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Overexpression of *sprA* and *sprB* Genes is Tightly Regulated in *Streptomyces griseus*

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Abstract The *sprA* and *sprB* genes, encoding the chymotrypsin-like proteases *Streptomyces griseus* protease A (SGPA) and *Streptomyces griseus* protease B (SGPB), and the *sprT* gene that encodes *Streptomyces griseus* trypsin (SGT) were cloned from *S. griseus* and were overexpressed in various strains of *S. griseus*. When the *sprT* gene was introduced into *S. griseus*, trypsin activity increased 2-fold in the A-factor deficient mutant strain, *S. griseus* HH1, and increased 4-fold in the wild strain, *S. griseus* IFO13350. However, there was no detectable increase of chymotrypsin activity in the transformants of *S. griseus* with either *sprA* or *sprB*, in contrast to the results obtained from *S. lividans* as a heterologous host. To solve the negative gene dosage effects in *S. griseus*, either the *sprA* or the *sprB* genes with their own ribosome binding sites were linked to the downstream of the entire *sprT* gene, and the coexpression efficiency was examined in *S. lividans* and *S. griseus*. The transformants of *S. lividans* with either pWHM3-TA (*sprT*+*sprA*) or pWHM3-TB (*sprT*+*sprB*) showed 3-fold increase of trypsin activity over that of the control, however, only the transformant of pWHM3-TB demonstrated 7-fold increase in chymotrypsin activity, indicating that the pWHM3-TB has a successful construction for the overexpression of chymotrypsin in *Streptomyces*. When the coexpression vectors were introduced into *S. griseus* IFO 13350, the trypsin level sharply increased by more than 4-fold, however, the chymotrypsin level did not increase. These results strongly suggest that the overexpression of the *sprA* and *sprB* genes is tightly regulated in *S. griseus*.

Key words: SGPA, SGPB, SGT, regulation, coexpression, *S. griseus*

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Streptomyces griseus is one of the most important industrial microorganisms because it can produce many kinds of antibiotics and enzymes, and the study on the biosynthetic genes for the secondary-metabolites formation has become the hottest issue in streptomycetes works especially for the polyketides [11] and aminoglycoside antibiotics [9, 10, 23]. Streptomycetes have unique characteristics in their life cycle, leading to aerial mycelium and spore formation, and many kinds of regulatory factors involved in this complex differentiation process have been intensively studied [16, 21]. A-factor (2-isocapryloyl-3-R-hydroxy-methyl-γ-butyrolactone) is a microbial hormone that is known as a positive regulator for the physiological and morphological differentiation in *S. griseus* [5, 6]. Therefore, *S. griseus* HH1, an A-factor-deficient mutant strain derived from strain IFO 13350 by NTG-mutagenesis, resulted in loss of ability for streptomycin production and sporulation [7].

Genes such as *sprA*, *sprB*, *sprC*, *sprD*, and *sprT*, 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* trypsin (SGT) [4, 13, 22]. All the proteases produced from *S. griseus* belong to a bacterial serine protease that has a common catalytic mechanism, involving a triad of the residues serine, histidine, and aspartic acid [17, 24]. Four chymotrypsin-like serine proteases, SGPA, SGPB, SGPC, and SGPD, are closely related to the mammalian Asp-Ser-Gly serine proteases, and SGT is a bacterial serine protease that is more similar to a mammalian protease, trypsin [18, 20, 24].

