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Overproduction and Secretion of β-Glucosidase in *Bacillus subtilis*

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Abstract Overproduction of intracellular β-glucosidase was attempted by modifying the promoter region of a β-glucosidase gene cloned from *Cellulomonas fimi* and expressing it in *Bacillus subtilis* DB104. A strong engineered promoter, BJ27UΔ88, was fused to the β-glucosidase gene after removing its native promoter. An effective Shine-Dalgarno sequence (gene10 of phage T7) was inserted between the promoter and the β-glucosidase structural gene. The modified gene was overexpressed in *B. subtilis* and produced 1121.5 units of β-glucosidase per mg protein which is about 12% of total intracellular protein. Secretion of overproduced intracellular β-glucosidase was attempted by using the signal sequence of the *Bacillus* endoglucanase gene as well as an in-frame hybrid protein of endoglucanase. The hybrid protein was normally secreted into the culture medium and still retained β-glucosidase activity.

Key words: β-Glucosidase, endoglucanase, overproduction, secretion, *Bacillus subtilis*

The enzymatic degradation of cellulose, which is the most abundant reusable organic compound in the world, requires the synergistic action of at least three enzymes: endoglucanase (EC3.2.1.4), cellobiohydrolase (EC3.2.1.91) and β-glucosidase (EC 3.2.1.21). Cellobiose is one of the main products of cellulase-catalyzed break down of cellulose, and the enzyme β-glucosidase catalyzes the conversion of cellobiose to glucose and can be the rate-limiting enzymatic step in cellulose degradation [2, 3]. Although *Trichoderma reesei* is one of the best sources of the first two enzymes, its β-glucosidase production is low [10, 11]. Attempts have been made to increase the β-glucosidase activity by controlling growth conditions [14] and it has been shown that the hydrolytic potential of the cellulase complex is greately enhanced by the addition of supplemental β-glucosidase [12, 14]. In this respect, efficient production of the β-glucosidase separately in a suitable bacterial host and then supplementing the enzyme or the strain would be an another plausible approach.

Several microorganisms are being used as an expression system for the production of important biological peptides and proteins. Those useful properties for biotechnology include reliable gene manipulation, adaptation to various nutritional and physiological conditions of growth, genetic stability during fermentation, mass productivity of the desired products, and nonpathogenecity to humans. *Bacillus subtilis*, a gram-positive bacterium, has the potential to be a useful system for producing proteins, because it has most of characteristics listed above and, very significantly, can secrete proteins into the culture medium efficiently. Thus, under the proper conditions, *B. subtilis* has the capacity to synthesize and secrete specific foreign proteins relatively free of other cellular proteins. To overexpress specific genes in *B. subtilis* during vegetative growth, we previously cloned and characterized many strong constitutive promoter fragments of *B. subtilis* chromosome. One of them, the strong promoter BJ27UΔ88, which is a modified form of a parental promoter BJ27, was successfully used for the overproduction of endoglucanase in *B. subtilis* [6, 7].

In this study, we attempted overproduction and secretion of β-glucosidase in *B. subtilis*. The β-glucosidase gene was previously cloned from *Cellulomonas fimi* in *Escherichia coli* and its nucleotide sequence and protein characteristics were determined [5]. To construct the β-glucosidase expression cassette (pβ) that was to

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be transferred into the *Hind*III site of the expression vector pBJ27UΔ88E [6], the native promoter and Shine-Dalgarno (SD) sequence of the β-glucosidase gene were replaced by an engineered strong promoter, BJ27UΔ88 [7], and a SD sequence from gene 10 of phage T7. Using the *B. subtilis* DB104 strain harboring pBJ27UΔ 88β, overproduction of intracellular β-glucosidase was attempted. We also attempted the secretion of overproduced intracellular β-glucosidase in *B. subtilis* using the signal sequence of the endoglucanase gene from *B. subtilis* as well as an in-frame hybrid protein of endoglucanase.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

The bacterial strains and plasmids used, including their pertinent attributes and derivations, are summarized in Table 1. The LB medium was used for routine bacterial culturing and kanamycin was added to the medium at a final concentration of 30 μg/ml.

DNA Manipulation

DNA manipulations were carried out with restriction enzymes and T4 DNA ligase (Boehringer Manheim Co.,

Table 1. Bacterial strains and plasmids used.

