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Streptomyces griseus Trypsin (SGT) Has Gelatinase Activity and Its Proteolytic Activity Is Enhanced by Manganese

Won-Jae Chi[1], Yoon-Hee Kim[1], Jong-Hee Kim[1], Dae-Kyung Kang[2], Sang-Soon Kang[3], Joo-Won Suh[1], and Soon-Kwang Hong[1,*]

[1]Department of Biological Science, College of Natural Science, Myongji University, San 38-2, Nam-Dong, Yongin, Kyunggi-do 449-728, Korea
[2]Bio-Resources Institute, Easy Bio System Inc., Chonan, Chungnam 330-820, Korea
[3]Division of Science Education, Chungbuk National University, Chongju 361-763, Korea

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Gelatinase is a proteolytic enzyme that hydrolyzes gelatin. Gelatinolytic activity was detected from culture broths of *Streptomyces griseus* IFO13350 and HH1 by paper disc assays on 0.5% agar plates containing 1% gelatin. The concentrated extracellular protein from the *S. griseus* was analyzed by SDS polyacrylamide gel, and two proteins, with molecular weights of 30 and 28 kDa, respectively, were identified to have gelatinase activity by gelatin zymography. The protein with a molecular weight of 28 kDa was confirmed to be *S. griseus* trypsin (SGT). The effects of metal ions and metal chelators on the protease activity of the SGT were studied. Of the metal ions tested, only manganese was found to enhance the protease activity, 2.6 times, however, Co^{2+}, Cu^{2+}, and Zn^{2+}, and metal chelators, such as EDTA and EGTA, inhibited the SGT activity. When the protease activity of the SGT was measured at various pHs, in the presence of 5 mM $MnCl_2$, its highest activity was at pH 11.0, whereas only 60% of the maximum activity was observed between pHs 4.0 and pH 6.0, and almost 80% activity between pHs 7.0 to pH 10.0. The protease activity was measured at various temperatures in the presence of 5 mM $MnCl_2$. The SGT was found to be stable up to 60°C for 30 min, while only 16% of the enzyme activity remained at 60°C, and at 80°C almost all the activity was lost. The optimal temperature for the protease activity was 50°C.

Key words: *Streptomyces griseus* Trypsin, SGT, serine protease, gelatinase

The genus *Streptomyces* is comprised of gram-positive soil bacteria with a complex life cycle. In addition to the complex morphological differentiation, *Streptomyces* are able to produce many kinds of secondary metabolites, including antibiotics and biologically active substances. *Streptomyces griseus* is a representative strain, and has been extensively studied for its regulatory cascade concerning streptomycin production and morphological differentiation (Uhnmee *et al.*, 1998). A-factor (2-isocapryloyl-3-γ-hydroxy-methyl-butyrolactone) is a microbial hormone that plays a role as a positive regulator for the productions of streptomycin and sporulation in *S. griseus* (Vujaklija *et al.*, 1991). The regulatory network in the cell, starting from A-factor, has almost elucidated (Horinouchi, 2002).

According to our results, certain proteases are produced in different manners in *S. griseus* strains IFO13350 and HH1, the later being an A-factor deficient strain. In addition, some serine protease and metalloprotease inhibitors induce the retardation of spore formation. With regard to these data, it has been suggested that certain proteases may be involved in the *Streptomyces* differentiation processes (Kim *et al.*, 2000).

S. griseus produces, not only a mixture of proteases, sold under the commercial name of Pronase, but also many kinds of secondary metabolites (Awad *et al.*, 1972). Many genes, such as *sprA*, *sprB*, *sprC*, *sprD*, *sprE*, and *sprT*, that encode *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), respectively, have also been cloned and analyzed (Olfason *et al.*, 1975; Kim *et al.*, 1991). All the proteases reported from *S. griseus* belong to the bacterial serine proteases that catalyze the hydrolysis of amides and esters, through a common catalytic mechanism, and involve a triad of the residues serine, histidine and aspartic acid (Narahashi *et al.*, 1968; Trop *et al.*, 1970; Lee *et al.*, 2000).

SGT is a bacterial serine protease with greater similarity to a mammalian protease than to either *S. griseus* proteases A and B (Trop *et al.*, 1968; Nishikata *et al.*, 1981).

