J. Microbiol. Biotechnol. (2005), **15**(5), 1125–1129

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Identification of the *sprU* Gene Encoding an Additional *sprT* Homologous Trypsin-Type Protease in *Streptomyces griseus*

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Received: January 24, 2005
Accepted: February 26, 2005

Abstract Cloning of a 6.6-kb *Bam*HI digested chromosomal DNA from *S. griseus* IFO13350 revealed the presence of an additional gene encoding a novel trypsin-like enzyme, named SprU. The SprU protein shows a high homology (79% identity, 88% similarity) with the SGT protease, which has been reported as a bacterial trypsin in the same strain. The amino acid sequence deduced from the nucleotide sequence of the *sprU* gene suggests that SprU is produced as a precursor consisting of an amino-terminal presequence (29 amino acid residues), prosequence (4 residues), and mature trypsin consisting of 222 amino acids with a molecular weight of 22.94 kDa and a calculated pI of 4.13. The serine, histidine, and aspartic acid residues composing the catalytic triad of typical serine proteases are also well conserved. When the trypsin activity of the SprU was spectrophotometrically measured by the enzymatic hydrolysis of the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-ρ-nitroanilide, the *S. lividans* transformant with pWHM3-U gave 3 times higher activity than that of control. When the same recombinant plasmid was introduced into *S. griseus*, however, the gene dosage effect was not so significant, as in the cases of other genes encoding serine proteases, such as *sprA*, *sprB*, and *sprD*. Although two trypsins, SprU and SGT, have a high degree of homology, the pI values, the gene dosage effect in *S. griseus*, and the gene arrangement adjacent to the two genes are very different, suggesting that the biochemical and biological function of the SprU might be quite different from that of the SGT.

Key words: SprT, SprU, *Streptomyces griseus*, bacterial trypsin

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Streptomyces griseus is one of the best-studied strains in the genus *Streptomyces*, because it can produce many kinds of secondary metabolites and various proteases. Streptomycin and Pronase are the representatives of the metabolites produced by *S. griseus*. The regulatory cascade concerning streptomycin production and morphological differentiation has been extensively studied in this strain, and the A-factor (2-isocapryloyl-3-R-hydroxy-methyl-γ-butyrolactone) has been known to play a central role in these regulatory cascades through switching on the transcription of *adpA* encoding a transcriptional activator by binding to ArpA, the A-factor receptor protein, and dissociating the DNA-bound ArpA from the target DNA [6]. AdpA activates a number of genes required for morphological and physiological differentiation, composing an AdpA regulon [6]. The *strR* (a pathway-specific transcriptional activator for streptomycin biosynthetic genes), *adsA* (ECF sigma factor of RNA polymerase essential for aerial mycelium formation), *sgmA* (a metalloendopeptidase involved in degradation of substrate hyphae), *ssgA* (a small acidic protein for spore septum formation), and *amfR* (essential for aerial hyphae formation) genes have been identified as members of the AdpA regulon [8].

Streptomyces griseus trypsin (SGT) is a bacterial serine protease with greater similarity to a mammalian protease, trypsin [19, 21]. The *sprT* gene (GenBank accession No. M64471) encoding SGT has been isolated from a *S. griseus* ATCC 10137 genomic library [12]. The amino acid sequence deduced from the nucleotide sequence of the *sprT* gene suggests that *S. griseus* trypsin is produced as a precursor consisting of an amino-terminal presequence (32 amino acid residues), prosequence (4 residues), and mature trypsin consisting of 223 amino acids with molecular weight of 23.1 kDa and pI value of 6.73. SGT catalyzes

the hydrolysis of amides and esters like other serine proteases, and a triad of the residues including serine, histidine, and aspartic acid is involved in its catalytic activity [2, 21].

In a previous study, the current authors optimized the overexpression system of the *sprT* gene in *S. lividans* TK24 under various conditions and characterized its enzymatic properties [15, 16]. The introduction of the *sprT* gene induced morphological changes in the colony shape without affecting the antibiotic production in *S. lividans*, which strongly suggested that the SGT might play some important role in the morphological differentiation of *Streptomyces* [1].

A-factor-deficient *S. griseus* mutant HH1 shows much diminished level of extracellular trypsin-like activity, and addition of A-factor to the mutant restored its productivity to the level of the wild-type strain [11]. Recently, it was found that the expression of *sprT* gene is dependent on A-factor via binding of AdpA protein to the *sprT* promoter region [8]. From these results, it was strongly suggested that the expression of the *sprT* gene might be related with morphological differentiation in *S. griseus*. Therefore, an experiment for disruption of the chromosomal *sprT* gene was carried out by inserting a kanamycin resistance gene in the *sprT*-coding sequence to elucidate the exact role of SGT as a member of the AdpA regulon. Correct disruption was checked by Southern hybridization with the appropriate fragment within the *sprT*-coding sequence. As expected, a 4.4-kb signal was deleted in the mutant strain; however, an unexpected 6.6-kb signal was observed with expected 4.4-kb signal in the *Bam*HI-digested chromosomal DNA [8]. It was assumed that the strong 6.6-kb signal is for another homologue of the *sprT* gene; therefore, subsequent cloning of the 6.6-kb fragment was carried out.

