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Streptomyces griseus HH1, An A-factor Deficient Mutant, Produces Diminished Level of Trypsin and Increased Level of Metalloproteases

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A-factor is a microbial hormone that can positively control cell differentiation leading to spore formation and secondary metabolite formation in *Streptomyces griseus*. To identify a protease that is deeply involved in the morphological and physiological differentiation of *Streptomyces*, the proteases produced by *S. griseus* IFO 13350 and its A-factor deficient mutant strain, *S. griseus* HH1, as well as *S. griseus* HH1 transformed with the *afsA* gene were studied. In general, *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. In particular, the specific activity of the trypsin of *S. griseus* IFO 13350 was greatly enhanced more than twice compared with that of *S. griseus* HH1 in the later stage of growth. The specific activity of the metalloprotease of *S. griseus* HH1 was greatly enhanced more than twice compared with that of *S. griseus* IFO 13350, and this observation was reversed in the presence of thiostreptone. However, *S. griseus* HH1 transformed with the *afsA* gene showed a significantly decreased level of trypsin and metalloprotease activity compared with that of the HH1 strain. There was no significant difference between *S. griseus* IFO 13350 and HH1 strain in their chymotrypsin and thiol protease activity, yet the level of leu-aminopeptidase activity was 2 times higher in *S. griseus* HH1 than in strain IFO 13350. *S. griseus* HH1 harboring *afsA* showed a similar level of enzyme activity, however, all the three protease activities sharply increased and the thiol protease activity was critically increased at the end of the fermentation. When a serine protease inhibitor, pefabloc SC, and metalloprotease inhibitor, EDTA, were applied to strain IFO 13350 to examine the *in vivo* effects of the protease inhibitors on the morphological differentiation, the formation of aerial mycelium and spores was delayed by two or three days.

Key words: *Streptomyces griseus*, A-factor, Trypsin, Metalloprotease

Streptomycetes are a group of Gram-positive soil bacteria, which have been studied for their importance in the production of various secondary metabolites and enzymes. Streptomycetes have a unique feature in their growth; one spore germinates and develops into multinucleated substrate mycelia and then into aerial mycelia and spores (9). The development of aerial mycelia and spores starts at a specific stage in the life cycle and some signal from an environmental and intracellular changes induce this type of morphological differentiation (6, 7, 17). The formation of secondary-metabolites is another characteristic feature of Streptomycetes, and in general it begins at the same stage as morphogenesis, which suggests that they are tightly regulated by some common regulatory gene(s) (10, 12, 24).

A-factor (2-isocapryloyl-3-R-hydroxy-methyl-γ-butyrolactone) is a microbial hormone which is known as a positive regulator for the production of streptomycin and sporulation in *Streptomyces griseus* (23). The A-factor deficient mutant, *S. griseus* HH1, can neither produce streptomycin nor sporulate, yet the external addition of A-factor in culture medium can restore the phenotype into the wild type. The regulatory network from the A-factor to streptomycin production includes global regulatory proteins such as, the A-factor receptor protein (ARP), A-factor dependent protein (ADP), and the StrR protein that is known as a pathway specific activator for streptomycin production (9). The regulatory cascade leading to sporulation under the control of A-factor has also been studied and many regulatory proteins involved in this regulation have been reported (23). Majority of the research concerning the A-factor cascade have mainly focused on intracellular regulatory proteins and it has been suggested that many intracellular proteins could be induced by the A-factor.

S. griseus can produce copious amounts of secondary metabolites and proteases. Pronase is the commercial name for many proteases produced by *S. griseus* (19). In general, many proteases are produced in the later stage of cell

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growth concomitant with morphogenesis and secondary metabolite formation. The current authors have studied the production of proteases in *S. griseus* (15), and found that the overexpression of *S. griseus* protease genes in *Streptomyces lividans* and even in *S. griseus* induced morphological changes in the host cell, which leads to the current study of the functional role of proteases in morphogenesis. To obtain a clue for the involvement of proteases in morphogenesis, two strains, *S. griseus* IFO 13350 and *S. griseus* HH1, an A-factor-deficient mutant, were selected, and the difference in their protease production was studied using specific artificial substrates. *S. griseus* HH1 transformed with the *afsA* gene, which can restore the A-factor producing ability and morphological differentiation in this mutant, was used as the control.

Several synthetic compounds and microbial metabolites are known to inhibit various types of proteases and the use of adequate inhibitors helped us to clarify the exact function of the proteins (25). Certain protease inhibitors were selected and their effect on the morphology of *S. griseus* was studied (26). This report describes the effect of various protease inhibitors on the developmental differentiation of *S. griseus*.