* To whom correspondence should be addressed.
(Tel) 82-31-330-6198; (Fax) 82-31-335-8249
(E-mail) skhong@mju.ac.kr

The *sprT* gene (Genebank accession No. M64471) encoding *S. griseus* trypsin has also been isolated from a *S. griseus* genomic library (Kim *et al.*, 1991). In a previous study, the current authors isolated the *sprT* gene from *S. griseus* ATCC10137, optimizing the overexpression system in *S. lividans* TK24 under various conditions (Koo *et al.*, 1998).

More recently, the current authors also reported that the overexpression of the *sprD* gene in *S. griseus* IFO13350 induced the production of the pigmented antibiotic, actinorhodin, in *S. lividans* TK24, with no morphological change, thereby implying SGPD to be deeply involved in the physiological differentiation in the host strain (Choi *et al.*, 2001). The introduction of the *sprA* gene into *S. lividans* TK24 also triggered the biosynthesis of the pigmented antibiotics, actinorhodin and undecylprodigiosin, and induced significant morphological changes in the colonies in Benedict, R2YE, and R1R2 media. In addition, the introduction of the *sprT* gene also induced morphological changes in the colony shape, without affecting the antibiotic production, thereby implying that certain proteases appear to play very important and specific roles in secondary-metabolites formation and morphological differentiation in *Streptomyces* (Chi *et al.*, 2001).

The gelatinolytic activity in culture broth of *S. griseus*, was studied. Of the proteins, identified from the gelatin zymography, one was confirmed to be SGT. In this report, the gelatinolytic activity of the purified SGT, and its enzymatic characteristics under various conditions, are described.

Materials and Methods

Bacterial strains and culture conditions

The *S. griseus* IFO 13350 and HH1 (Vujaklija *et al.*, 1991) were obtained from Prof. S. Horinouchi (University of Tokyo, Japan) and the *S. lividans* TK24 (Hopwood *et al.*, 1985) was from the John Innes Institute, United Kingdom. The *S. griseus* HH1 is an A-factor deficient mutant strain derived from the IFO 13350 strain by NTG-mutagenesis (Vujaklija *et al.*, 1991). All the streptomycetes strains were maintained on a R2YE agar, that containing, 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 [pH 7.2], 2 ml of trace elements solution, and 2.2% agar per liter (Hopwood *et al.*, 1985). To measure the gelatinase activity, 1% gelatin instead of 2.2% agar, was used. The *S. griseus* and *S. lividans*, transformed with pWHM-T, carrying the *sprT* gene coding for SGT, were grown in an R2YE liquid broth at 28°C, with vigorous shaking, and used for purification of the SGT.

Enzymes and chemicals

The artificial chromogenic substrates for the protease assay were all obtained from Boehringer Mannheim GmbH, Germany. Other fine chemicals used for preparing the buffers and enzyme assays were obtained from Sigma Chemical Co.

Quantitation and SDS-PAGE analysis of protein

The protein concentration was determined by Bradford's method (Bradford *et al.*, 1976), using bovine serum albumin (BSA) as a standard. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed, with a minigel apparatus (SE250 Unit of Hoeffer Scientific Instruments), according to the method of Laemmli (Laemmli, 1970). The protein samples were mixed with an appropriate volume of 5× sample buffer, boiled for 3 min and loaded onto the gels. After the electrophoresis at 40 mA, the gels were stained with Coomassie Brilliant Blue R-250.

Purification of SGT from S. lividans TK24 transformant

The *S. lividans* TK24 transformant harboring the pWHM-T, was grown in C5/L medium (5 l) for 4 days, at 28°C, under aerobic conditions, as described previously (Koo *et al.*, 1999). The culture broth was centrifuged at 6,000×*g* for 15 min at 4°C to separate the cells and supernatant. Ammonium sulfate was added to the supernatant, to 25% saturation, to precipitate the unwanted proteins. After centrifugation, further ammonium sulfate was added to the supernatant to attain 55% saturation. The precipitate was collected by centrifugation and dissolved in small quantities of buffer A (10 mM Na-acetate [pH 5.0], 10% glycerol, and 1 mM EDTA). The solution was dialyzed against buffer A for 12 h, changing the buffer several times.

