The Journal of Microbiology, December 2001, p.305-313 Vol. 39, No. 4

Overexpression of the *spr*D Gene Encoding *Streptomyces griseus* Protease D Stimulates Actinorhodin Production in *Streptomyces lividans*

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(Received September 31, 2001 / Accepted October 18, 2001)

The *spr*D gene encoding *Streptomyces griseus* protease D (SGPD), a chymotrypsin-like protease, was cloned from *Streptomyces griseus* IFO13350 and sequenced. Most of the amino-acid sequence deduced from the nucleotide sequence is identical to that of *Streptomyces griseus* IMRU3499 except that one amino acid has been deleted and Trp369 has been substituted into Cys369 in the SGPD from *S. griseus* IFO13350 without affecting the protease activity. The *spr*D gene was overexpressed in *Streptomyces lividans* TK24 as a heterologous host. Various media with different compositions were also used to maximize the productivity of SGPD in the heterologous host. The SGPD productivity was best when the transformant of *S. lividans* TK24 was cultivated in R2YE medium. The relative chymotrypsin activity of the culture broth measured with an artificial chromogenic substrate, *N*-succinyl-ala-ala-pro-phe-p-nitroanilide, was 16 units/ml. A high level of SGPD was also produced in YEME and SAAM media but it was relatively lower than in R2YE medium, and negligible amounts of SGPD were produced in GYE, GAE and Benedict media. The growth of *S. lividans* reached the maximum level of cell mass at days 3 and 4 of the culture, but SGPD production started in the stationary phase of cell growth and kept increasing till the 10th day of culture in R2YE and YEME medium, but in GYE media the productivity reached maximum level at 8 days of cultivation. The introduction of the *spr*D gene into *S. lividans* TK24 triggered biosynthesis of the pigmented antibiotic, actinorhodin, which implies some protease may play a very important role in secondary-metabolite formation in *Streptomyces*.

Key words: *sprD*, *S. griseus*, protease, actinorhodin, *S. lividans*

Streptomyces griseus produces many kinds of secondary metabolites and a mixture of proteases that have been sold under the commercial name of Pronase which have a variety of industrial uses. Many proteases have been purified from Pronase and their biochemical properties studied (1, 20). Based on the N-terminal sequences of the purified proteases, many genes such as *sprA*, *sprB*, *sprC*, *spr*D, *sprE* and *sprT*, that encode for *S. griseus* protease A(SGPA), *S. griseus* protease B(SGPB), *S. griseus* protease C(SGPC), *S. griseus* protease D(SGPD), and *S. griseus* protease E(SGPE), and *S. griseus* trypsin(SGT), have been cloned (5, 12, 19). All the proteases reported from *S. griseus* belong to the bacterial serine protease that catalyzes the hydrolysis of amides and esters by a common catalytic mechanism involving a triad of the residues serine, histidine and aspartic acid. SGPA, SGPB, SGPC and SGPD, four chymotrypsin-like serine proteases, are closely related to the mammalian Asp-Ser-Gly serine proteases and are very stable in unusual conditions such as 6M guanidine (5, 19). The SGT from the same bacterium with an active serine sequence Asp-Ser-Gly has more similarity to mammalian trypsin (17, 21).

The *spr*D gene (Genebank accession No. L29018) encoding SGPD has been isolated from a *S. griseus* IMRU3499 genomic library (19). The recombinant SGPD expressed in *Bacillus subtilis* forms a very stable α_2 dimer and the amino acid sequence of the protein in the region of the specificity pocket is similar to SGPA, SGPB and SGPC. The purified SGPD has been reported to have a primary specificity for large aliphatic or aromatic amino acids.

In a previous study, we isolated the *sprT* gene that encodes the SGT from *S. griseus* IFO13350 and set up an overexpression system in a heterologous host in various

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conditions (12, 13). We have been studying protease in *S. griseus* to develop a high production system for the protease and to elucidate the exact cellular function of the protease in relation to morphogenesis. For this reason, we cloned the *spr*D gene from *S. griseus* IFO13350 based on the PCR method and constructed an overexpression system of the *spr*D gene using *S. lividans* TK24 as a heterologous host. In this report, the differerence between the *spr*D genes from two different strains, the conditions for overexpression, and the effect of *spr*D gene overexpression on antibiotic production in *S. lividans* is described.

Materials and Methods

Bacterial strains and plasmids

S. lividans TK24 which has low levels of milk protein-hydrolyzing proteolytic activity was obtained from the John Innes Institute, United Kingdom (6). *S. griseus* IFO13350 was obtained from the the University of Tokyo, Japan. *E. coli* strain DH5α was used for subcloning. The *Streptomyces-E. coli* shuttle vector pWHM3 was obtained from C. R. Hutchinson (University of Wisconsin, U.S.A.). Plasmids used in this study are listed in Table 1.