In the previous report, we described that *sprA*, *sprB*, and *sprT* genes were cloned from *S. griseus* and successfully overexpressed in *S. lividans* TK24 as a heterologous host. The chymotrypsin activity of the culture broth measured with the artificial chromogenic substrate was 5 and 7 times

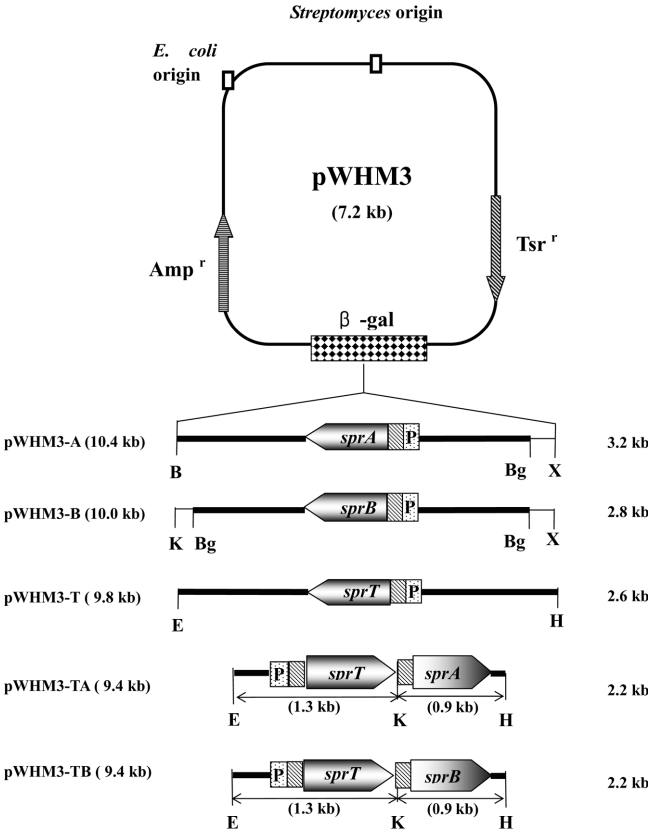

Fig. 1. Restriction maps of expression vector pWHM3-A, pWHM3-B, pWHM3-T, pWHM3-TA, and pWHM3-TB containing insert of entire *sprA*, *sprB*, *sprT*, *sprA* linked to *sprT*, and *sprB* linked to *sprT* genes, respectively.

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 only the parts from the multicloning sites introduced by the DNA manipulation procedure are depicted by a thin line. The organization of the structural genes, with pre-pro peptide (▨) and mature protease (□), is shown on the maps. Abbreviations: amp^r, ampicilline resistance; tsr^r, thiostreptone resistance; β-gal, β-galactosidase; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I.

higher in the transformants harboring either *sprA* or *sprB* gene than that of the control, respectively [2]. In addition, the transformant of *S. lividans* TK24 with pWHM3-T containing the *sprT* gene produces 15 times more SGT in R2YE medium than the *S. lividans* control, and 3.5 times more than *S. griseus* 10137, from which the *sprT* gene originated [14, 15]. In addition, the introduction of the *sprA* gene into *S. lividans* triggered the biosynthesis of the pigmented antibiotics, actinorhodin and undecylprodigiosin, and the introduction of *sprA* and *sprT* genes induced significant morphological changes in the colonies [2]. These results imply that certain proteases play very important roles in secondary-metabolites formation and morphological differentiation in *Streptomyces.*

Based on the successful overexpression and resulting physiological and morphological changes in the *S. lividans* host system, the same recombinant plasmids such as pWHM3-A, pWHM3-B, and pWHM3-T, containing the *sprA*, *sprB*, and *sprT* genes, were reintroduced into *S. griseus* HH1, an A-factor deficient mutant, using the protoplast transformation method [8, 19]. Then, the plasmids were reisolated from *S. griseus* HH1 and used for the transformation of *S. griseus* IFO 13350 to avoid the strong restriction system of *S. griseus* wild strain. The transformants were cultured in an R2YE broth [8] containing 20 μg/ml of thiostrepton, and then the chymotrypsin proteolytic activity was analyzed using the artificial substrate, N-succinyl-Ala-Ala-Pro-Phe-ρ-nitroanilide, as described [2].