Strains or plasmid	Description
E. coli JM109	Transformable host for plasmid constructions before transfer to *B. subtilis* [8]
B. subtilis DB104	Transformable host for studying expression of plasmid constructions as well as double mutant deficient in extracellular alkaline and neutral protease [4]
Plasmids	
pUC19	Vector for plasmid constructions in *E. coli* [8]
pUCβ	Starting plasmid with SD sequence of gene 10 of phage T7, β-glucosidase structural gene from *C. fimi*, and noncoding regions (this work)
pβ	*Hind*III cassette of β-glucosidase gene that was to be transferred into pBJ27UΔ88; pUCβ was treated with *Sal*I, self-ligated and amplified with primers 1 and 2 (prepn 1); prepn 1 was treated with *Eco*RI, *Hind*III and ligated with *Eco*RI-*Hind*III treated pUC19, introduced in *E. coli* and selected by restriction mapping (prepn 2); prepn 2 was treated with *Kpn*I and ligated with *Kpn*I fragment of pUCβ (this work)
pENDO	*Hind*III casette of endoglucanase gene that was to be transferred into pBJ27UΔ88 (this work)
pSEC	Plasmid containing signal sequence of endoglucanase; pENDO was treated with *Hinc*II and *Bgl*II linker was attached to fix the reading frame of the β-glucosidase gene (this work)
pSECβ	*Hind*III secretion cassette of β-glucosidase that was to be transferred into pBJ27UΔ88; pSEC was opened by *Bgl*II and *Xba*I, and ligated to the *Bam*HI-*Xba*I fragment of pβ (this work)
pENDO/β	*Hind*III cassette of endoglucanase-β-glucosidase hybrid that was to be transferred into pBJ27UΔ 88; *Bam*HI linker was attached into the *Ssp*I fragment of pENDO and the resulting DNA was opened by *Bam*HI and combined to the *Bam*HI fragment of pβ (this work)
pBJ27UΔ88	*E. coli–B. subtilis* shuttle expression vector with BJ27UΔ88 promoter; pBR322 and pUB110 origins, kanamycin and ampicillin resistance, multiple cloning site derived from pUC8 [6, 7]
pBJ27UΔ88E	*E. coli–B. subtilis* shuttle expression vector with the *Hind*III casette of endoglucanase gene under the control of the BH27UB88 promoter [6, 7]
pBJ27UΔ88β	*E. coli–B. subtilis* shuttle expression vector with the *Hind*III casette of β-glucosidase gene under the control of the BJ27UΔ88 promoter (this work)
pBJ27UΔ88SECβ	*E. coli–B. subtilis* shuttle expression vector with the *Hind*III secretion casette of β-glucosidase gene under the control of the BJ27UΔ88 promoter (this work)
pBJ27UΔ88ENDO/β	*E. coli–B. subtilis* shuttle expression vector with the *Hind*III cassette of endoglucanase-β-glucosidase hybrid gene under the control of the BJ27UΔ88 promoter (this work)

Manheim, Germany) according to the instructions of the suppliers. Purification of plasmids and DNA fragments were done as described by Maniatis *et al.* [8]. The oligodeoxynucleotide was prepared by a DNA synthesizer (Pharmacia LKB Gene Assembler system) according to the manufacturer's recommendation.

Polymerase Chain Reaction (PCR)

The PCR reaction was carried out in a Hybaid thermal reactor (HBTR1, Hybaid Inc., U.K.) in a total volume of 100 μl using 1 unit of *Taq* polymerase (New England BioLabs, Beverly, U.S.A.). Primer 1 (5'-GGGGTTCGA-AAGTCCCGACCTACCAG-3') and primer 2 (universal forward primer) were used as PCR primers. The reaction was performed for 30 cycles in standard conditions. The PCR product was extracted with phenol/chloroform and then precipitated with ethanol.

Transformation of *B. subtilis*

The transformation of *B. subtilis* cells was performed by the competent-cell method [13].

Enzyme Assay

β-Glucosidase activities were determined by measuring the liberation of *p*-nitrophenol (*p*NP) from paranitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate. The *p*NPG was dissolved in a 0.05 M sodium phosphate buffer at a final concentration of 1 mM. Crude extracts or supernatants of culture broth were mixed with 1 ml of 1 mM *p*NPG solution in sodium phosphate buffer and incubated for 15 min at 37°C. The reaction was stopped by the addition of 2 ml of 1 M sodium carbonate solution. The released *p*NP was determined by reading OD_{400}. One unit was defined as the amount of enzyme which hydrolyzed 1 nmol of *p*NP per min at 37°C.

Quantitative assays of endoglucanase were performed in a 50 mM citrate buffer (pH. 5.5) at 55°C and activities were measured according to the reducing sugar method [9]. One unit of activity was defined as an amount of enzyme that released 1 μmole of glucose equivalent per min. The endoglucanase activity was expressed in units per ml of supernatant.

Protein concentration was determined by the Bradford method [1] and crystalline bovine serum albumin was used as a standard. All assays were repeated a minimum of three times for each strain and the results were highly reproducible.