Determination of trypsin activity

The release of ρ-nitroaniline, due to the enzymatic hydrolysis of the artificial chromogenic substrate, Nα-benzoyl-DL-arginine-ρ-nitroanilide (Koo *et al.*, 1998), was used as a measure of the trypsin activity, and was recorded spectrophotometrically. 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 Nα-benzoyl-DL-arginine-ρ-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 the addition of 400 μl of 30% acetic acid in dioxane, and the absorbance at 405 nm recorded. One unit of trypsin activity was defined as the amount of enzyme that gave a 0.1 increase in the absorbance under the above conditions (Koo *et al.*, 1999)

Determination of protease activity

The protease activity of the SGT was measured spectrophotometrically by the azocasein hydrolysis method, a modification to the Secades's method (Secades *et al.*, 1999). The reaction mixture, composed of 480 μl reaction

buffer (25 mM Tris·Cl [pH 7.6], 5 mM $MgCl_2$ and 1% (wt/vol) azocasein) was prewarmed for 5 min at 30°C, rapidly mixed with 120 µl of the enzyme solution and incubated for 30 min. The reaction was stopped by the addition of 600 µl of 10% trichloroacetic acid, and centrifuged for 10 min at 15,000 rpm. The supernatant (800 µl) was neutralized by the addition of 200 µl of 1.8 N NaOH solution, and the absorbance at 420 nm recorded. However, the same molar concentration of various chemicals were added in place of the 5 mM $MgCl_2$, to check the effects on the protease activity of SGT, but 5 mM $MnCl_2$ was used in all the experiments to test the effects of pH and temperature on the activity.

Determination of gelatinolytic activity on agar plates

S. griseus was inoculated onto the R2YE solid medium, containing 1% gelatin instead of agar, and incubated at 30°C. The gelatinolytic activity of the *S. griseus*, depending on cultural time, was checked everyday, after staining with Coomassie Brilliant Blue R250. The paper disc assay was used to test the gelatinolytic activity of the culture broth of the 0.5% agarose gel plate containing 1% gelatin.

The strain was cultured in R2YE liquid medium, in 500 ml baffled flasks, at 28°C, with vigorous shaking. After 1 day of cultivation, 10 ml of the culture broth was sampled everyday. After centrifugation at 6,000×*g* at 4°C for 10 min, the resulting supernatant was used to measure the protein concentration and gelatinase activity, and the cell pellet, after cell disruption by sonication, was used for the quantification of the protein concentration. The paper disc, immersed in 70 µl of the culture broth, was placed on the assay plate, incubated for overnight at 30°C, and then halo of the gelatinolysis observed after staining with Coomassie Brilliant Blue R250.

Gelatin zymography

Zymography was performed according to the methods of Nakamura *et al.*, 2002. The *S. griseus* was grown in R2YE medium for 3 days, at 28°C, under aerobic conditions. The culture broth was centrifuged at 6,000×*g* at 4°C for 15 min to separate the cells and supernatant. Trichloroacetic acid was added to the supernatant to final concentration of 10%. After 30 min on ice, the precipitate was collected by centrifugation, washed twice with cold acetone, and then dissolved in a small quantity of buffer A. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-12% PAGE), containing 0.1% gelatin, was performed using a minigel apparatus. The protein samples were mixed with an appropriate volume of 5×SDS sample buffer, boiled for 3 min and loaded onto the gels. After gel electrophoresis at 40 mA, the gels were washed twice with 2.5% Triton X-100, immersed into the reaction buffer (50 mM TrisCl [pH 7.5], 10 mM $CaCl_2$, and 0.01% NaN_3) for 12-16 h, and then stained with Coomassie Brilliant Blue R-250.

N-terminal Sequencing of proteins

The protein showing gelatinolytic activity in the gelatin zymography was transferred onto nitrocellulose paper, by electrotransfer of the gel at 0.65 mA/cm^2 for 2 h, in transfer buffer (0.1% Tris, 1.44% glycine, and 20% methanol). An N-terminal analysis of blotted protein was performed at the Korea Basic Science Institute.