The growth curve of each transformant was compared for the protein concentration of the cell lysates by the method of Bradford [1]. Although there were small fluctuations in the growth curves of each strain, significant differences were not observed (Figs. 2A, 2C). In contrast to the results from *S. lividans*, the chymotrypsin activity in the transformants of *S. griseus* with pWHM3-A and pWHM3-B was almost the same as that of the control, and no gene dosage effect could be observed in any of the host strains used (Figs. 2B, 2D). This fact suggests that the expression of chymotrypsin-like protease genes, such as *sprA* and *sprB*, is repressed by some factor(s) in its original strain, which is absent in *S. lividans*. Although the chymotrypsin activity of the *S. griseus* IFO 13350 transformed with pWHM3-A was more than 2 times higher than the control during the earlier stage of growth, the difference disappeared after 6 days of cultivation (Fig. 2D).

The transformants with pWHM3-T were also cultivated under the same conditions, and their trypsin activities were measured, as shown in Fig. 3. The trypsin activity was spectrophotometrically measured, based on the release of ρ-nitroaniline caused by the enzymatic hydrolysis of the artificial chromogenic substrate, N-α-benzoyl-DL-arginine-ρ-nitroanilide [2]. In all the strains, gene dosage effects were observed, but to different degrees. The transformant of *S. griseus* IFO 13350 showed 7-fold increase in trypsin activity and the transformant of *S. griseus* HH1 showed 2-fold increase. This observation suggests that the *sprT* gene can be overexpressed in *S. griseus*, as in *S. lividans*, and its activity is higher in A-factor-positive strains, such as *S. griseus* IFO 13350 and *S. griseus* HO1, than in the A-factor-negative strain *S. griseus* HH1 (data in *S. griseus* HO1 is not shown).

The repression of the gene dosage effect in *S. griseus* was observed in the case of another chymotrypsin-like protease gene, *sprD*, which was also well overexpressed in *S. lividans* (data not shown). The common feature of promoter regions for *sprA*, *sprB*, and *sprD* genes was compared (Fig. 4). Several conserved regions were found, and one possible explanation for this repression might be that some repressor protein might recognize one of those conserved regions and control its transcription.