RESULTS AND DISCUSSION

Construction of *Hind*III Expression Cassette, pβ

A plasmid with a β-glucosidase gene that was to be transferred to the *B. subtilis* expression vector pBJ27UΔ88

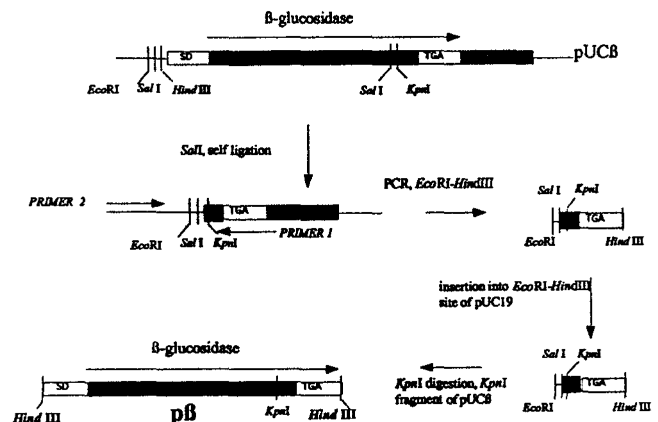


Fig. 1. Construction scheme of the β-glucosidase *Hind*III expression cassette, pβ.

was constructed (Fig. 1). The pUCβ contains an SD sequence of gene 10 of *E. coli* phage T7, the β-glucosidase structural gene, and noncoding regions. To remove the noncoding region and introduce a *Hind*III site at the end of the termination codon, a PCR was performed. To minimize the fidelity error during PCR, a small 300 bp fragment of DNA that comprises a portion of the 3' end of the β-glucosidase gene and noncoding region was subcloned into pUC19. This fragment was subsequently used as a template for PCR. The sequences of primers 1 and 2 are described in the Materials and Methods section. The PCR product was confirmed by restriction mapping and nucleotide sequencing. This PCR product was digested with *Eco*RI and *Hind*III and transferred into the *Eco*RI-*Hind*III sites of pUC19. To assemble the β-glucosidase gene, the construct was opened by *Kpn*I and ligated with the *Kpn*I fragment of pUCβ to construct pβ. The plasmid pβ, a *Hind*III cassette that was to be transferred into the expression vector pBJ27UΔ88, contains the *Hind*III fragment carrying the SD sequence from gene 10 of phage T7 and β-glucosidase structural gene from *C. fimi*.

Expression of the β-Glucosidase Gene in *B. subtilis*

From the plasmid pβ a *Hind*III fragment containing the engineered β-glucosidase gene was transferred to an expression vector pBJ27UΔ88 to obtain pBJ27UΔ88β. *B. subtilis* DB104 was transformed with pBJ27UΔ88β and a new strain harboring pBJ27UΔ88β, *B. subtilis* (pBJ 27UΔ88β), was constructed. Another strain harboring pBJ27UΔ88, *B. subtilis* (pBJ27UΔ88), which carries the vector plasmid alone, was also constructed for comparison.

Expression of the β-glucosidase gene in the host cell was observed. SDS-PAGE analysis of a total cell extract of *B. subtilis* (pBJ27UΔ88β) showed a major distinct protein band (Fig. 2). It was shown that the protein band corresponds to β-glucosidase by pNPG blotting (data not

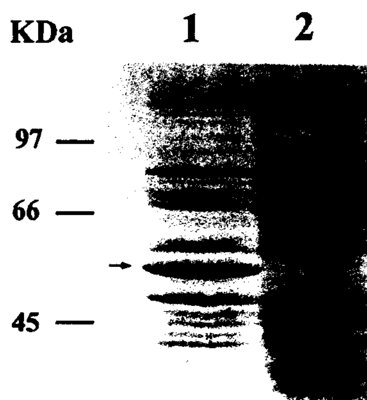


Fig. 2. SDS-PAGE analysis.
B. subtilis DB104 strains harboring pBJ27UΔ88(β) (lane 1) or empty expression vector (pBJ27UΔ88) (lane 2) were grown for 12 h at 37°C in LB medium. 20 μl of cell extracts were applied to a 8% polyacrylamide gel. An arrow indicates overproduced β-glucosidase.

shown). Total activities of the β-glucosidase were localized to the cytoplasm, suggesting that most of the enzyme produced was not secreted into the culture medium. The specific activity in this case was 1125.6 units per mg protein which accounts for about 12% of the total cell protein by densitometric scanning (data not shown).