To clone the 6.6-kb fragment, the genomic DNA was isolated from *S. griseus* IFO 13350. *S. griseus* was maintained on R2YE agar, routinely grown in R2YE liquid broth at 28°C, and was used for isolation of the chromosomal DNA [5]. DNA preparation and manipulation were performed in *E. coli* by the method of Sambrook *et al.* [20] and in *Streptomyces* by the method of Hopwood *et al.* [5]. The chromosomal DNA was completely digested with *Bam*HI restriction enzyme, and a pool of 6.6-kb fragment was used for the construction of a mini-library in pUC19 [7]. One positive clone from 360 colonies was obtained by PCR. PCR primers (forward primer: 5'-ggsaaggactgggcsctsatcaag-3'; reverse primer: 5'-catsggscscsswgtcscctggca-3') were designed from the conserved amino acid sequences (GKDWALIK and CQGDSGGPM) derived from the SGT homology of *S. fradae, S. glaucences,* and *S. griseus* [9], and the expected length of the fragment was 290 bp (Fig. 1). The clone was found to have a 6.6-kb *Bam*HI insert as expected and was named as pSH101 (Fig. 2). The 2.7-kb *Sac*I/*Bam*HI fragment was confirmed to have the corresponding *sprT* homologue and was subcloned into pUC19 to give pSH102 (Fig. 2).

The 1.7-kb *Hin*dIII-*Bam*HI fragment from pSH102 was subcloned, sequenced, and the nucleotide sequence has been assigned as GenBank accession no. AY588948. Frame-plot analysis (ver. 2.3.2) of the nucleotide sequence revealed one complete open reading frame (ORF) starting with a 693th GTG start codon to 1,457th stop codon. The ORF contained 255 amino acids with a predicted molecular mass of 25.64 kDa and calculated pI of 4.25, and the corresponding gene for the ORF was named as *sprU*. The SprU protein shows a high homology (79% identity, 88% similarity) with the SGT protease reported in the same strain. The amino acid sequence deduced

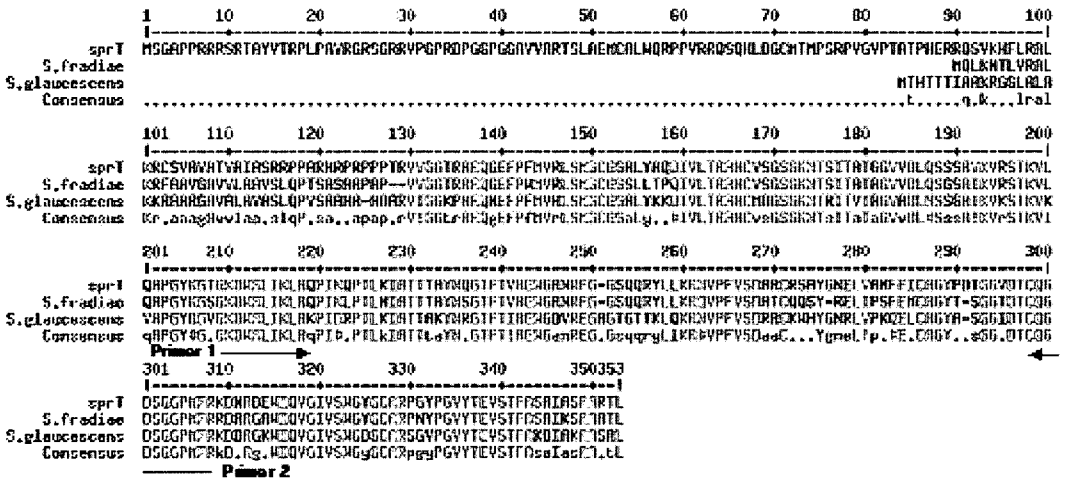

Fig. 1. Comparison of the amino acid sequences among the SGT homolog of *S. fradae*, *S. glaucences*, and *S. griseus*.
Forward primer (primer 1, 5'-ggsaaggactgggcsctsatcaag-3') and reverse primer (primer 2, 5'-catsggscscsswgtcscctggca-3') were designed from the conserved amino acid sequences GKDWALIK and CQGDSGGPM, respectively.

