at 550 nm. One unit of enzyme activity corresponded to 1 μmole of glucose equivalents liberated per min from CMC.

ConA-Sepharose affinity chromatography

The fractionated solutions were applied to a ConA-Sepharose column (1.0×5 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 M KCl. After the column was washed with the same buffer, the enzyme was eluted with 0.25 M methyl-α-D-mannoside in the same buffer and its CMCase activity was measured.

Native gel electrophoresis

Polyacrylamide gel electrophoresis was performed in 9% polyacrylamide gel in the absence of SDS as described by Laemmli [11]. Enzyme samples (10 μl) were applied to the gel and electrophoresis was conducted at room temperature at a constant current of 40 mA for 2 hr. Upon completion of electrophoresis, the gels were submerged in 50 mM citrate buffer (pH 5.5) for 30 min. The equilibrated gel was blotted on CMC agar replica containing 0.5 % CMC and 1 % agarose in 50 mM citrate buffer (pH 5.5) and then incubated at 40°C for 1 hr. The CMC agar replica was stained with Congo red solution (0.5 mg/ml) for 30 min to visualize light yellowish activity bands [14].

Results and Discussion

Construction of hybrid plasmid

S-score, C-score and Y-score analysis of 30 N-terminal amino acids showed that S-score gradually increased to maximal value at the 10th Lys and then decreased to the lowest value at the 25th Leu (data not shown), and the 25th Leu had the highest C-score of 0.665 and Y-score of 0.439 (Fig. 1). These analyses strongly suggest that most likely signal sequence cleavage site is between the 24th Ala and the 25th

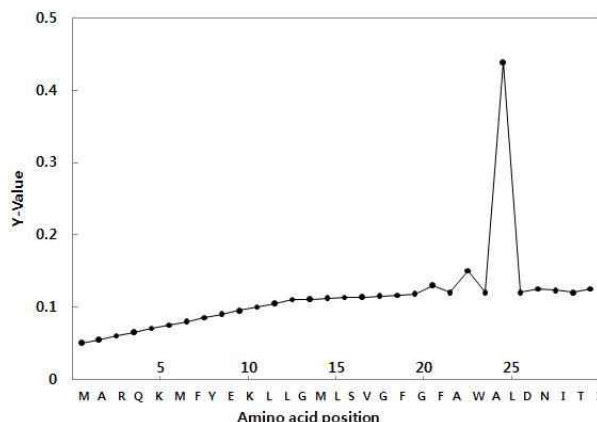


Fig. 1. Prediction of signal peptide cleavage site of the SGA. The numbering of the amino acid residues begins at the translation initiation codon, methionine. The signal sequence cleavage site predicted by cleavage probabilities is between the 24th Ala and the 25th Leu.

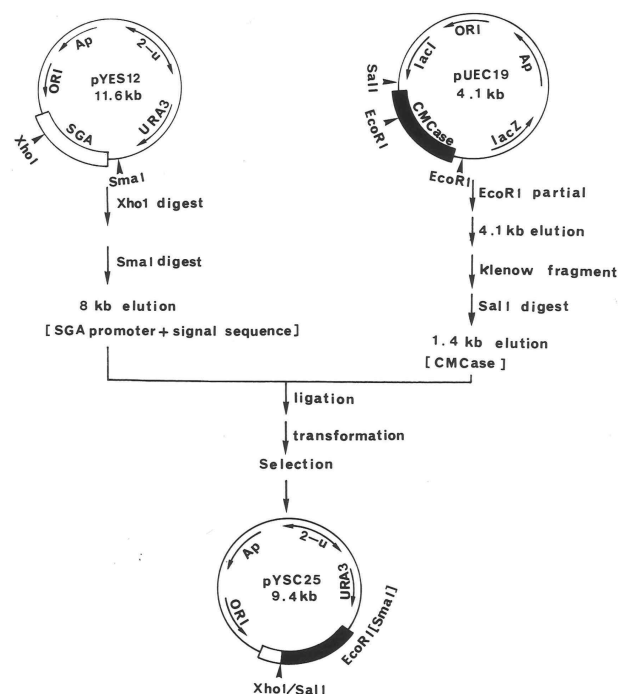


Fig. 2. Construction of a recombinant plasmid, pYSC 25. The open bar represents the open reading frame of the SGA gene and the closed bar the structural gene of *B. subtilis* CMCase.

Leu and the signal peptide of the SGA consists of 24-amino acid residues [10]. Moreover, the Ala at the 22nd and the 24th codon are consistent with the (-3, -1) rule [18].