Media and culture conditions

E.coli DH5 was maintained on M9 minimal agar and cultured in LB medium at 37°C with agitation. *Streptomyces* strains were maintained on R2YE agar and *S. lividans* TK24 grown in R2YE liquid broth at 28°C and used for the preparation of protoplasts and the isolation of plasmid DNAs (6). Various media listed in Table 2 were used to overproduce SGPD from the transformant of *S. lividans* TK24.

Enzymes and chemicals

Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs, Inc., and other fine chemicals were from Sigma Chemical Co. Primers for PCR-based cloning were purchased from Atman BioScience Inc., Korea.

DNA manipulations

DNA preparation and manipulation was performed by the method of Maniatis *et al.* in *E. coli* and Hopwood *et al.* in *Streptomyces* (6). DNA samples were digested with restriction endonucleases and ligated with T4 DNA ligase according to the supplier's recommendations. Plasmid DNAs and their digests were analyzed by horizontal agarose gel electrophoresis with the TAE buffer system.

Transformation procedure

Competent cells of *E. coli* strains for transformation were routinely prepared according to the frozen storage protocol and transformation was done by the method described by Hanahan (4).

Protoplasts of *S. lividans* TK24 were prepared as described by Hong *et al* (11) and Okanishi *et al* (16). Cells were grown in 100 ml of R2YE containing 0.5% glycine in a 500 ml baffled flask and incubated for 18 to 24 hr. The obtained cells were treated with 20 ml of P buffer containing 80 mg of lysozyme, and the resulting protoplasts were suspended in P buffer at a concentration of ~10^9/ml and frozen at -70°C. *Streptomyces* protoplasts were transformed by using 100 μl of protoplasts (~10^8), 1 μg of plasmid DNA in 20 μl of TE buffer, and 500 μl of 25% PEG1000 in P buffer. Samples (100 μl) were plated in 2.5 ml of 0.6% soft R2YE agar on R2YE regeneration plates. After incubation at 28°C for 18 to 24 hr, the plates were overlaid with 2.5 ml of 0.6% soft R2YE agar containing 25 μg/ml of thiostrepton. Transformants were visible after incubation for an additional 3 to 5 days at 28°C.

Cloning of the sprD gene and construction of recombinant plasmid

S. griseus IFO13350 was the original source of genomic DNA for the cloning experiments. On the basis of the *spr*D sequence data from *S. griseus* IMRU3499 (accession number L29018), 2 primers designated as 5-GGATCCGCGCCGTCGAATACCGGACA-3', which correspond to the 5'-end, and 5'-AAGCTCCGGCCGGTGACAGCCACGG-3', which corresponds to the complementary sequence of the 3'-end. These primers were used to

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Source or Reference
Strains		
S. lividans TK24	*Str*-6	Hopwood (6)
S. griseus IFO13350	Wild type; Streptomycin producer, A-factor$^+$	IFO[a]
E. coli DH5αF'	*sup*E44 Δ*lac*U169(ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*96 *thi*-1 *rel*A1	Hanahan (4)
Plsmids		
pWHM3	High-copy, tsr^r, amp^r, *E. coli-Streptomyces* shuttle vector	Hutchinson
pT7Blue[®]	*E. coli* vector, pUC19 backbone, T-cloning flank, amp^r	Novagen[b]

[a]Institute of Fermentation, Osaka, Japan
[b]Novagen, Inc., Madison, Wisconsin, USA

amplify DNA fragments from the chromosomal DNA of *S. griseus* IFO13350.

The PCR mixture contained 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM $MgCl_2$) supplied by Takara Co. Ltd. (Japan). Taq polymerase (2U, Takara) and approximately 100 ng target DNA were added in a final reaction volume of 50 μl. Amplification was performed in a thermal cycler (model 480, Perkin Elmer Cetus, CT) by denaturing the samples at 94°C for 4 min, subjecting them to 30 cycles of denaturing (98°C, 20s), annealing (67°C, 1 min), and then by elongating at 72°C for 10 min (7). The 1.4-kbp PCR products were recovered using 1.5% agarose gel electrophoresis and ligated into pT7Blue®. The recombinant plasmid pT7-*spr*D was digested with *EcoRI/PstI* then subcloned into the *EcoRI/PstI* site of pWHM3, a *Streptomyces-E. coli* shuttle vector, by 2-fragment ligation and transformed into *E. coli* DH5α. The resulting recombinant plasmid, pWHM3-*spr*D was purified from *E. coli* cells and used for transformation into *S. lividans* TK24.