Materials and Methods

Bacterial strains and plasmids

The *S. griseus* IFO 13350, *S. griseus* HH1, and plasmid pAFB1 were all obtained from Prof. S. Horinouchi (University of Tokyo, Japan). *S. griseus* HH1 is an A-factor deficient mutant strain derived from strain IFO 13350 by NTG-mutagenesis (9). Plasmid pAFB1 is a derivative of pIJ702 with a 9.0 kb insert DNA containing the A-factor biosynthetic gene (*afsA*).

Media and culture conditions

The *E. coli* DH5α was maintained on M9 minimal agar and cultured in LB medium at 37°C with agitation (16). The *Streptomyces* strains were maintained on R2YE agar that contained per liter, 103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 10 g glucose, 0.1 g casamino acid, 5 g yeast extract, 10 ml of 0.5% K_2HPO_4, 80 ml of 3.68% $CaCl_2 \cdot 2H_2O$, 15 ml of 20% L-proline, 100 ml of 5.73% of TES (pH7.2), 2 ml of trace elements solution, and 2.2% agar (8). *S. griseus* grown in R2YE liquid broth without agar at 28°C with vigorous shaking was used for the preparation of the protoplasts and the isolation of the plasmid DNAs (11). R2YE broth was used for the protease assay.

Enzymes and chemicals

DNA modifying enzymes were purchased from Takara Biomedical Inc., Japan. The artificial chromogenic substrates for the protease assay and protease inhibitors such as, antipain, aprotinin, bestatin, chymostatin, E-64, EDTA, leupeptin, pefabloc SC, pepstatin, and phosphoramidon, were all purchased from Boehringer Mannheim GmbH, Germany. Other fine chemicals used for preparing the buffer and enzyme assay were purchased from Sigma Chemical Co.

DNA Manipulations

The DNA preparation and manipulation were performed using the method of Hopwood *et al.* in *E. coli* and *Streptomyces* (11). The plasmid DNAs and their digests were analyzed by horizontal agarose gel electrophoresis with a TAE buffer system (16).

Transformation procedure

Cells from the *E. coli* strains competent for transformation were routinely prepared according to the frozen storage protocol and the transformation was performed using the method described by Hananhan (3).

Protoplasts of *S. griseus* IFO 13350 and HH1 were prepared by the treatment of lysozyme (15). The cells were grown in 100 ml of R2YE containing 0.5% glycine in a 500 ml baffled flask and incubated for 18 to 24 h. The resulting cells were treated with 20 ml of P buffer containing 80 mg of lysozyme, and the resulting protoplasts were suspended in the P buffer at a concentration of 10^9/ml and frozen at -70°C. The *Streptomyces* protoplasts were transformed using 100 μl of the protoplasts, 1 μg of the plasmid DNA in 20 μl of the TE buffer, and 500 μl of 25% PEG1000 in the 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 h, the plates were overlaid with 2.5 ml of a 0.6% soft R2YE agar containing 25 μg/ml of thiostrepton. The transformants were visible after incubation for an additional 3 to 5 days at 28°C.

Sample preparation for protease assay

S. griseus IFO 13350 and HH1, and the transformant of *S. griseus* harboring the pAFB1 recombinant plasmid were grown in 100 ml of R2YE medium in 500 ml baffled flasks at 28°C with vigorous shaking. After 1 day of cultivation, 10 ml of the culture broth were sampled everyday. After centrifugation at 6,000 rpm for 10 min, the resulting supernatant was used for measuring the protein concentration and protease activity, and the cell pellet was used for the quantitation of the protein concentration after cell disruption by sonication. The protein concentrations were determined using the method of Bradford and bovine serum albumin was used as the standard (1).

Determination of total protease activity

The total protease activity was measured spectrophotometrically by casein hydrolysis (21). 500 μl of a casein solution (0.6% hammarstein in a 50 mM potasium phos-

phate buffer (pH 7.0) prewarmed for 5 min at 37°C was mixed with 100 μl of the enzyme solution and incubated at 37°C for 30 min for saturation. The reaction was stopped by adding 500 μl of a TCA solution (0.1 M TCA, 0.22 M Na-acetate, 0.33 M acetic acid) and incubating at 37°C for 30 min. The reaction mixture was then centrifuged at 12,000 rpm for 15 min, and the absorbance of the supernatant at 275 nm was recorded. One unit of protease was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions.

Determination of trypsin activity

The trypsin activity was measured spectrophotometrically by the release of *p*-nitroaniline due to the enzymatic hydrolysis of the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-*p*-nitroanilide (15). The assay reaction mixture, composed of 890 μl of a reaction buffer (50 mM Tris-HCl (pH 8.0), 20 mM $CaCl_2$) and 10 μl of 50 mM N-α-benzoyl-DL-arginine-*p*-nitroanilide in DMSO, was prewarmed for 5 min at 37°C, rapidly mixed with 100 μl of the enzyme solution, and incubated for 15 min. The reaction was stopped by adding 400 μl of 30% acetic acid in dioxane, and the absorbance at 405 nm was recorded. One unit of trypsin was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions (30).