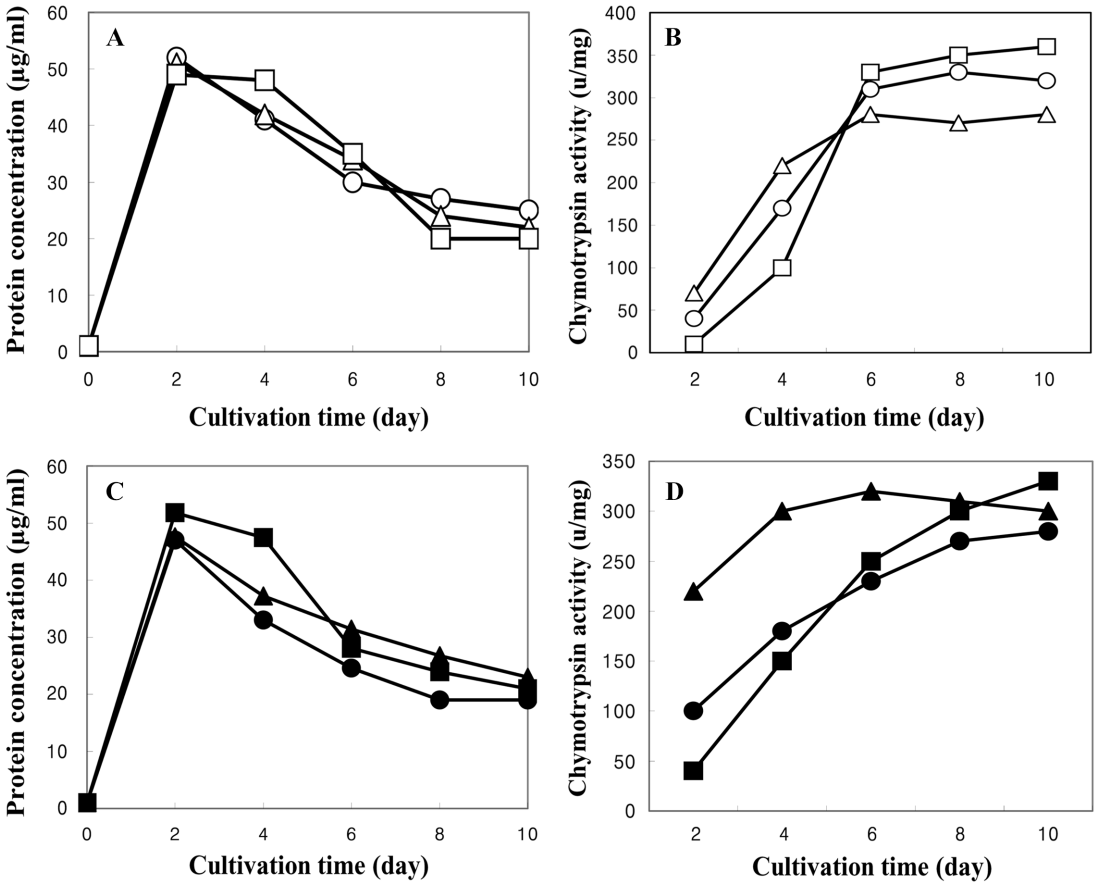

Fig. 2. Comparison of the growth curve and chymotrypsin activity, produced by each transformant of *S. griseus* HH1 (A, B) and IFO 13350 (C, D) in R2YE medium.

(A, C) The concentration of cellular protein in the transformants was measured using the Bradford method after the cells were disrupted by sonication, and expressed in mg/ml. (B, D) The chymotrypsin activity of the cultural filtrate prepared from the transformant was expressed in unit/mg of cellular protein, as described in Materials and Methods. □—□, HH1 control with pWHM3; △—△, HH1 transformant with pWHM3-A; ○—○, HH1 transformant with pWHM3-B; ■—■, IFO 13350 control with pWHM3; ▲—▲, IFO 13350 transformant with pWHM3-A; ●—●, IFO 13350 transformant with pWHM3-B.

The above data indicate that the *sprT* gene could be successfully overexpressed in all the streptomycetes strains tested, thus prompting us to construct a coexpression system for *sprA* and *sprB* genes by using the *sprT* promoter. For the construction of the coexpression system, the *sprA* and *sprB* genes respectively were amplified with its ribosome-binding site by polymerase chain reaction (PCR) [3]. The primers with appropriate restriction enzyme sites are listed in Table 1. The amplified *sprA* and *sprB* genes were connected to the downstream of the entire *sprT* gene, which resulted in pWHM3-TA and pWHM3-TB, respectively, as depicted in Fig. 1. At first, two plasmids were respectively introduced into *S. lividans* TK24, and the chymotrypsin and trypsin activities were measured.

After the cultivation for 9 days, the trypsin activities of the transformants harboring either pWHM3-TA or pWHM3-TB were 3.5 and 2.5 times higher than those of the transformant harboring pWHM3 (Fig. 5A). This result suggests that the constructs are operating well to express the *sprT* gene. The chymotrypsin activities of the transformants, harboring either pWHM3-TA or pWHM3-A, were almost the same, but much higher than those of the transformant with pWHM3 (Fig. 5B). Surprisingly, the chymotrypsin activities of the transformants, harboring either pWHM3-B or pWHM3-TB, were 7.1 and 6 times higher than those of the transformant with pWHM3 (Fig. 5B). In addition, the transformant with either pWHM3-A or pWHM3-B showed much higher chymotrypsin activity than those of pWHM3-TA or pWHM3-TB, possibly reflecting the polar effect of the linked gene. These data indicate that at least the pWHM3-TB was correctly constructed for successful cotranscription and translation of chymotrypsin and trypsin by using the promoter of *sprT* in *S. lividans* TK24.