Overexpression of the sprD gene in S. lividans

Because the genes originated from Streptomycetes could not be properly expressed in the *E. coli* host system, the recombinant plasmid pWHM3-*spr*D prepared from *E. coli* was introduced into *S. lividans* TK24 by the protoplast transformation method. Transformants of *S. lividans* TK24 selected by thiostreptone were tested on skim milk plates for secretion of protease. A clear zone representing the degradation of milk protein surrounded each transformant that contained pWHM3-*spr*D. The plasmid pWHM3-*spr*D was reisolated from *S. lividans* transformant for futher use.

Sample preparation for protease assay

The transformant of *S. lividans* TK24 harboring the pWHM3-*spr*D recombinant plasmid was grown in 100 ml of R2YE media in 500 ml baffled flasks at 28°C with vigorous shaking at 250 rpm. After 2 days cultivation, 10 ml of culture broth was used for inoculation into 100 ml of various liquid media listed in Table 2 in 500 ml baffled flasks in the same condition. 5 ml of culture broth were sampled everyday and centrifuged at 5,000 rpm for 10 min. The supernatant was used for the measurement of protein concentration and protease activity, and the cell pellet was used for the quantitation of proteins after cell disruption by sonication. Protein concentrations were measured by the method of Bradford and bovine serum albumin was used as the standard (2).

Determination of chymotrypsin activity

Chymotrypsin activity was measured spectrophotometrically by the release of p-nitroaniline using N-succinyl-ala-ala-pro-phe-p-nitroanilide as an artificial chromogenic substrate (11). The reaction mixture containing 890 μl of reaction buffer (100 mM Tris-HCl [pH 8.0], 10 mM $CaCl_2$) and 10 μl of 30 mM N-succinyl-ala-ala-pro-phe-p-nitroanilide in DMSO was used for reaction with 100 μl of enzyme solution in the same manner. After the reaction for 15 min, the chymotrypsin activity was measured at 405 nm. One unit of chymotrypsin was defined as the

Table 2. Protease production media used in this study

Medium	Ingredient	Amount (g/l)	Medium	Ingredient	Amount (g/L)
SAAM (23) (pH 6.8)	glucose	15	GAE (22) (pH 7.2)	glucose	10
	soybean meal	15		asparagine	1
	NaCl	5		yeast extract	4
	yeast extract	1		$MgSO_4 \cdot 7H_2O$	0.5
	$CaCO_3$	1		$FeSO_4 \cdot 7H_2O$	0.1
	glycerol	2.5 ml		K_2HPO_4	0.5
GYE (3) (pH 7.2)	glucose	12.5	R2YE (7)	sucrose	103
	yeast extract	5.0		K_2SO_4	0.25
	$CaCO_3$	8.0		$MgCl_2 \cdot 6H_2O$	10.12
	NaCl	5.3		glucose	10
				casamino acid	0.1
				yeast extract	5
				K_2HPO_4 (0.5%)	10 ml
				$CaCl_2 \cdot 2H_2O$ (3.68%)	80 ml
				L-proline (20%)	15 ml
				TES (5.73%, pH 7.2)	100 ml
				trace element	2 ml
Benedict (23) (pH 7.0)	yeast extract	2	YEME (7) (pH 7.2)	malt extract	10
	malt extract	2		$MgCl_2 \cdot 6H_2O$	2
	casamino acid	4		glucose	4
	maltose	10		sucrose	300
				yeast extract	4
				glycine	10

amount of enzyme corresponding to 0.1 increase in absorbance in the above conditions.

Antibiotic assays

Assays for the detection of actinorhodin and undecylprodigiosin were performed as previously described (14). A stationary culture of *S. lividans* TK24 was transferred to 100 ml of R2YE or YEME medium in a 500 ml baffled flask, and incubated at 30°C on a reciprocal shaker. Portions (5 ml) of the culture broth taken out at intervals were extracted with 5ml of chloroform for 30 min at room temperature, with shaking. Then, 5 ml of 1N NaOH was added, and the tubes were vortexed and spun in a microcentrifuge for 15S. The aqueous phase contained actinorhodin, which is blue at an alkaline pH. The A_{590} of the aqueous phase was determined. The chloroform phase