Determination of chymotrypsin activity

The chymotrypsin activity was also measured spectrophotometrically by the release of *p*-nitroaniline using N-succinyl-ala-ala-pro-phe-*p*-nitroanilide as the artificial chromogenic substrate (4). The reaction mixture containing 890 μl of a 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 the reaction with 100 μl of the enzyme solution in the same manner as outlined above. After reacting for 15 min, the chymotrypsin activity was measured at 405 nm. One unit of chymotrypsin was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions.

Determination of leu-aminopeptidase activity

The artificial chromogenic substrate, L-leu-*p*-nitroanilide, was used for the measurement of the leu-aminopeptidase activity (30). The reaction conditions were the same as those used for measuring the trypsin activity except for the use of 10 μl of 100 mM L-leu-*p*-nitroanilide in 95% ethanol. After reacting with 100 μl of the enzyme solution for 15 min, the reaction was stopped with 400 μl of 30% acetic acid in dioxane, and the absorbance at 405 nm was recorded. One unit of leu-aminopeptidase was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions.

Determination of thiol protease activity

The artificial chromogenic substrate, P-glu-phe-leu-p-nitroanilide was used for determining the thiol protease activity (2). The reaction mixture, which was composed of 890 μl of reaction buffer (100 mM phosphate buffer (pH 6.5), 0.3 M KCl, 0.1 mM EDTA, 3 mM DTT) and 10 μl of 50 mM P-glu-phe-leu-*p*-nitroanilide in DMSO, was prewarmed for 5 min at 37°C, rapidly mixed with 100 μl of the enzyme solution, and incubated for 15 min. The reaction was then stopped by adding 400 μl of 30% acetic acid in dioxane, and the absorbance at 405 nm based on the release of *p*-nitroaniline due to the enzymatic hydrolysis was recorded. One unit of thiol protease was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance under the above conditions.

Mycelial growth and scanning electron microscopy

The *in vivo* influence of protease inhibitors on *S. griseus* IFO 13350 was determined. Spores or protoplasts prepared from strain IFO 13350 were grown on a solid R2YE medium with paper discs containing different concentrations of protease inhibitors. The aerial mycelium formation, sporulation and pigment production were examined after 3 to 7 days of growth at 28°C and further examined by scanning electron microscopy (5). Agar pieces with mycelium and spores were cut and fixed under a 1% osmium tetroxide vapor for 15 h. These fixed specimens were then dehydrated and dried with a freeze-dryer for another 15 h. After being coated with platinum-gold under a vacuum by sputter coating for 5 min, the resulting mycelium and spores were observed under a Hitachi Model S4000 scanning electron microscope.

Results and Discussion

Comparison of protease production by S. griseus IFO 13350 and S. griseus HH1

S. griseus IFO 13350 and its A-factor deficient mutant strain, *S. griseus* HH1, were cultured in R2YE liquid medium and their cell growth, total protease activity, and various specific protease activities produced were spectrophotometrically measured.

The growth of the *S. griseus* was monitored by measuring the protein concentration of the cell-free extract prepared by the ultrasonic disruption of the cell. The growth of *S. griseus* IFO 13350 and strain HH1 reached a maximum level of growth after 1 day of cultivation, and the amount of total proteins was 1.3 times higher in the wild type than in that of the A-factor deficient mutant (Fig. 1A).

When the total protease activity of the culture broth was measured using the caseinolytic method, the specific activity continually increased until the end of the fer-