Results and Discussions

Detection of gelatinolytic activity

Gelatinase, a proteolytic enzyme that hydrolyzes gelatin, is produced by bacteria, yeasts, and fungi. Recently, many important metalloproteases, such as pepsin B, collagen metalloprotease, and matrix metalloprotease, were reported to have gelatinolytic activity in eukaryotes. The *S. griseus* strains, IFO13350 and HH1, were cultured in R2YE liquid medium, and the culture broth taken to check the gelatinolytic activity by paper disc assays on 0.5% agar plates, containing 1% gelatin. The gelatinolytic zone around the disc was clearly observed after staining with Coomassie Brilliant Blue R-250, and the gelatinolysis area sharply increased with culture time (Fig. 1A). To identify the protein that gave the gelatinolytic activity, the concentrated extracellular protein from the *S. griseus* was analyzed by electrophoresis on SDS polyacrylamide gel, containing 0.1% gelatin. After renaturation of the gel, gelatin zymography was performed, as explained in materials and methods. Two distinct proteins, with molecular weights of 30 and 28 kDa, respectively, were identified to have gelatinase

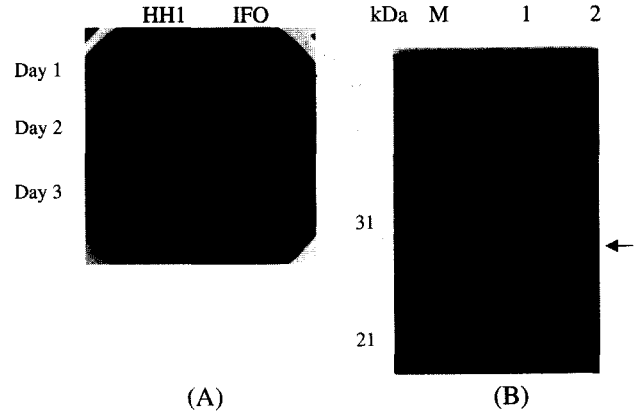

Fig. 1. Agar plate assay (A) and gelatin zymography (B) for gelatinolytic activity. (A) The *S. griseus* strains IFO13350 and HH1, were cultured in R2YE liquid medium, as described in materials and methods. The supernatant was in the paper disc assay to check for gelatinolytic activity on the 0.5% agar plates containing 1% gelatin. HH1, *S. griseus* HH1; IFO, *S. griseus* IFO13350. (B) The extracellular protein in a 3 day culture broth of *S. griseus* IFO13350 was precipitated with trichloroacetic acid, and gelatin zymography performed, as mentioned in materials and methods. M, Molecular weight size marker; Lane 1, extracellular protein of *S. griseus* IFO13350; Lane 2, SGT protein purified from *S. lividans* transformed with pWHM-T. The SGT protein, with a molecular weight of 28 kDa, is indicated by the thick arrow.

activity (Fig. 1B). When the authentic SGT, purified from *S. lividans* TK24 transformed with pWHM-T, was comigrated in the same gel, the lower band, with a molecular weight of 28 kDa exactly coincided with SGT (Fig. 1B). The amino acid sequence of the protein of 28 kDa was determined as Val-Val-Gly-Gly-Thr, which was identical to the sequence of the purified SGT protein (Koo *et al.*, 1998). All these results clearly indicate that *S. griseus* can produce proteases with gelatinolytic activity, and that SGT is one of the gelatinolytic enzymes.

Effects of metal ions and metal chelators on the protease activity of SGT

The proteolytic activity of gelatinase requires metal ion cofactors especially zinc ion in eukaryotes (Tsuyski *et al.*, 1991). Our data from the paper disc assay and gelatin zymography clearly show that SGT has gelatinolytic activity. Therefore, the effects of metal ions and metal chelators, on the gelatinase activity of the SGT, were examined using azocasein as a substrate. Because of the difficulties in solubilizing gelatin, azocasein or collagen has frequently been used to measure the protease activity, as gelatinase or metalloprotease, in a quantitative way (Secades *et al.*, 1999; Nakamura *et al.*, 2002). Of the metal ions tested, only manganese enhanced the protease activity, by 2.6 times, however, Co^{2+}, Cu^{2+}, and Zn^{2+} inhibited the activity by 51, 16, 23%, respectively, when measured under standard reaction conditions, with no additional elements (Fig. 2). Metal chelators, such as EDTA and EGTA, showed significant inhibitory effects, but did not give complete inhibition. This data shows that the SGT activity can be increased by the presence of manganese, and this enhancing effect is only slightly influenced by metal chelators, which suggests that SGT is not an absolute metalloprotease. However, studying how the manganese can enhance the SGT activity will be a very interesting field to understand its real function. A similar result was reported for a serine protease from *Bacillus amyloliquefaciens* HTP-8 (Lim *et al.*, 2002). Based on the results of the paper cited above, all the following experiments were carried out in the presence of 5 mM $MnCl_2$ in the reaction buffer.