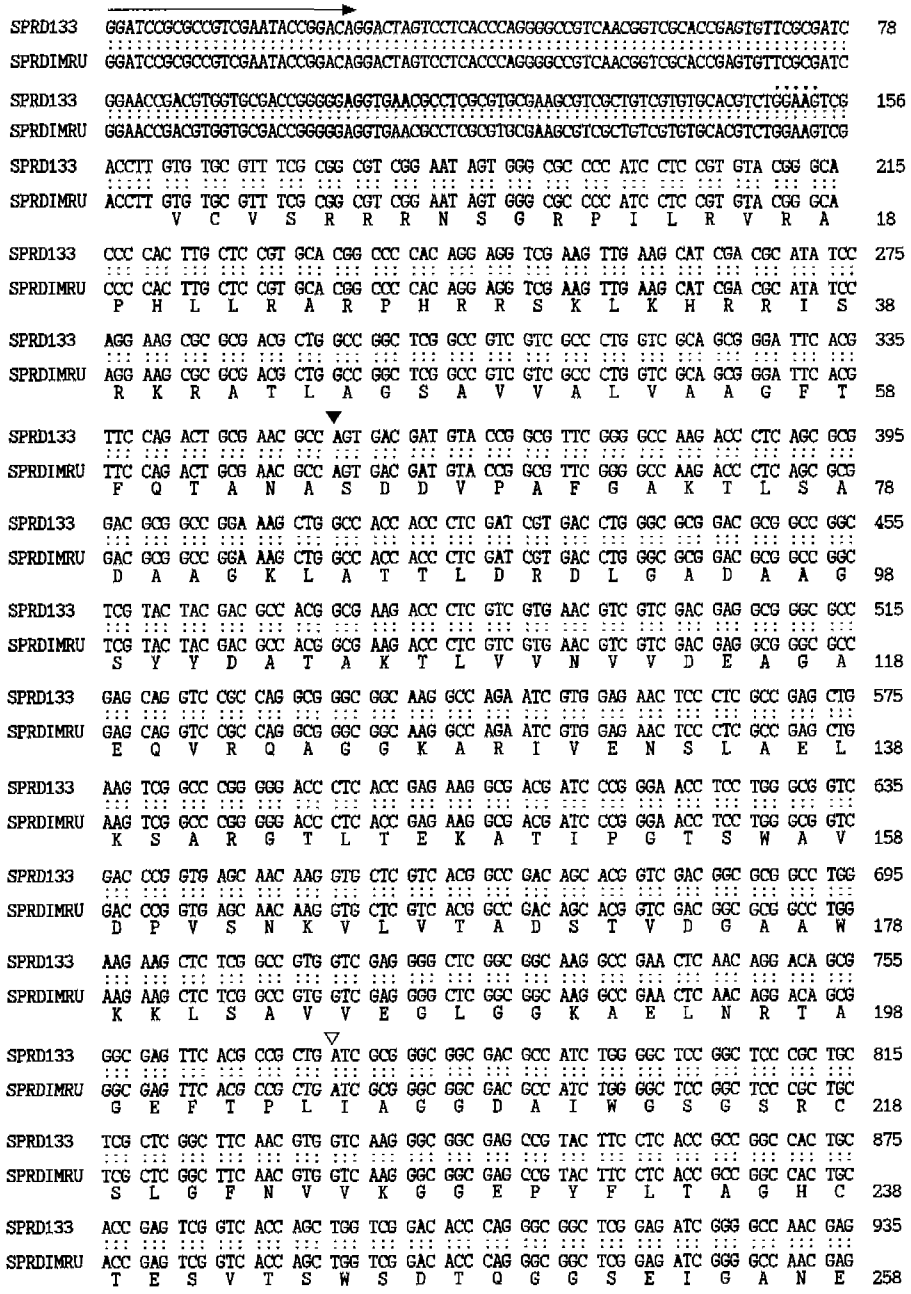

Fig. 1. Comparison of the nucleotide and amino acid sequences of the *spr*D gene obtained from *S. griseus* IFO13350 (SPRD133) and *S. griseus* IMRU3499 (SPRDIMRU). The *spr*D gene was cloned by the PCR from *S. griseus* IFO13350 and the nucleotide sequences used for designation of primers are depicted with P1(forward primer) and P2(reverse primer). The numbering to the right of the sequences is relative to the first nucleotide in the sequence and to the first amino acid coded by the gene. A putative ribosome binding site is indicated by a series of dots preceding the initiation codon. Junctions between the pre- and proregions and pro- and mature regions are indicated by a closed and an open triangle, respectively. The alteration of the nucleotide and amino acid sequences originated from two strains are boxed.