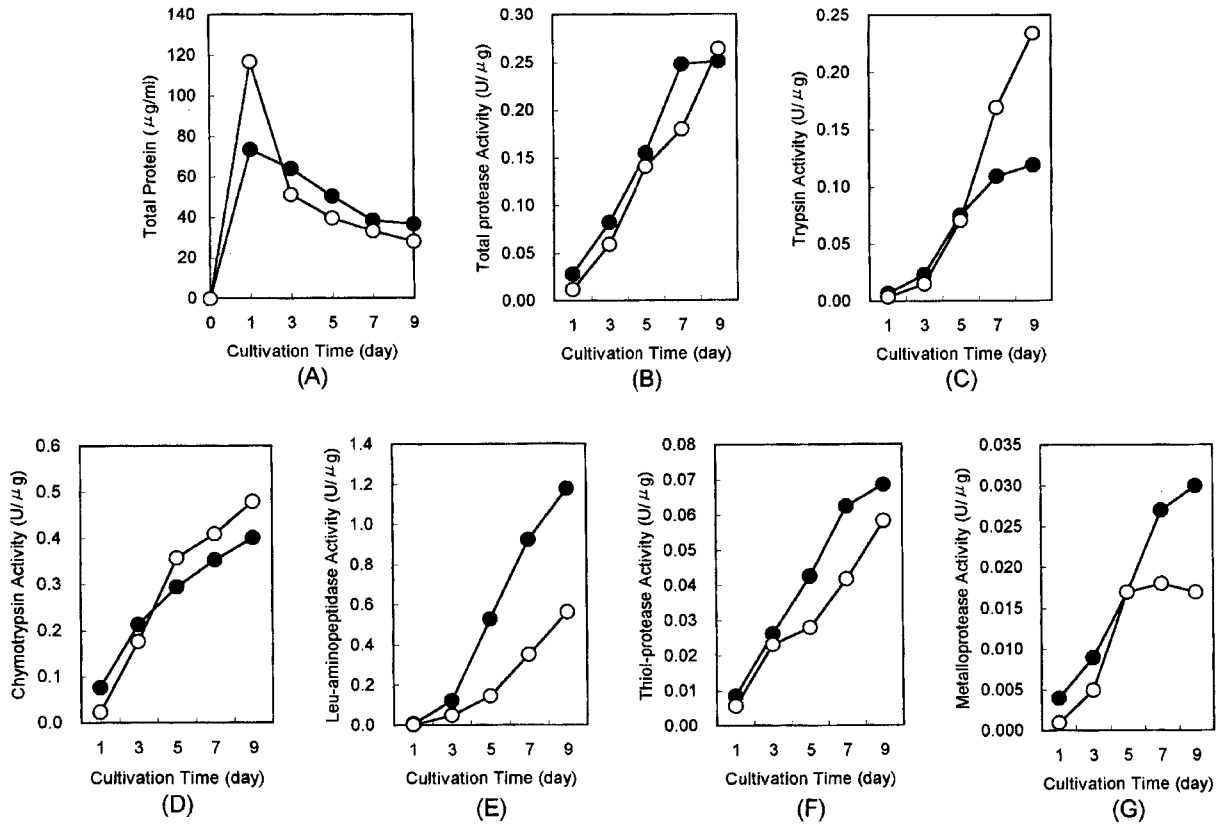

Fig. 1. Comparisons of growth (A), total protease activity (B) and trypsin (C), chymotrypsin (D), leu-aminopeptidase (E), thiol-protease (F), and metalloprotease (G) activities produced by *S. griseus* IFO 13350 (○ – ○) and *S. griseus* HH1 (● – ●). Two strains were cultured in an R2YE broth medium with vigorous shaking and samples from the culture broth were taken everyday and used for measuring the cellular protein concentration and enzyme activities as explained in Materials and Methods. The amount of enzyme produced was expressed in units per μg of cellular protein.

mentation (Fig. 1B). This fact implies that *S. griseus* can produce proteases in a stable manner or the protease itself remains in an active form for a long period of time. Interestingly, the specific activity of the protease produced by *S. griseus* HH1 was slightly higher than that by *S. griseus* IFO 13350 in spite of its poor growth.

The trypsin activity was measured using the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-*p*-nitroanilide (Fig. 1C). Up to the 5 th day of cultivation the level of the trypsin activity was almost the same in both strains, however, after 5 days of growth the specific activity of the trypsin in *S. griseus* IFO 13350, was greatly enhanced and reached 0.23 U/μg of cellular proteins, which corresponds to twice the activity shown by *S. griseus* HH1.

The chymotrypsin Leu-aminopeptidase, and thiol protease activities were measured spectrophotometrically based on the release of *p*-nitroaniline using N-succinyl-ala-ala-pro-phe-*p*-nitroanilide, L-leu-*p*-nitroanilide, and P-glu-phe-leu-*p*-nitroanilide as artificial chromogenic substrates according to the method described in Materials and Methods. There was no significant difference between the *S. griseus* IFO 13350 and HH1 strains in their chymotrypsin activity (Fig. 1D) and thiol protease activity (Fig. 1F), however, the level of leu-aminopeptidase activity was 2 times higher in *S. griseus* HH1 than in the strain IFO 13350 (Fig. 1E).

The metalloprotease activity (22) was measured spectrophotometrically by a casein hydrolysis in the presence or absence of EDTA (Fig. 1G). The value of the metalloprotease activity was calculated by subtracting the value of the caseinolytic activity in the presence of EDTA from the value in the absence of EDTA (total protease activity). *S. griseus* HH1 showed metalloprotease activity which was 2 times higher than that of strain IFO 13350, and the difference in the enzyme activity became apparent in the later stage of growth.

Effect of mass production of A-factor on the production of proteases in S. griseus

The A-factor-negative mutant, *S. griseus* HH1, can neither produce secondary metabolites nor develop into aerial mycelium. The external addition of the A-factor or the introduction of the *afsA* gene that is responsible for A-factor biosynthesis can restore all of these deficiencies in *S. griseus* HH1. To find proteases whose expression is related to A-factor, the *afsA* gene placed on pIJ702 was introduced into *S. griseus* HH1 and the difference in the protease activity was examined. *S. griseus* IFO 13350 and

strain HH1 with the pIJ702 vector were cultured and used as the controls in the presence of thiostrepton at a final concentration of 25 μg/ml.