Next, the coexpression vectors, pWHM3-TA and pWHM3-TB, were respectively introduced into *S. griseus* IFO 13350. As shown in Fig. 6A, the trypsin level increased sharply by more than 4 to 5 times than that of the control (transformant with pWHM3) after cultivation for 10 days.

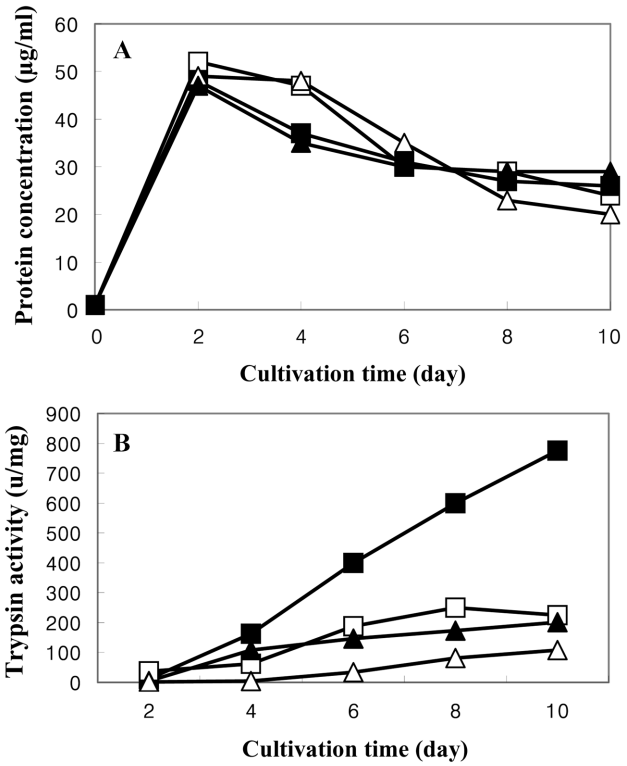

Fig. 3. Comparison of the growth curve (A) and trypsin activity (B), produced by each transformant of *S. griseus* HH1 and IFO 13350 in R2YE medium.

(A) The concentration of cellular protein in the transformants was measured using the Bradford method after the cells were disrupted by sonication, and expressed in mg/ml. (B) The trypsin activity of the cultural filtrate prepared from the transformant was expressed in unit/mg of cellular protein, as described in Materials and Methods. □—□, IFO 13350 control with pWHM3; △—△, HH1 control with pWHM3; ■—■, IFO 13350 with pWHM3-T; ▲—▲, HH1 with pWHM3-T.

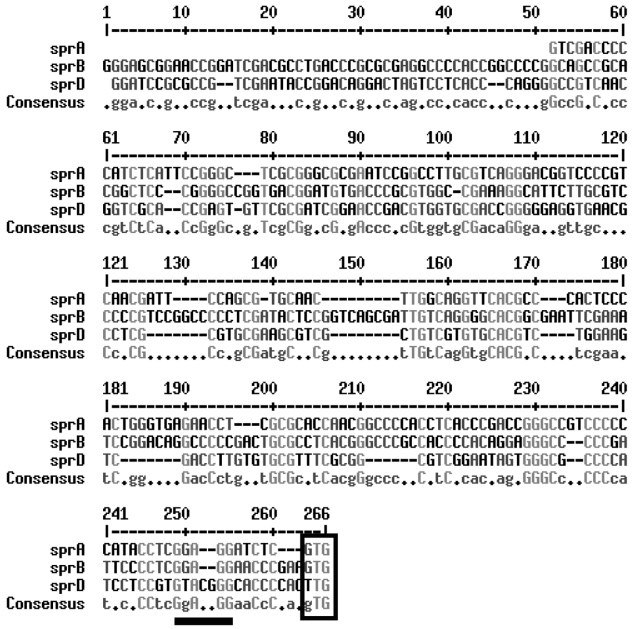

Fig. 4. Comparison of promoter regions of three chymotrypsin genes, *sprA*, *sprB*, and *sprD*.