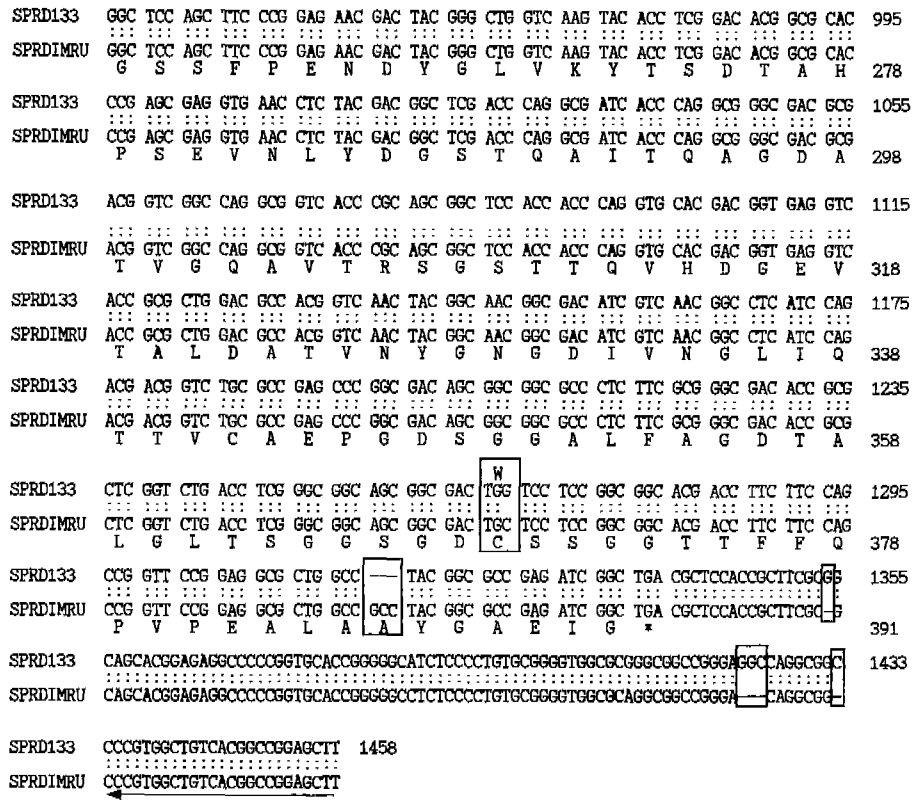

Fig. 1. Continued.

contained the undecylprodigiosin, which was yellow. For the absorbance measurements of undecylprodigiosin, the chloroform layer was acidified with HCl, and the A_{530} of the now-red chloroform phase was then determined.

Results and Discussion

Cloning and sequencing of the sprD gene

The *spr*D gene amplified from the chromosomal DNA of *S. griseus* IFO13350 was cloned based on the PCR method in pT7 PCR vector and then sequenced (Fig. 1). The amino acid sequence deduced from the nucleotide sequence revealed that the *spr*D gene of *S. griseus* IFO13350 encodes a polypeptile of 391 amino acids with a molecular weight of 40 kDa. However, the *spr*D gene previously reported in *S. griseus* IMRU3499 encodes a polypeptide of 392 amino acids with duplicated Ala residues such as Ala385-Ala386. This fact implies that one of the Ala residues in Ala385-Ala386 was deleted in the *Spr*D protein of *S. griseus* IFO13350. In addition, Trp369 in the SGPD from *S. griseus* IMRU3499 was substituted into Cys369 in that of *S. griseus* IFO13350. Interestingly, it was confirmed that the deletion and the substitution themselves did not affect the enzymatic activity of the SGPD. The nucleotide and deduced amino acid sequences of the two *spr*D genes were exactly the same except for the one deletion and one substitution in the coding region, but the down-stream region of the termination codon showed some differences in their sequences. This fact implies that the coding region of the *spr*D genes is relatively highly conserved compared to the noncoding region.

Overexpression of the sprD gene in S. lividans TK24

To overexpress the *spr*D gene in *Streptomyces*, the *E. coli-Streptomyces* shuttle vector pWHM3 was double-digested with *EcoR1* and *PstI* and ligated with the entire *spr*D gene including one promoter digested with the same retriction enzymes (Fig. 2). The resulting recombinant plasmid pWHM3-*spr*D prepared from *E. coli* was introduced into *S. lividans* TK24 by the protoplast transformation method. All the transformants were tested on skim milk plates for the production of protease in the presence of thiostreptone. Subsequently, two transformants were selected from the 80 transformants that had large and obviously clear proteolytic zones. The two transformants were cultured in YEME broth containing 20 μg/ml of thiostrepton and then the chymotrypsin proteolytic activity was analyzed by using artificial substrate, N-succinyl-Ala-Ala-Pro-Phe-ρ-nitroanilide. Release of the chromophoric substrate, ρ-nitroanilide group was monitored as an activity of chymotrypsin protease. Two transformants showed higher