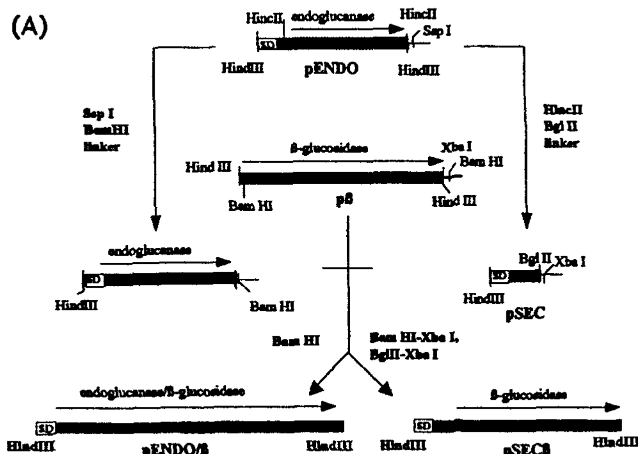
Construction of *Hind*III Secretion Cassette, pSECβ and pENDO/β

To secrete overproduced β-glucosidase into the culture medium of *B. subtilis*, we introduced two approaches. The first was to fuse the signal sequence of the endoglucanase gene to the β-glucosidase gene and the second was to construct a hybrid protein in which the endoglucanase gene is fused in frame to the β-glucosidase gene.

To fuse the signal sequence of the endoglucanase gene to the β-glucosidase gene, plasmid pENDO [4] was digested by *Hinc*II, and a *Bgl*II linker (5'-CAGATCTG-3') was attached to fix the reading frame of the β-glucosidase gene. Self-ligation of this DNA fragment produced pSEC. Subsequently, pSEC was opened by *Bgl*II and *Xba*I, and ligated to the *Bam*HI-*Xba*I fragment of pβ to make a *Hind*III secretion cassette pSECβ (Fig. 3). To make a hybrid protein of β-glucosidase which is fused to the endoglucanase structural gene, a 12-mer *Bam*HI linker (5'-CGCGGATCCGCGC-3' was inserted into the 3' end of the *Ssp*I fragment of pENDO. The resulting DNA was opened by *Bam*HI and combined with the *Bam*HI fragment of pβ to construct another *Hind*III secretion cassette pENDO/β (Fig. 3).

Secretion of β-Glucosidase in *B. subtilis* Using pENDO/β and pSECβ

The *Hind*III secretion cassette, pENDO/β or pSECβ, was transferred to the *Hind*III opened expression



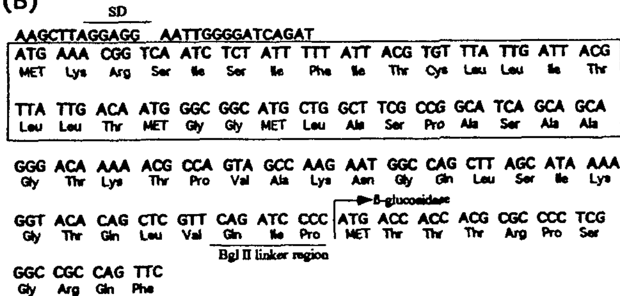


Fig. 3. Construction scheme of the *Hind*III secretion cassettes (A) and the nucleotide sequence of signal sequence-β-glucosidase fusion (pSECβ) (B).
The boxed region indicates the N-terminal signal sequence of endoglucanase gene.

vector pBJ27UΔ88 to obtain pBJ27UΔ88ENDO/β and pBJ27UΔ88SECβ, respectively. *B. subtilis* DB104 was transformed and a new strain harboring each plasmid was constructed. Expression and secretion of β-glucosidase in the host cells was observed. SDS-PAGE analysis and enzyme assay of the culture supernatant of *B. subtilis* (pBJ27UΔ88SECβ) showed neither protein band corresponding to β-glucosidase or β-glucosidase activity. Also, inside of the host cell, we could detect neither a protein band of β-glucosidase or β-glucosidase activity. On the other hand, the culture supernatant of *B. subtilis* (pBJ27UΔ88 ENDO/β) revealed β-glucosidase activity, although the level of enzyme activity was reduced. The specific activity in this case was 271 units per mg protein. The β-glucosidase activity was not detected in the cytoplasm of the host cell. However, the level of endoglucanase activity in the culture supernatant was very close to the level in an overexpression system containing endoglucanase gene alone [6], suggesting that the signal sequence of the endoglucanase gene in hybrid protein functioned efficiently. We could not have achieved the extracellular production of β-glucosidase

by simple fusion of the signal sequence. This may be caused by denaturation which results from agitation, and the denaturation of β-glucosidase could lead to increased susceptibility to proteolysis in *B. subtilis*. Obviously, more studies are required on the unstable characteristics of β-glucosidase, especially in the extracellular environment of *B. subtilis*. We previously noticed the intrinsic instability of the β-glucosidase as a purified form. In this report, we found again that such unstable properties are still present in the extracellular environment of *B. subtilis*. However, the observed extracellular production of β-glucosidase as a hybrid protein of endoglucanase which is secreted into the culture supernatant raises the possibility of enhanced extracellular production of β-glucosidase in *B. subtilis*.

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