The hybrid plasmid, pYESC25 was made according to the procedures shown in Fig. 2. This plasmid contains the promoter and signal sequence of the SGA and structural region

of *B. subtilis* CMCase. The nucleotide and amino acid sequences around the fusion site between the SGA signal peptide and the CMCase gene are shown at Fig. 3. The 24-amino acid residues from the initiation codon, Met to the 24th codon, Ala are the putative signal peptide of the SGA. The structural region of the CMCase starts at the 32nd codon. There are more 6 amino acid residues between the signal peptide and structural CMCase, which were originated from the SGA and polylinker cloning sites of the vector, pUC19. The correctly recombined plasmid, pYEC25 was selected by restriction enzyme mapping and nucleotide sequencing analysis and then introduced into *S. diastaticus* YIY345 by transformation.

Secretion and glycosylation of *Bacillus* CMCase

When transformants containing the pYSC25 were grown on YNBD plate containing 1% CMC and stained with Congo red, the yellowish halos were shown around the colonies (Fig. 4B). These halos indicate that the CMCase was secreted into culture medium by the aid of the signal sequence of the SGA. However, there were no halos around host strain, *S. diastaticus* YIY345 (Fig. 4A).

To assay CMCase activity in the different cellular regions, we cultured transformant cells in 200 ml of YPD broth and then fractionated culture into extracellular, periplasmic and cytoplasmic CMCase according to the methods described in Materials and Methods. First, these prepared enzyme solutions (20 μ l) were loaded in wells on agar plates containing

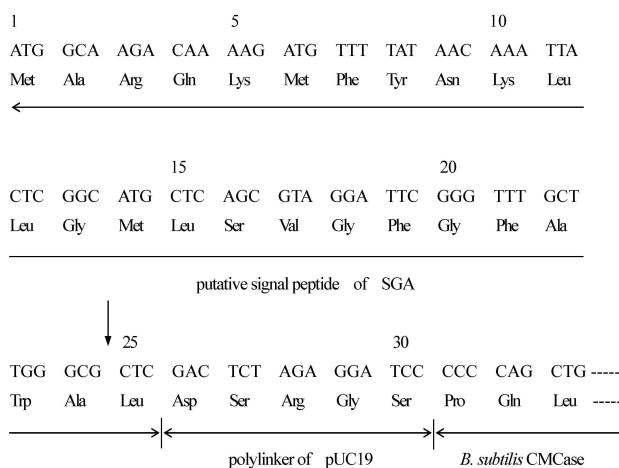


Fig. 3. The nucleotide and amino acid sequences around the fusion sites of the SGA and the CMCase gene. The corresponding amino acid sequence is given in three-letter code. The arrow indicates probable cleavage site of the SGA signal sequence.

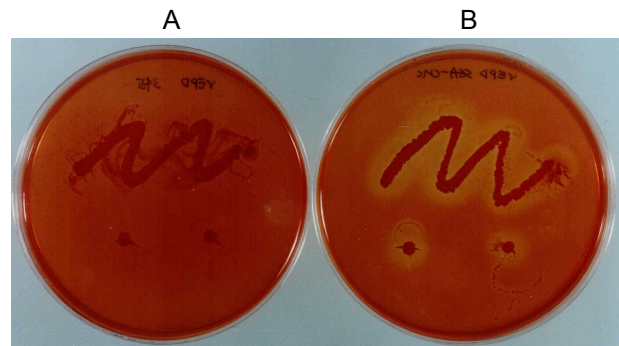


Fig. 4. Secretion of CMCase by yeast transformant. Recipient cells, *S. diastaticus* YIY 345 (A) and transformant cells containing pYSC 25 (B) were cultured on minimal agar media supplemented with 1% CMC and stained with Congo red. The yellowish halos indicate that CMCase was secreted into culture medium by the signal sequence of the SGA.

1% CMC and then incubated at 40°C for 30 min. The halos around wells appeared after Congo red staining (data not shown). CMCase activity was detected in all fractionated sample; extracellular, periplasmic and cytoplasmic region. The enzyme activity of each region was shown in Table 1. The total activity was lower than expected, which may be due to the weak promoter activity of the SGA gene. Regulatory region like upstream activator sequence could be missing in relatively short promoter in the cloned SGA gene [8]. Because of the absence of regulatory region, we could purify the SGA from transformant containing the SGA gene at the vegetative stage [5].

The CMCase contains two potential N-glycosylation sites (Asn-X-Ser/Thr). N-linked glycosylation of proteins occurs during the passage through endoplasmic reticulum (ER) and Golgi apparatus which are components of yeast secretion pathway. If the CMCase in the culture medium and periplasmic region was glycosylated, it would strongly suggest that the enzyme is secreted through the yeast secretory pathway. This was confirmed by ConA-Sepharose affinity chromatography and native gel electrophoresis. The CMCase from extracellular, periplasmic and cytoplasmic region was loaded onto ConA-Sepharose column. The enzymes in all regions were tightly bound to the resin and specifically eluted with methyl- α -D-mannoside (Table 1). This implies that yeast CMCase was glycosylated and putative SGA signal sequence functions in targeting bacterial CMCase to ER, the first step of yeast secretion pathway.