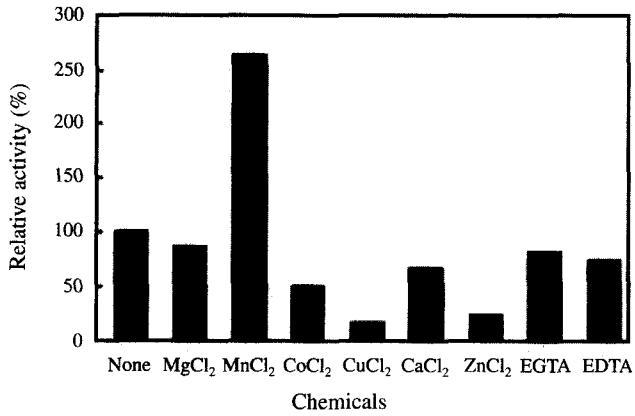

Fig. 2. Effects of metal ions and metal chelators on the protease activity of SGT. The protease activity of the purified SGT was measured, as described in materials and methods, with each chemical added to each reaction to a final concentration of 5 mM. The protease activity, with no chemicals, was considered as 100% when calculating the relative activities. All analyses were performed at least twice, in duplicate, and the data are presented as the average values obtained within standard deviations of 10-15%.

Effects of pH on the protease activity of SGT

The protease activity was measured at various pHs, using azocasein as the substrate, in the presence of 5 mM $MnCl_2$. The effect of the pH on the gelatinase activity is shown in Fig. 3. The SGT exhibited its highest activity at pH 11.0, whereas only 60% of the maximum activity was observed between pHs 4.0 and 6.0, and 80% between pHs 7.0 to 10.0. When the trypsin activity of SGT was measured in a standard reaction buffer, with the artificial chromogenic substrate, BAPNA, but without $MnCl_2$, the maximum activity appeared at pH 8.0. In contrast, our data showed that the protease activity of the SGT in the presence of manganese was maintained over a broad range of alkaline pHs, which suggests that the SGT has characteristics similar to those of alkaline serine protease under the conditions used. The alkaline proteases from *Bacillus sp.* KUN-17 (Hwang, 1995), *Pseudomonas* (Lee *et al.*, 2000), and alkalophilic *Bacillus sp.* KSM-K16 (Kobayashi *et al.*, 1995) are known to have optimums at pH 10.5, 10.0, and 12.3, respectively.

Effects of temperature on the protease activity of SGT

The protease activity was measured at various temperatures, using azocasein as the substrate, in the presence of

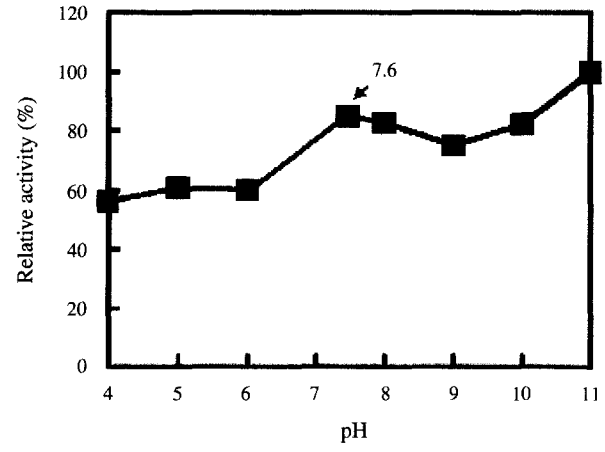

Fig. 3. Effects of pH on the protease activity of the SGT. The protease activities of the SGT were quantitatively measured under various pH conditions using the enzymatic release of azo dye from azocasein. 25 mM piperazine buffer, 25 mM PIPES buffer, 25 mM Tris·HCl buffer, 25 mM CHAPS buffer, and 25 mM carbonate buffer were used for pHs 4.0-5.0, 6.0, 7.6-8.0, 10, and 11, respectively. The highest protease activity was considered as 100% when calculating the relative activities. The relative activities are the average for three independent experiments.