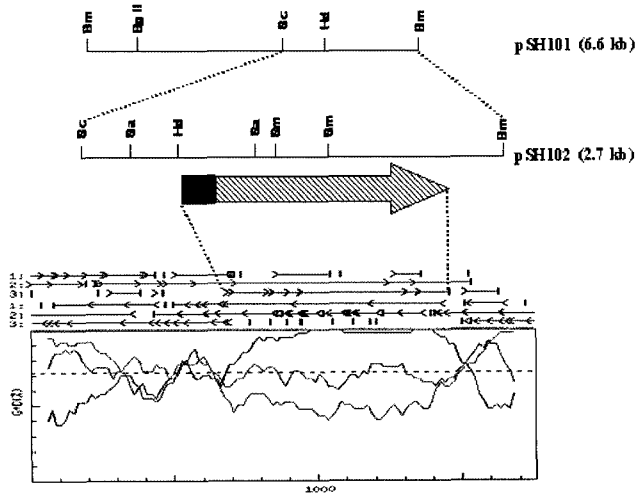

Fig. 2. Restriction map and FramePlot anaysis of the DNA fragment including the *sprU* gene.
(Upper) Restriction map of the clone. Insertion of a 6.6-kb fragment into pUC19 yielded pSH101, and subcloning of the *Sac*I-*Bam*HI-digested fragment (2.7-kb) into pUC19 gave pSH102. Restriction site abbreviations: Hd, *Hin*dIII; Bm, *Bam*HI; Sc, *Sac*I; BgII, *Bgl*II. (Lower) Frame analysis of the 1.7-kb *Hin*dIII-*Bam*HI fragment from pSH102. The arrowhead on the frame plot indicates the putative ORF named as SprU. The filled area and the slashed area in the arrowhead represent the pre-pro-region and the mature protein of the translated product, respectively.

(A)

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                                   ▽  ▼
  1 MKLLSVLKRC SVGIAAVALA TVSLQPASAA PQPIVGGERA AQGEFPFMVR  50
 51 LSMGCGGALY AQDIVLTAAH CVDGSGDDTS ITVTGGVADL QSPDAVEVQS 100
101 TKVLQAPGYN GTGKDWALIK LAQPIDQPTL KIATDGAYDE GTFTVAGWGA 150
151 DKEGGSQQRH LLKADVPFVT DADCQGAYGN LVADEELCAG LLDTGGVDSC 200
201 QGDSGGPMFR KDDADEWVQV GIVSWGQGCA RPNYPGVYTQ VSHFAGDIAS 250
251 AADSL                                                 255
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(B)

SprU	29	4	222 (pI=4.13, 22.94 kDa)
SprT	32	4	223 (pI=6.73, 23.11 kDa)

Fig. 3. Deduced amino acid sequence of the SprU (A) and comparison of two trypsin proteases (B).
(A) The translated product of the *sprU* gene is composed of 255 amino acids and the probable N-terminus after processing is indicated by an open triangle, and the probable site cleaved by signal peptidase is shown by a closed triangle. The amino acids that form a His-Asp-Ser catalytic triad are indicated by bold letters. (B) The pre-, pro-, mature-peptides of the SprU and SprT were compared with the numbers of composing amino acids. Although two proteins share high homology in their amino acid sequence, the pI value of the SprU (pI=4.13) was quite lower than that of the SprT (pI=6.73).

from the nucleotide sequence of the *sprU* gene suggests that SprU is produced as a precursor, consisting of an amino-terminal presequence (29 amino acid residues), prosequence (4 residues), and mature trypsin consisting of 222 amino acids with a molecular weight of 22.94 kDa and calculated pI of 4.13 [Fig. 3(A), 3(B)]. It was supposed that SprU catalyzes the hydrolysis of amides and esters by a common catalytic mechanism of serine proteases including SGT, and the serine, histidine, and aspartic acid residues composing the catalytic triad are also well conserved [4].

To confirm the trypsin activity of the SprU protein, the entire *sprU* gene was introduced into *Streptomyces* with its own promoter. The 2.7-kb *Sac*I-*Bam*HI fragment was ligated into pWHM3, an *E. coli-Streptomyces* shuttle vector, and used for the protoplast transformation [10, 14, 18] into *S. lividans* TK24 and *S. griseus* IFO 13350 [Fig. 4(A)]. The transformant was selected by 20 μg of thiostrepton/ml, and the culture broth was taken everyday for measuring the trypsin activity. The trypsin activity was spectrophotometrically measured, based on the release of ρ-nitroaniline by enzymatic hydrolysis of the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-ρ-nitroanilide [19]. The trypsin activity of the *S. lividans* transformant with pWHM3-U gave 3 times higher activity than that of control, clearly showing that the *sprU* gene encodes for a trypsin-like protease [Fig. 4(B)]. When the same recombinant plasmid was introduced into *S. griseus*, the relative activity was increased by 1.3 times that of control [Fig. 4(C)]. Many genes encoding serine proteases, such as *sprA*, *sprB*, *sprD*, and *sprT*, were cloned from *S. griseus* and introduced into the original strain on high-copy number plasmid; however, a high level of expression could not be attained, except for the *sprT* gene [3, 13]. These results strongly suggest that the gene dosage effects of many protease-encoding genes, including the *sprU* gene, are tightly regulated in *S. griseus*, and the regulation for the expression of the *sprT* might be different from that of *sprU*.