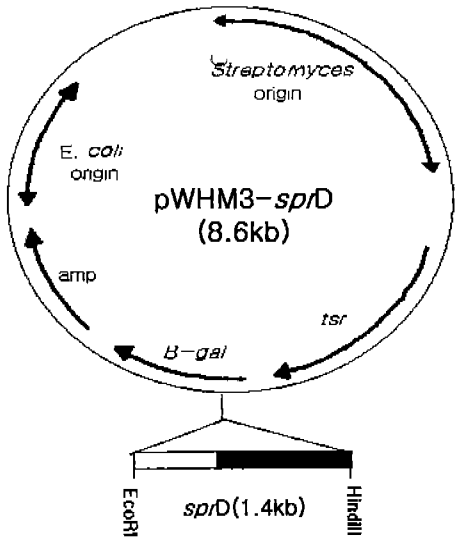

Fig. 2. Restriction map of expression vector pWHM3-*spr*D containing an insert of the entire *spr*D gene at *EcoR*I and *Pst*I sites. The thick line indicates the *spr*D coding region. The organization of the structural gene, with pre-pro peptide (204 amino acids, ■) and mature protease (187 amino acids, □) is shown below the map. Abbreviations: amp, ampicilline resistance; *tsr*, thiostreptone resistance; β-gal, β-galactosidase.

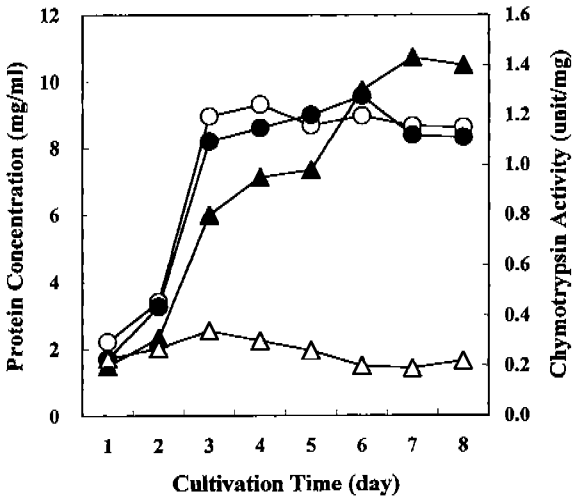

Fig. 3. Overexpression of the SGPD in *S. lividans* TK24 transformed with pWHM3-*spr*D. The transformant was cultured in YEME broth as described in 'Materials and Methods'. The SGPD activity of the cultural filtrate prepared from the transformant (▲–▲, pWHM3-*spr*D; △–△, pWHM3) was expressed in unit/mg of cellular protein. The concentration of cellular protein of the transformants (●–●, pWHM3-*spr*D; ○–○, pWHM3) was measured by Bradford's method after cell disruption by sonication.

protease activity in a similar degree and the average values of the measured activity were compared to that of *S. lividans* TK24 haboring pWHM3 as a control (Fig. 3). Protease activities increased sharply from day 3 to day 8 of cultivation. The productivity of the chymotypsin was

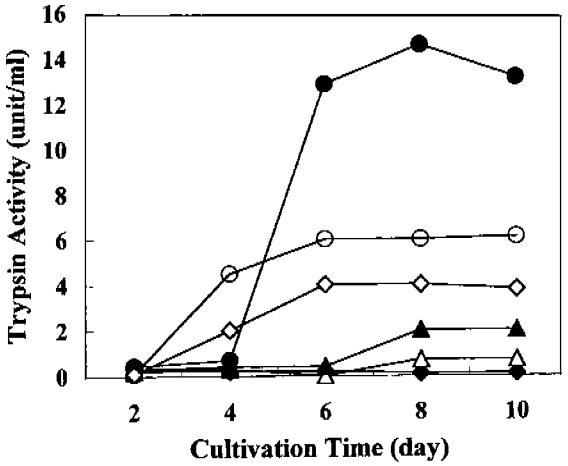

Fig. 4. Comparison of SGPD activity produced by *S. lividans* TK24 haboring pWHM3-*spr*D cultivated in a variety of media. The amount of enzyme produced is expressed in units/ml of cultural filtrate depending on cultivation time because specific activity could not be calculated owing to insoluble ingredients in production media. ●–●, R2YE medium; ○–○, YEME medium; ◇–◇, SAAM medium; ▲–▲, Benedict medium; △–△, GYE medium; ◆–◆, GAE medium.