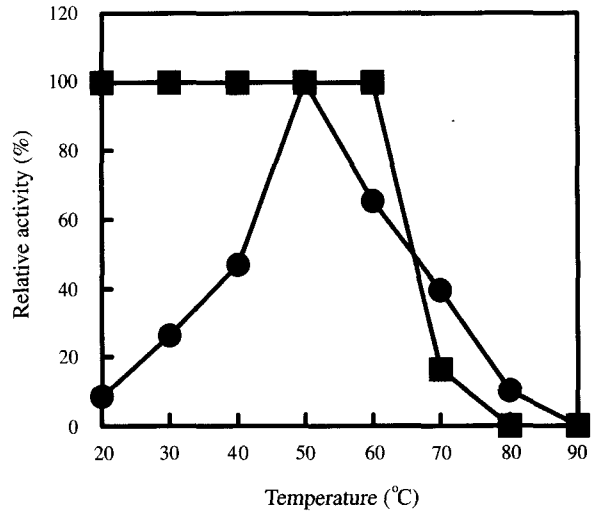

Fig. 4. Effects of temperature on the protease activity (A) and the stability (B) of the SGT. The reaction was carried out at pH 7.6, for 30 min, at various temperatures. The values obtained at 50°C were taken to be 100%. The relative activities are the averages from three independent experiments. For thermal stability, the enzyme was preincubated for 1 h in 25 mM pH 7.6 Tris·HCl buffer, at various temperatures, with the residual protease activity measured after incubation for 30min at 30°C. The residual protease activities are represented as percentages in relation to the untreated sample. ●, protease activity; ■, thermal stability.

5 mM $MnCl_2$. The SGT was found to be stable up to 60°C for 30 min, while only 16% of the enzyme activity remained at 60°C, with almost all the activity was lost at 80°C. The optimal temperature for the protease activity was 50°C. When the trypsin activity of SGT was measured in a standard reaction buffer, with the artificial chromogenic substrate, BAPNA, the maximum activity appeared at the same temperature. All these data suggest that the optimal temperature for the SGT activity was not influenced by the presence of 5 mM $MnCl_2$ or by the kind of substrate used.

SGT is a member of the serine proteases, and has trypsin-like enzyme activity (Trop *et al.*, 1970). Serine proteases are one of a group of endoproteases of both animal and bacterial sources, and share a common reaction mechanism that is based on formation of an acyl enzyme intermediate on a specific active serine residue. This group of enzymes includes those active in digestion, blood clotting, immune reactions, and fertilization of the ovum. Trypsin, chymotrypsin and the bacterial enzyme subtilisin, are examples of these types of enzyme, with many gelatinases also included in the serine family of protease.

When the protein samples from *S. griseus* were applied to the SDS polyacrylamide gel, without prior heat treatment, many gelatinolytic bands were observed in the gelatin zymography (data not shown). However, only two bands were detected after complete boiling with one confirmed as SGT (Fig. 1). The protease activity of the SGT was greatly enhanced by the presence of 5 mM $MnCl_2$, and its stability at various pHs was critically changed in the presence of manganese.

In previous experiments the specific activity of the trypsin of *S. griseus* IFO 13350 was found to be greatly enhanced, more than twice, compared with that of *S. griseus* HH1 in the later stage of growth. When a serine protease inhibitor, pefabloc SC, and a metalloprotease inhibitor, EDTA, were applied to the IFO 13350 strain, the formations of aerial mycelium and spores were delayed by two or three days. These results support the idea that some trypsin-like protease, or metalloprotease, whose production appears to depend on A-factor, was involved in the aerial mycelial and spore formations of *S. griseus* (Chi *et al.*, 2001).

The formation of a ring with abundant sporulation was reported on the application of manganese to plates inoculated with *S. griseus* and *S. coelicolor* (Hong *et al.*, 1993; Hong *et al.*, 1998). The manganese caused more fragmented aerial mycelium and smaller, denser spore formation in both strains. The addition of manganese also caused elevated phosphorylation, not only of AfsR, but also several other protein kinases in *S. griseus* and *S. coelicolor* (Horiniuchi *et al.*, 1990; Hong *et al.*, 1991; Matsumoto *et al* 1994). From all of these data, it is postulated that the elevated phosphorylation induced by the manganese might cause the abundant sporulation. However, the possibility that the enhancement of the SGT activity by manganese ion can not be ruled out as one of the critical factors that leads to the abundant sporulation in *S. griseus*.

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