Because the SprU product has a high degree of identity with that of the putative trypsin reported in *S. avermitilis* MA-4680, the arrangement of the genes upstream of both trypsin genes were compared. Interestingly, similar proteins with unknown functions were arranged in both strains, implying that those two trypsin genes would have the same evolutional origin and similar cellular function (Fig. 5). However, the gene arrangement near the *sprT* gene was quite different from that of *sprU* [8].

Here, we report another novel bacterial trypsin gene that would be a homologue of the *sprT* gene in *S. griseus*. The two trypsins have a high degree of identity (79%) and similarity (88%), but the pI values calculated from the deduced amino acids are quite different. In addition, the difference in the gene dosage effect in *S. griseus* and the gene arrangement adjacent to the two genes

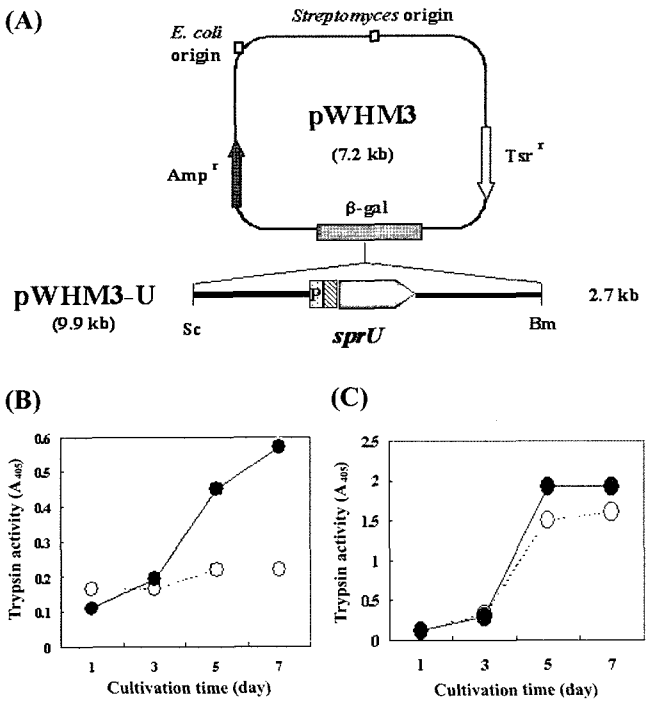

Fig. 4. Restriction map of expression vector pWHM3-U (A), and comparison of the trypsin activity produced by each transformant of *S. lividans* TK24 (B) and *S. griseus* IFO 13350 (C) in R2YE medium.

(A) The restriction enzyme sites used for cloning are placed at both ends of the inserts. The DNA fragments derived from *S. griseus* are indicated by a thick line, and the organization of the structural genes with pre-pro peptide (▨) and mature protease (□) is shown on the maps. Abbreviations: amp^r, ampicillin resistance; tsr^r, thiostrepton resistance; β-gal, β-galactosidase; Bm, *Bam*HI; Sc, *Sac*I. (B, C) The trypsin activity of the cultural filtrate prepared from the transformant was expressed as absorbance at 405 nm. ○—○, control with pWHM3; ●—●, transformant with pWHM3-U.

led us to conclude that the biochemical and biological function of the SprU might be quite different from that of the SGT.

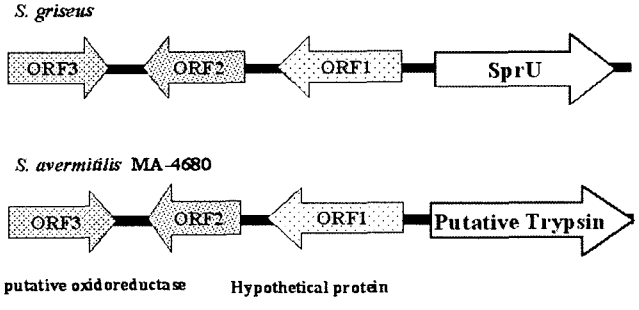

Fig. 5. Gene organizations in the cloned fragments containing *sprU* from *S. griseus* and a *sprT* homologue from *S. avermitilis*.

The positions and directions of open reading frames predicted by the Frame-Plot analysis of the nucleotide sequence are indicated by arrows. Similar ORFs in two fragments are shown to be organized in the same directions.

Acknowledgment

This work was supported by a grant from Korea Science and Engineering Foundation (KOSEF, R01-2000-00109).

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