For the comparison of molecular size of glycosylated

Table 1. Subcellular localization of CMCase activity in *S. diastaticus* YIY345 transformant

Subcellular fraction	CMCase activity (unit) ^a	Relative activity (%)	Glycosylation ^b
Extracellular	33	62.7	+
Periplasmic	7	13.3	+
Intracellular	12.6	24.0	+

Yeast transformant strain was cultured in 200 ml of YPD medium at 30°C for 2 days.

^aOne unit was defined as the amount of enzyme releasing 1 μ mole of glucose equivalents from CMC per minute.

^bThe degree of glycosylation was verified by Con-A Sepharose affinity chromatography and CMCase activity assay of flow-through and eluted fraction.

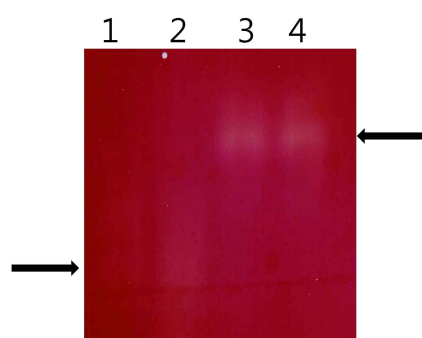


Fig. 5. Native polyacrylamid gel electrophoresis of CMCase. Each sample (10 μ l) was loaded on 9.0 % polyacrylamide gel and electrophoresis was conducted for 2 hr. The polyacrylamid gel was blotted on CMC agar replica and the replica was stained with Congo red. Lane 1, *B. subtilis* CMCase; 2, intracellular fraction; 3, periplasmic fraction; 4, extracellular fraction.

CMCase from yeast with unglycosylated CMCase from *B. subtilis*, native gel electrophoresis was carried out and CMCase was detected with activity staining. Fig. 5 showed that extracellular and periplasmic CMCase from yeast were much larger than the CMCase from bacteria. This means that the CMCase from yeast was hyper-glycosylated and molecular weight increased to more than 150 kDa (Fig. 5. right upper arrow). The MW of the CMCase from *B. subtilis* was reported to be 33 kDa [11]. Cytoplasmic CMCase was partly glycosylated and the size of MW was similar to bacterial CMCase (Fig. 5. left lower arrow).

In conclusion, we proved that the SGA has secretory potential in that bacterial CMCase directed by the putative SGA signal sequence was secreted to extracellular region via yeast secretion pathway. This might be the first report about heterologous protein secretion using putative signal sequence of the SGA. The SGA signal sequence could be utilized for the secretory production of other heterologous proteins in *Saccharomyces* species.

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초록 : 효모에서 포자형성 특이 글루코아밀라제의 분비서열에 의한 세균 endo-1,4-β-D-glucanase의 분비

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효모 *Saccharomyces diastaticus*가 포자형성기에 세포질에서 생산된다고 알려진 포자형성 특이 glucoamylase (SGA)가 세포 외로 분비되는 단백질을 증명하고자 *S. diastaticus*의 SGA promoter와 예상되는 분비신호서열 다음에 reporter gene으로 사용한 고초균의 CMCase 구조유전자를 융합한 재조합 플라스미드 pYSC25를 제작하고 수주세포인 *S. diastaticus* YIY345에 형질전환 하였다. 형질전환체를 1% CMC를 포함하는 최소한천배지에서 배양한 후 Congo red 염료로 염색하여 생성된 투명환으로부터 SGA의 분비서열에 의해 세균의 CMCcase가 효모세포 외로 분비되는 것을 확인하였다. 효모세포부위 별 CMCcase의 활성분포를 측정하여 SGA 분비서열의 분비효율을 추정하기 위해 효모세포 배양액을 배양상등액, periplasmic 및 세포질 분획으로 나눈 다음 효소활성을 측정한 결과 CMCcase 활성의 76%가 배양상등액과 periplasmic 부위에 존재하였으며 N-연결형 당쇄가 일어났으므로 SGA 분비서열은 효과적으로 작용함을 알 수 있었다. 대조균인 고초균에서 생산된 CMCcase에서는 당쇄가 일어나지 않은 것을 확인하였다. 이상의 결과로부터 SGA는 아미노 말단에 존재하는, 24개의 아미노산으로 구성된 분비서열을 보유한 분비성 단백질을 확인하였다.