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Escherichia coli Signal Peptidase Recognizes and Cleaves the Signal Sequence of Xylanase from a Newly Isolated *Bacillus subtilis* Strain R5

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Abstract—A gene encoding the xylanase from *Bacillus subtilis* strain R5 containing the native signal sequence was cloned and expressed in *Escherichia coli*. The heterologous expression of the gene resulted in the production of the recombinant protein in the cytoplasm as well as its secretion into the culture medium. The xylanase activity in the culture medium increased with time after induction up to 90% of the total activity in 14 h. Molecular mass and N-terminal amino acid sequence determinations of the purified recombinant xylanase revealed that the native signal peptide was cleaved off by *E. coli* signal peptidases between Ala28 and Ala29.

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Proteins contain intrinsic signals that govern their transport and localization in the cell. Protein secretion in bacteria is one of the important fields in biological research. The secretion system of the Gram-negative bacterium *Escherichia coli* has been studied in detail [1-3] compared to Gram-positive bacteria such as *Bacillus* species [4, 5]. Although the structure of the cell membrane and cell wall of *E. coli* and *Bacillus* sp. are significantly different, the molecular mechanisms for protein export of both types of bacteria share a number of common features [6, 7]. Their exported proteins are synthesized by membrane bound ribosomes, they use proton motive force to transport the protein, and the structure of the signal peptides of both *Bacillus* and *E. coli* are quite similar [7]. They are composed of three distinct regions: a positively charged region (N-domain), a hydrophobic core region (H-domain), and a hydrophilic signal peptidase recognition site (C-domain) [8]. A main limitation of using *E. coli* for secretion of recombinant proteins is that not every protein can be secreted efficiently even using the *E. coli* signal sequences [3].

We isolated *B. subtilis* strain R5 from an oily material coagulated in the drains of a restaurant in Osaka, Japan. The strain R5 produces several extracellular enzymes including amylase, cellulase, lipase/esterase, protease, and xylanase [9]. Xylanase from this strain has been cloned and characterized [10].

In this paper we report that the *E. coli* secretion system recognizes the signal sequence of xylanase from *B. subtilis* strain R5, cleaves it, and secretes out the mature protein into the culture medium.

MATERIALS AND METHODS

DNA manipulation. Enzymes were purchased from Fermentas (USA) and used according to the instructions of the manufacturer. Genomic DNA and plasmid DNA were isolated by using Fermentas Genomic and Plasmid DNA isolation kits, respectively (Fermentas). A DNA purification kit (Fermentas) was used to recover DNA fragments from agarose gels. Transformations were carried out using a calcium chloride method [11].

Cloning and expression of xylanase gene. A xylanase gene from *B. subtilis* strain R5 was amplified by polymerase chain reaction (PCR) by using a set of forward (5′-GGAGGTAACATATGTTTAAG-3′) and reverse (5′-GCTACCCCTGATTAAGGATG-3′) primers. The primers

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were designed on the basis of the N- and C-terminal amino acid sequence of the precursor xylanase (accession No. AB457186). The PCR amplified DNA fragment was inserted into pT7-7 expression vector utilizing the *Nde*I and *Eco*RI restriction sites. The resulting plasmid was used to transform *E. coli* strain BL21(DE3) CodonPlus-RIL. Heterologous expression of the xylanase gene was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant protein was purified and analyzed as described previously [10].

Molecular weight determination. The molecular mass of the purified protein was analyzed by matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry (MALDI-TOF MS). The purified enzyme was desalted by a Microspin TM G-25 column (GE Healthcare, USA). Analyte was prepared in sinapinic acid made in a diluent (1/3 volume of acetonitrile, 2/3 volume of 0.1% trifluoroacetic acid in water). Sinapinic acid (10-15 mg) was dissolved in 1 ml of the diluent. The solution was centrifuged at 12,000*g* for 2 min at room temperature. The salt-free protein (2 μg/μl) was mixed with sinapinic acid solution (6 mg/ml) in a 1 to 10 ratio, respectively, and 2 μl applied to the target plate of an Auto Flex III SmartBeam Mass Spectrometer (Bruker Daltonics GmbH, Germany). The sample was allowed to dry at room temperature for 15-25 min. The spectrum was obtained by striking 200 shots in a detection range of 12,000-100,000 Da, and the final spectrum was subjected to smoothing, baseline subtraction and centroiding.

N-Terminal amino acid sequence determination. To determine the N-terminal amino acid sequence, the recombinant protein after SDS-PAGE analysis was electroblotted onto a polyvinylidene difluoride membrane (PVDF from Millipore, USA). The membrane was stained for 5 min with Amido Black solution, which was prepared by dissolving 5% amido black in 40% methanol and 10% acetic acid. The membrane was destained with the solution consisting of 40% methanol, 10% acetic acid, and N-terminal amino acid residues were commercially determined (Alta Bioscience, UK).