The translational start codons are indicated by the box and the putative ribosomal biding sites are represented by the thick underline.

However, the chymotrypsin level did not show any significant difference among all the transformants after cultivation for 6 days, even though a big difference was observed in the earlier stage of the growth (Fig. 6B).

These observations imply that the overexpression of *sprA* and *sprB* cannot be attained in *S. griseus*, in contrast to *S. lividans*, by increasing the copy number of the gene and by replacing the promoter region. This fact strongly suggests that there are some special devices regulating the level of SGPA and SGPB in *S. griseus*. One possibility is the transcriptional or posttranscriptional regulation by some factor(s), and the other possibility is the production of a protease inhibitor that is specific for chymotrypsin, but not for trypsin. The posttranslational modification of the expressed chymotrypsins, SGPA and SGPB, into inactive ones, or the limited processing of premature forms of SGPA and SGPB into active forms, can also be considered.

Recently, some of the present authors studied the proteases produced by *S. griseus* IFO13350 and *S. griseus* HH1 [12], showing higher degree of 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* IFO13350 was greatly enhanced in the later stage of growth, which coincides with our data. Interestingly, the formation of aerial mycelium and spores was delayed by the treatment of the serine protease inhibitor, pefabloc SC, and the metalloprotease inhibitor, EDTA, in *S. griseus* IFO13350 [12]. These observations suggest that certain proteases may be very important for differentiation, and that

Table 1. Primers used in this study.

Genes	Forward	Reverse
sprT	atcgcccgcc***gaattc***gcaacatct---(219 nts)---**GTG**	acgctc**TGA**cggca***ggtacc***ggca
sprA	tccccc***ggtacc***tcggaggatctc**GTG**	**TGA**gcgcctacggggcaacggtcctgtagccggtgccaccg***aagctt***cgggct
sprB	ggcccccc***ggtacc***cctcggaggaacccgaa**GTG**	**TGA**ccggccccgccccggtcgggtacgga***gcatgc***cgtacaaacgt

Only the underlined sequences were used for primers, and the restriction enzyme sites used for the cloning are represented in italicized bold letters. Start codons (GTG) and stop codons (TGA) are depicted in the capital letters to show the relative positions.