1.4 and 0.2 units/mg in the transformants with pWHM3-*spr*D and pWHM3, respectively, at day 8 of cultivation in YEME broth. Judging from the similar growth curve, this result clearly shows that the *spr*D gene was successfully overexpressed by using own promoter in *S. lividans* TK24 as a heterologous host. The relationship between the cell growth and SGPD production was studied in YEME medium. The growth of *S. lividans* reached the maximum cell mass at 3 days culture, but SGPD production started in the stationary phase of cell growth and kept increasing till the 8th day of cultivation (Fig. 3).

Production of SGPD in various media

The transformant of *S. lividans* TK24 harboring pWHM3-*spr*D was cultured in various liquid media to study the best composition for media for the SGPD overproduction. Because of the many insoluble ingredients in the fermentation media, it was difficult to measure exactly the cell growth and the concentration of extracellular proteins, therefore the SGPD activity produced could not be compared for specific activity. When the relative activities of the SGPD were compared in unit volume, the chymotrypsin activity of the culture broth was 16 units/ml and 6 units/ml in R2YE and YEME media, respectively (Fig. 4). In SAAM, Benedict, GYE and GAE media, the level of SGPD activity was relatively very low. This data shows that the productivity of the chymotrypsin can be influenced significantly by the medium composition. R2YE was the best medium for the production of SGPD among the tested media.

It was known that the production of protease can be regulated by many factors, especially medium component. In