RESULTS AND DISCUSSION

The calculated molecular mass of the enzyme, based on amino acid sequence, was 23,354 Da. When the purified recombinant xylanase was subjected to SDS-PAGE analysis it appeared at a molecular weight equivalent to 20 kDa (Fig. 1). We, therefore, analyzed the protein using a MALDI-TOF spectrometer. The results of MALDI-TOF spectrometry analysis revealed that the purified recombinant protein had a molecular mass of 20,443 Da (Fig. 2). The analysis of the amino acid sequence of the enzyme for the presence of a signal peptide using a signal peptide prediction program (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.htm) revealed a signal peptide of

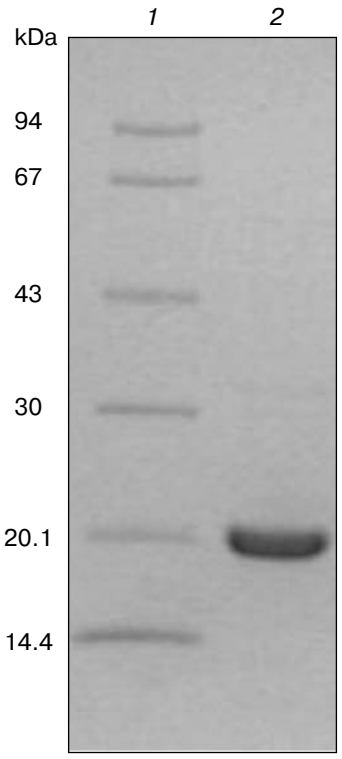

Fig. 1. Coomassie brilliant blue stained 0.1%SDS/14%PAGE demonstrating recombinant xylanase purified to apparent homogeneity. Lanes: *1*) molecular weight standards; *2*) purified xylanase after hydrophobic column (6.5 μg).

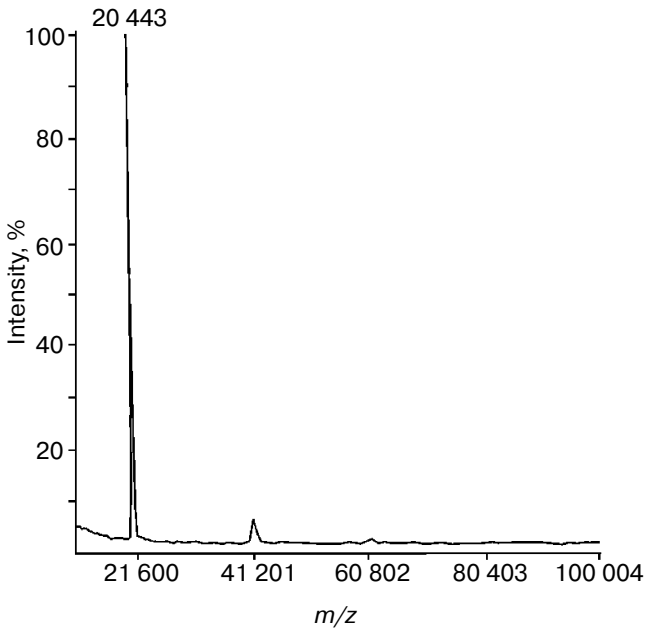

Fig. 2. Molecular mass determination of recombinant xylanase by MALDI-TOF mass spectrometry. Mass of recombinant xylanase is shown at the top of the peak (20,443 Da).

28 residues consisting of mostly uncharged and hydrophobic residues. The predicted signal peptide ended at Ala28 with a cleavage site between Ala28 and Ala29. The calculated molecular mass of the 185-residue mature protein (20,380 Da) was very close to the molecular mass of the recombinant protein determined by MALDI-TOF spectrometry analysis (20,443 Da). These results indicated that although the xylanase was cloned in the expression vector with signal peptide, the host *E. coli* signal peptide peptidase recognized this sequence and cleaved off the signal peptide releasing the mature xylanase. When the purified recombinant protein was subjected to N-terminal amino acid sequence analysis, the following six amino acids were determined at the N-terminal of the protein: ASTDYW. These amino acids matched exactly with the xylanase sequence after the 28th amino acid. This finding confirmed that the *E. coli* signal peptidase recognized the xylanase signal sequence and cleaved it between Ala28 and Ala29.

To optimize the secretary expression, different inducing times and inducer concentrations were used to induce the expression, followed by examining the enzyme activity. There were 70% extracellular and 30% intracellular activities after 4 h induction at 37°C. The highest extracellular secretion of the active enzyme was obtained when *E. coli* cells were induced by 0.5 mM IPTG at 37°C for 14 h, and the resulting extracellular activity reached 91% of the total activity.

Bacillus species are important sources and have long been used for the production of various industrial enzymes since these bacteria have a high secretion capacity [4]. The signal peptides from *B. subtilis* and *E. coli* share common features, which are a basic N-terminus, a central hydrophobic region, and a polar C-terminal region. Secretion efficiency depends not only on the signal peptide but also on the sequence of the mature protein especially at the N-terminus [8, 12]. There have been previous reports on the secretion of native *Bacillus* hydrolytic enzymes including subtilisin [13], α-amylase [14, 15], chitinase [15], and mannanase [15, 16] in *E. coli*. It seems possible that the xylanase signal peptide could be used for the secretion of other recombinant proteins as well, and our finding could be applied equally well to the cloning and extracellular production of other enzymes using an *E. coli* expression system. Further work on proteins, which were difficult to be produced in *E. coli* [17], utilizing this signal sequence, is in progress in our laboratory.

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