S. griseus under the stress of thiostrepton showed poorer growth than that exhibited in the thiostrepton-negative medium (Fig. 2A). *S. griseus* IFO 13350 showed greater growth than the A-factor negative HH1 strain, however, the transformant of the strain HH1 containing the *afsA* gene showed the greatest growth. The introduction of the *afsA* gene on a high-copy number plasmid such as pIJ702 induced A-factor biosynthesis from a very early phase of cell growth and affected many physiological regulations resulting in the retardation of sporulation and streptomycin production. Based on this data, it appears that A-factor production can induce a better adaptability for antibiotic exposure such as thiostrepton, yet it is still not certain whether A-factor influences the expression of the *tsr* gene.

When the total protease activity in the culture broth was measured, *S. griseus* IFO 13350 showed caseinolytic activity which was 1.3 times higher than that of *S. griseus* HH1 (Fig. 2B). Furthermore, on the 9th day of cultivation *S. griseus* HH1 with *afsA* produced 1.7 and 1.3 times more protease activity than that produced by *S. griseus* HH1 and strain IFO 13350. This result clearly shows that A-factor can restore the ability of the A-factor deficient mutant to produce proteases at the same level as the wild type, and suggests that there is some protease(s) in *S. griseus* whose production is affected by the presence of the A-factor.

The trypsin activity was also compared in the three strains (Fig. 2C). Similarly, the trypsin activity produced by *S. griseus* IFO 13350 was 2.5 times higher than that produced by the A-factor deficient strain HH1 even in the presence of thiostrepton. Interestingly, *S. griseus* HH1 with the *afsA* gene showed a significant decrease in the level of trypsin activity compared with the HH1 strain. This fact implies that the wild-type IFO 13350 strain can produce a higher amount of trypsin compared to strain HH1, however the production of A-factor can not restore the ability to produce trypsin in HH1 as in the wild type. However, this result does not directly imply that *S. griseus* trypsin is not under the control of A-factor because the earlier mass production of the A-factor in *S. griseus* may change many of the physiological controlling systems including the regulatory cascades for protease production