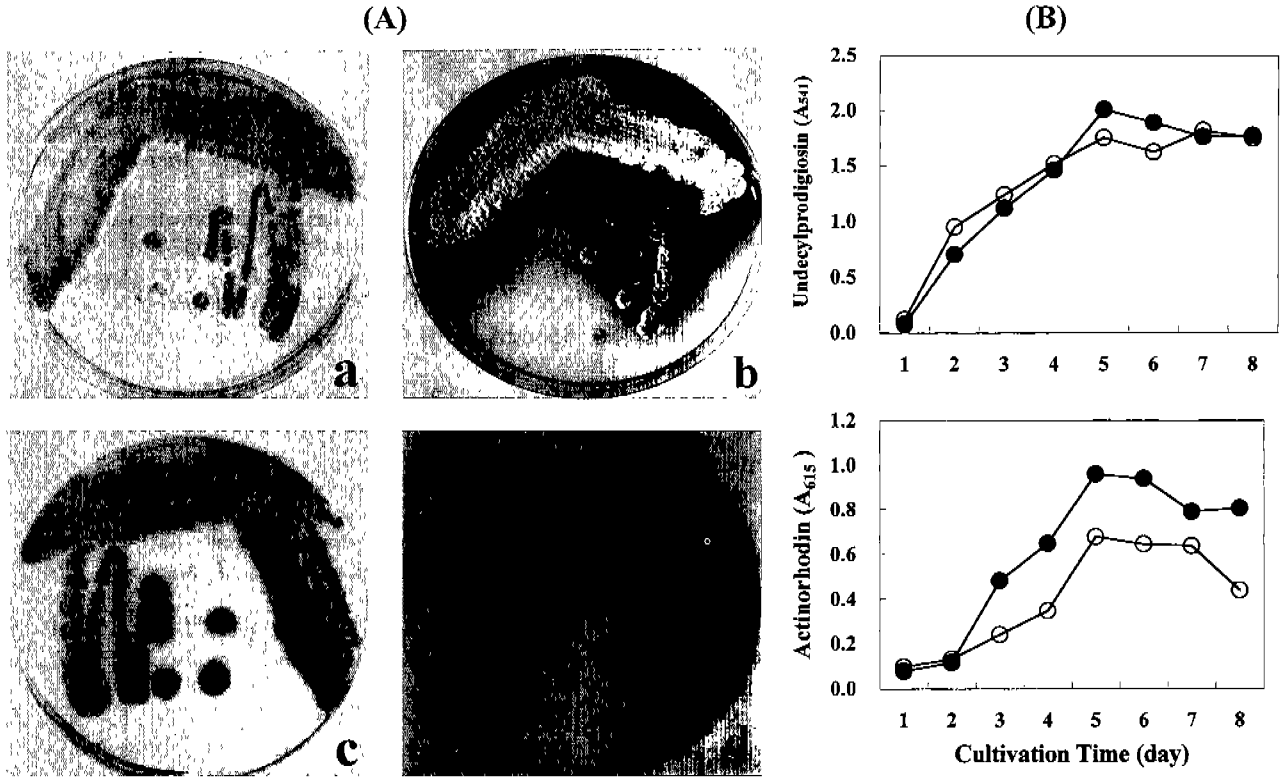

Fig. 5. Effects of SGPD overproduction on pigmented antibiotic production of *S. lividans* TK24. Photographs of the plates (A) and pigmented antibiotic production of *S. lividans* TK24 (B). (A) Upper, photographs of the surface of the plate; bottom, photographs of the reverse side of the plate. a, c, *S. lividans* TK24 harboring pWHM3 as a control; b, d, *S. lividans* TK24 harboring pWHM3-*spr*D, where massive production of blue pigment is clearly visible. (B) Time course of pigmented antibiotics production by *S. lividans* TK24. Portions sampled as described in 'Materials and Methods' were scanned with a spectrophotometer for the quantitation of undecylprodigiosin and actinorhodin. ○–○, transformant with pWHM3 as a control; ●–●, transformant with pWHM3-*spr*D.

a previous report we showed the subcloning of *sprT* that encodes the SGT into pWHM3 resulted in the overexpression of SGT in *S. lividans* TK24, which made it convenient to purify SGT (13). Through those experiments, we showed the selection of the host strain and an appropriate medium could be the most important factors for increasing the productivity of the recombinant strain; R2YE was found to be the best medium for the production of SGT in *S. lividans* TK24. At this time we do not understand which difference, the host strain or the medium composition, affect the production yield. However, the common feature of the media yielding higher productivity is the high concentration of sucrose as the carbon source. The proper combination of yeast extract with a large amount of carbon source such as sucrose or glucose and the presence of free amino acids or trace elements, and the buffering capacity of the medium seem to be very important factors in stabilizing the productivity of SGPD and SGT in *S. lividans* TK24.

Effect of the sprD gene on antibiotic production in S. lividans

The regulation of the secondary metabolites formation can be affected by many factors, such as nutrient limitations, environmental changes, global regulatory factors and pathway-specific regulatory factors (19). *S. lividans* has biosynthetic gene clusters for the production of pigmented antibiotics, actinorhodin and undecylprodigiosin (14). However, these gene clusters are present as sleeping genes and in usual conditions this strain is not able to produce those antibiotics. During this experiment, the transformant introduced with the *spr*D gene was found to produce massive amounts of the blue-pigmented antibiotic, actinorhodin, on R2YE solid medium (Fig. 5A). Therefore, we think that the overexpression of the *spr*D gene in *S. lividans* stimulated the expression of the gene cluster for the pigmented antibiotic production. When the transformant was cultured in YEME liquid medium, only the production of the actinorhodin between the two pigmented antibiotics increased 1.6 times (Fig. 5B). The difference in the actinorhodin production was most obvious in R2YE broth as could be visually differentiated on R2YE solid medium, however, the pigment was coprecipitated with many insoluble precipitates during the analytical process, which disturbed the quantitation of the pigments. The production of the red pigment undecylprodigiosin was not affected by

the overexpression of the *spr*D gene in the same conditions (Fig. 5A, 5B).

Proteases of the chymotrypsin superfamily are a vast group of enzymes and are known to have many important intracellular functions such as mammalian blood clotting cascades, digestive activity of the pancreas, regulation of the cell cycle, and maturation and secretion of other proteins (19). *S. griseus* produces many extracellular proteases and at least four genes that are responsible for chymotrypsin-like proteases have been reported. If we classify the protease into primary and secondary proteases depending on production time, the SGPD seems to be a secondary protease. In general, secondary proteases are produced after active cell growth has ceased because of nutritional depletion. When we consider that secondary metabolism or cell morphogenesis is also occurring after cell growth has stopped, it is plausible that these kinds of secondary proteases may have some functional relationship with differentiation (8, 9, 15, 22).

Recently we have studied the proteases produced by *S. griseus* IFO13350 and its A-factor deficient mutant strain (blocked mutant for sporulation), *S. griseus* HH1. *S. griseus* showed a higher degree of cell growth and protease activity in proportion to its ability to produce a higher amount of A-factor (11). In particular, the specific activity of the trypsin of *S. griseus* IFO13350 was greatly enhanced and that of the metalloprotease of *S. griseus* HH1 was greatly enhanced in later stages of growth. Interestingly, when a serine protease inhibitor, pefabloc SC, and metalloprotease inhibitor, EDTA, were applied to strain IFO13350 to examine the *in vivo* effects of the protease inhibitors on the morphological differentiation, the formation of aerial mycelium and spores was delayed. These facts tell us some serine- or metallo-proteases are playing very important roles for differentiation in *Streptomyces*. The result that the expression of the *spr*D gene can stimulate the biosynthetic gene cluster for secondary metabolites in a pathway-specific way, is evidence supporting the idea that some proteases are involved in differentiation.

Acknowledgment

This work was supported by a Seoil College Research grant.

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