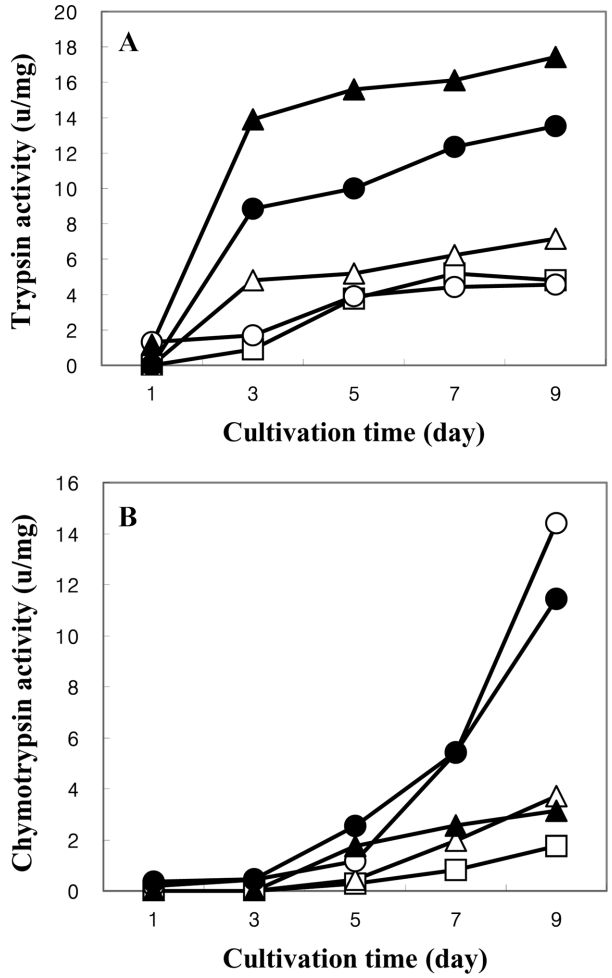

Fig. 5. Comparison of the trypsin activity (A) and chymotrypsin activity (B) produced by each transformant of *S. lividans* in R2YE medium.
(A) The trypsin activity of the cultural filtrate prepared from the transformant was expressed in unit/mg of cellular protein. (B) The chymotrypsin activity of the cultural filtrate prepared from the transformant was also expressed in unit/mg of cellular protein, as described in Materials and Methods. □—□, Control with pWHM3; △—△, transformant with pWHM3-A; ○—○, transformant with pWHM3-B; ▲—▲, transformant with pWHM3-TA; ●—●, transformant with pWHM3-TB.

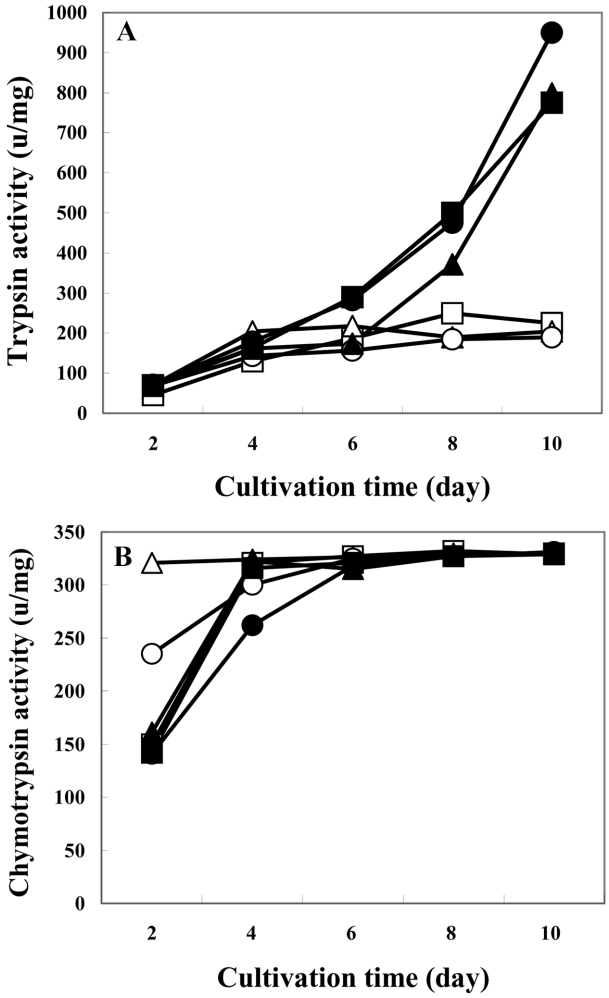

Fig. 6. Comparison of the trypsin activity (A) and chymotrypsin activity (B) produced by each transformant of *S. griseus* in R2YE medium.
(A) The trypsin activity of the cultural filtrate prepared from the transformant was expressed in unit/mg of cellular protein. (B) The chymotrypsin activity of the cultural filtrate prepared from the transformant was also expressed in unit/mg of cellular protein, as described in Materials and Methods. □—□, Control with pWHM3; △—△, transformant with pWHM3-A; ○—○, transformant with pWHM3-B; ▲—▲, transformant with pWHM3-TA; ●—●, transformant with pWHM3-TB; ■—■, transformant with pWHM3-T.

their expression may be precisely regulated in *Streptomyces*. Therefore, studies on the regulatory cascade of the protease production are expected to be very helpful to understand the cellular function of proteases.

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