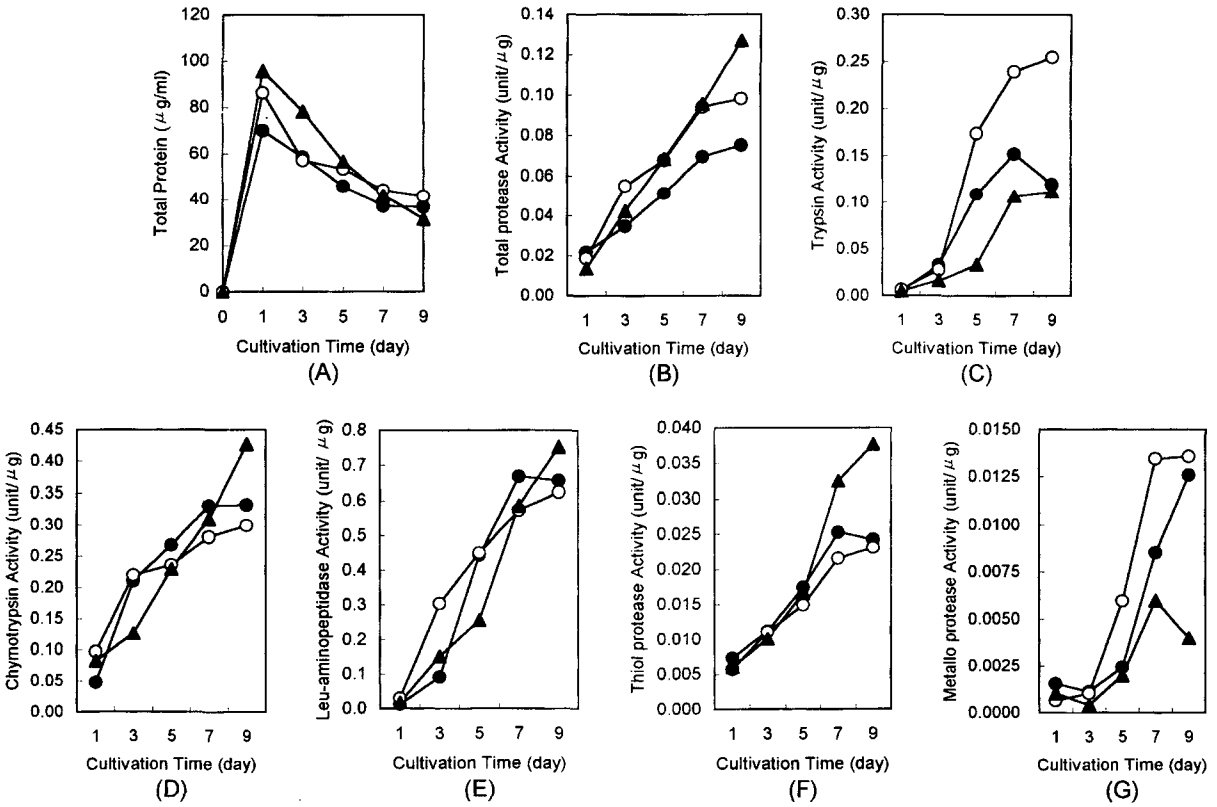

Fig. 2. Comparisons of growth curve (A), total protease activity (B), trypsin (C), chymotrypsin (D), leu-aminopeptidase (E), thiol-protease (F), and metalloprotease (G) activities produced by *S. griseus* IFO 13350 with pWHM3 (○–○), *S. griseus* HH1 with pWHM3 (●–●), and *S. griseus* HH1 with pAFB1 (▲–▲). Three strains were cultured in R2YE broth medium with vigorous shaking in the presence of thiostrepton at a final concentrations of 20 μg/ml of culture broth and samples from the culture broth were taken everyday and used for determining the cellular protein concentration and enzyme activities as described in Materials and Methods. The amount of enzyme produced was expressed in units per μg of cellular protein.

like trypsin.

The chymotrypsin activity, leu-aminopeptidase, and thiol protease activity were measured by the method outlined in Materials and Methods (Fig. 2D-F). There was no significant difference between *S. griseus* IFO 13350 and HH1 in these enzyme activities. *S. griseus* HH1 harboring *afsA* showed a similar level of enzyme activity, yet at the end of fermentation all three protease activities sharply increased and, in particular, the thiol protease activity substantially increased by 1.6 times compared with that in strains HH1 and IFO 13350.

The metalloprotease activities were also compared. *S. griseus* IFO 13350 showed a metalloprotease activity which was 2.4 and 1.6 times higher than that of HH1 on the 5th and 7th day of cultivation, respectively, in contrast to the results without thiostrepton (Fig. 2G). However *S. griseus* HH1 with *afsA* showed a significantly decreased level of metalloprotease activity, approximately one half or a fourth of that of *S. griseus* HH1 and *S. griseus* IFO 13350, respectively. Thiostreptone has been known to affect the expression of various genes in *Streptomyces,* and the production of the metalloproteases in *S. griseus* seems to be deeply influenced by the presence of thiostreptone.

The result of this study indicates that *S. griseus* IFO 13350 can produce more proteases than the A-factor-deficient mutant HH1, and this defect can be restored by the introduction of the *afsA* gene. Trypsin activity was significantly higher in wild type regardless of the presence of thiostreptone, and the metalloprotease activity was higher in the mutant strain in the absence of thiostreptone. However, the introduction of the *afsA* that leads to an earlier mass production of A-factor resulted in the suppression of both enzyme activities. Apparently, the different activity of trypsin and metalloprotease in an A-factor producer and decreased level of both enzyme activities in mass producer of A-factor suggests that the expression of these enzymes is somehow related to A-factor production. As explained in the regulation of streptomycin production by the A-factor, the concentration of A-factor in the cell may determine the expression level of the responsible protease-encoding genes. The restoration of the total protease activity in the *afsA* transformant may be mainly attributable to the thiol-protease and partly attributable to chymotrypsin, leu-aminopeptidase and other proteases which were not detected in the assay systems used in this study. Obviously, the thiol-protease activity was sharply increased in the transformant of *afsA*, and the chymotrypsin and leu-aminopeptidase activities showed a slight increase in the transformants in the later stage of the cell cycle. To obtain clearer evidence for the presence of an A-factor-dependent protease and its involvement in bacterial differentiation, all the genes encoding proteases from *S. griseus* will need to be cloned in future studies.

Effects of protease inhibitors on morphogenesis of S. griseus IFO 13350

Because of the distinct differences in the protease activities between the two strains of *S. griseus*, the *in vivo* effects of the protease inhibitors on morphology were examined. The protease inhibitors examined show the following patterns (25, 26). Antipain inhibits mainly papain, trypsin and cathepsin A and B, and aprotinin inhibits most of the serine proteases. Bestatin (((2S, 3R)-3-amino-2-hydroxyl-4-phenylbutanoyl)-L-leucine) is primarily, if not exclusively, an inhibitor of aminopeptidases like aminopeptidase B and leu-aminopeptidase, and chymostatin is a specific inhibitor for α-, β-, γ- and δ-chymotrypsin. E-64 (N-(N(L-3-trans-carboxirane-2-carbonyl)-L-leucyl)-agmatine) inhibits papain and other cysteine proteases like cathepsin B and L, and EDTA strongly inhibits metalloprotease. The synthetic peptide, leupeptin, inhibits serine and thiol proteases and pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride) is a specific, potent and irreversible inhibitor of serine protease. Pepstatin isolated from *Streptomyces* inhibits aspartic proteases like pepsin, renin and many microbial acid proteases, and phosphoramidon (N-(rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan) inhibits collagenase and metalloproteases from various microorganisms like *Bacillus subtilis, S. griseus* and *Pseudomonas aeruginosa.* A paper disc containing each of the inhibitors was placed on an R2YE agar after 6 hrs of incubation of the plates seeded with spores, the mycelial morphology was observed (Fig. 3).

When pefabloc SC (1-1.5 mg/disc) was applied to strain IFO 13350 and incubated at 30 for 3 days, the formation of aerial mycelium and spores around the disc was completely inhibited (Fig. 3A, i3). No growth inhibition, even in the area close to the disc, was observed, indicating that the pefabloc SC inhibited cellular differentiation without affecting the vegetative growth. The substrate mycelium grown in medium containing a high concentration of pefabloc SC was normal, as observed by scanning electron microscopy, however, a few small sprouts or buds which seemed to differentiate into aerial mycelia were seen (Fig. 3B, b).

EDTA (1-1.5 mg/disc) caused the formation of a growth inhibition zone around the disc indicating its killing effect at high concentrations (Fig. 3A, j3). A ring was formed outside the clear zone where only substrate mycelium was formed. This implied that EDTA at a certain concentration inhibited the aerial mycelium formation without exerting a killing effect. In fact, a smaller amount of EDTA (0.5 mg/disc) caused the appearance of a distinct zone around the disc where the aerial mycelium formation was completely inhibited (Fig. 3B, c).

Other protease inhibitors were also examined to study their effect on the morphogenesis of *S. griseus*, however, no distinct changes were found in their morphology even at a very higher concentration (Fig. 3A, a-h). The phe-

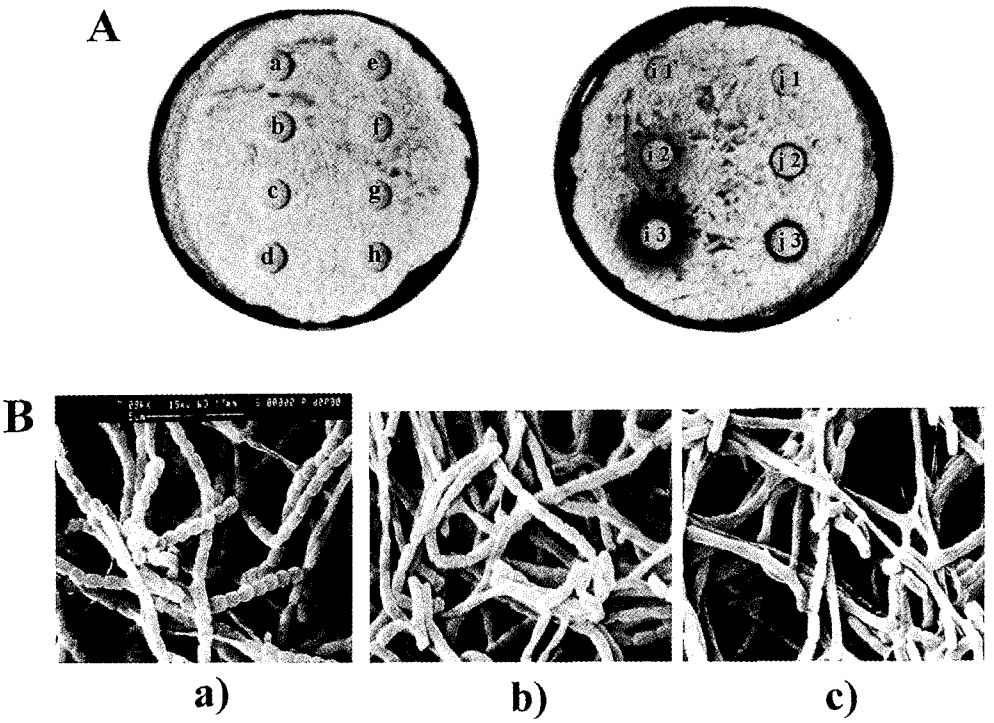

Fig. 3. Effects of protease inhibitors on the morphogenesis of S. griseus IFO 13350. Photographs of the plates (A) and scanning electron microscopic observations (B). (A) Photographs of the surface of the plate. Each paper disc contained the corresponding chemicals in the amounts indicated. a, antipain (1 mg); b, bestatine (0.25 mg); c, chymostatin (0.5 mg); d, E-64 (0.5 mg); e, leupeptin (0.05 mg); f, phosphoramidon (0.05 mg); g, pepstatin (1 mg); h, aprotinin (0.05 mg); i1, pefabloc SC(0.5 mg); i2, pefabloc SC (1.0 mg); i3, pefabloc SC (1.5 mg); j1, EDTANa2 (0.5 mg); j2, $EDTANa_2$ (1.0 mg); j3, $EDTANa_2$(1.5 mg). (B) a, S. griseus IFO 13350 as a control; b, taken from the inhibition zone caused by pefabloc SC (1.5 mg) (A-i3), where aerial mycelium formation is delayed; c, taken from the inhibition zone caused by $EDTANa_2$ (1.5 mg) (A-j3), where aerial mycelium formation is delayed.

notypic changes induced by pefabloc SC and EDTA could not be maintained for longer than 5 days of cultivation, however, delayed aerial mycelium formation by two or three days and relatively scarce sporulation were mainly observed. This fact implies that some proteases play very important roles in the formation of aerial mycelium formation and sporulation, and they may belong to a class of serine- or metallo-proteases.

Proteases are known to be involved in many important intracellular functions such as cell differentiation, cell to cell recognition, cell growth, virulence, apoptosis, and other processes. *Streptomyces* can produce many extracellular proteases which can be divided into primary and secondary proteases depending on production time (28). In general, secondary proteases are produced after active cell growth has ceased because of nutritional depletion. This kind of secondary protease is thought to have some functional relationship with secondary metabolism or cell morphogenesis which also occurs after cell growth has ceased (13, 18). The profile of trypsin and metalloprotease production depending on the cultivation time in *S. griseus* IFO 13350 and strain HH1 implies they may be necessary for the differentiation of *S. griseus* (15). In addition, the *in vivo* effects of protease inhibitors on the morphological differentiation clearly show the involvement *Streptomyces* proteases in the formation of aerial mycelia. Therefore, elucidating the exact biological function of proteases will offer valuable information to understand the complex and elaborate system of living things.

The importance of the trypsin-like protease and metalloprotease in the morphological differentiation of Streptomyces can be found in *Streptomyces exfoliatus* and *Streptomyces albogriseolus*. *S. exfoliatus* SMF13 sequentially produces leupeptin, leupeptin-inactivating enzyme (LIE) and trypsin-like protease (TLP). The inhibition of the morphological differentiation by the addition of bestatin, an inhibitor of LIE, is due to the inhibition of the TLP activity, which is essential for aerial-mycelium formation and is specifically inhibited by the remaining leupeptin that was not inactivated (13, 14). *S. albogriseolus* 444 was shown to constitutively synthesize an extracellular proteolytic complex with metalloprotease and trypsin-like activities defining the differentiation of the substrate mycelium to the aerial one as well as the spore (18).

Recently, many proteases have been investigated due to their importance in the morphological differentiation of *Streptomyces* (20). Two exocellular nucleases with molecular masses of 18 and 34 kDa, which reach their maximum

activity during aerial mycelium formation and sporulation, have been detected in *Streptomyces antibioticus*. Their function appears to be DNA degradation in the substrate mycelium. The 18-kDa nuclease has been shown to be a member of the cyclophilin family and is produced by a proteolytic processing from a less active protein precursor. The protease responsible has been identified as a serine protease. The inhibition of both of the nucleases or protease impairs aerial mycelium development in *S. antibioticus*. The biochemical features of cellular DNA degradation during *Streptomyces* development show significant analogies with the later steps of the apoptosis of eukaryotic cells.

The genes of *Streptomyces coelicolor* A3(2) encoding the catalytic subunits (ClpP) and regulatory subunits (ClpX and ClpC) of the ATP-dependent protease family *Clp* have already been characterized (27). The disruption of the *clpP1* gene in *S. lividans* and *S. coelicolor* blocks differentiation at the substrate mycelium step. The overexpression of *clpP1* and *clpP2* appears to accelerate aerial mycelium formation in *S. lividans*, *S. albus* and *S. coelicolor*.

The formation of an aerial mycelium by the filamentous bacterium *S. coelicolor* is determined in part by a small morphogenetic protein called SapB (29). The collection of representative bald mutants, which are blocked in aerial mycelium formation, are all defective in the production of this protein and regain the capacity to undergo morphological differentiation when SapB is supplied exogenously.

All of these reports strongly suggest that some proteases are involved in the morphogenesis of *Streptomyces*. The function of these proteases may be to generate a specific signal leading to morphological or physiological differentiation or to supplement the nutritional source, which is necessary for further development in *Streptomyces*. The results of this study support the idea that some trypsin-like protease or metalloprotease, whose production appears to depend on A-factor, are involved in the aerial mycelial and spore formation of *S. griseus*. Therefore, the characterization of some protease-encoding genes whose expression is dependent on the production of A-factor is currently under investigation.

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