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Purification and Characterization of *Streptomyces griseus* Trypsin Overexpressed in *Streptomyces lividans*

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Abstract *Streptomyces griseus* trypsin (SGT) is an extracellular proteinase produced by *S. griseus*. The *sprT* gene, which encodes premature SGT protein, was cloned into the plasmid pWHM3, a *Streptomyces–E. coli* shuttle vector. When the recombinant plasmid was introduced into *Streptomyces lividans* TK24, two proteins with molecular weights of 28 kDa and 42 kDa were detected. The 28-kDa protein was a SGT protein while the larger 42-kDa protein is thought to have been a premature form of the SGT protein. The SGT protein was purified to homogeneity via ammonium sulfate fractionation and many column chromatographies, including CM-sepharose chromatography, Mono-S chromatography, and Superose-12 chromatography, from the culture broth of *S. lividans* TK24 harboring the *sprT* gene. The N-terminal amino acid sequence, isoelectric points, and stabilities at various conditions of the SGT proteins purified from the Pronase and transformant were almost identical. The amount of the expressed SGT in *S. lividans* TK 24 was determined to be 5 times more than that of *S. griseus* based on the enzymatic activity against artificial substrate.

Key words: Purification, characterization, *sprT*, *Streptomyces griseus* trypsin (SGT), *Streptomyces lividans*

Proteinases of microbial origin are of considerable commercial value as a source of free or immobilized enzymes for use in food, pharmaceutical, and tanning industries or as additives to household detergents [30]. They are also applied in laboratory practices in the structural determination of protein-composed macromolecules or in removing proteinaceous material during the purification of certain biopreparations [21]. Actinomycete proteolytic complexes provide an excellent source of proteinases of various substrate specificities [6]. Of all the actinomycete proteinase complexes available, the most attention has given to Pronase obtained from the culture broth of a *S. griseus* strain [2, 29]. Pronase is a mixture of several proteinases and peptidases, ten of which have been purified to homogeneity and characterized [19, 26, 31].

Streptomyces griseus trypsin (SGT) is a bacterial serine proteinase that is more similar to mammalian proteinase than to *S. griseus* protease A and B, two other serine proteinases from the same bacterium [9, 22]. The general similarity of the substrate binding regions of SGT and bovine trypsin (BT) is consistent with the similarity of their interactions with substrates and inhibitors. SGT cleaves the oxidized B chain of insulin in the same manner as BT, as well as the synthetic substrate *N*-α-benzoyl-L-arginine ethyl ester [20]. The *sprT* gene (GenBank accession No. M64471) encoding *S. griseus* trypsin has been isolated from a *S. griseus* genomic library [13]. The amino acid sequence from its nucleotide sequence suggests that *S. griseus* trypsin is produced as a precursor consisting of three portions; an amino-terminal presequence (32 amino acid residues), a prosequence (4 residues), and the mature trypsin consisting of 223 amino acids with a molecular weight of 23 kDa.

In this study, we overexpressed the *sprT* gene of *S. griseus* in *S. lividans*, something that was not successful in *E. coli*. The purified SGTs from Pronase and *S. lividans* carrying the *sprT* gene were compared with respect to their enzymatic properties.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

S. lividans 1326 and TK24 were obtained from the John Innes Institute, United Kingdom [11]. Both strains have low levels of milk protein-hydrolyzing proteolytic

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Table 1. Compilation of plasmids used.

Plasmid	Marker	Features relevant to this study (size)	Reference
pUC19	Amp	*E. coli* multipurpose vector (2.9 kb)	[28]
pG4173	Amp	pUC19 derivative containing the 6.2-kb *Eco*RI/*Hin*dIII DNA fragment harboring *sprT* gene (8.9 kb)	[13]
pTrc99A	Amp	A derivative of pKK233-2 expression vector (4.1 kb)	[1]
ptrΔT	Amp	pTrc99A derivative containg 800 bp *Nco*I DNA fragment harboring a part of *sprT* gene (4.9 kb)	This study
pWHM3	Amp, Tsr	*Streptomyces–E. coli* shuttle vector which is high copy number in *Streptomycete* (7.2 kb)	[27]
pWHM-T	Amp, Tsr	pWHM3 derivative containing 2.6-kb *Eco*RI/*Hin*dIII DNA fragment harboring *sprT* gene (9.8 kb)	This study

Abbreviations: Amp, ampicilline resistance; Tsr, thiostreptone resistance.

activity. *S. griseus* 10137 was obtained from the American Type Culture Collection. *E. coli* strains JM109 and DH5α were 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 JM109 and DH5α were maintained on M9 minimal agar and cultured in LB medium at 37°C with agitation [17]. *Streptomyces* strains were maintained on R2YE agar and the *S. lividans* TK24 grown in R2YE liquid broth at 28°C was used for the preparation of protoplasts and the isolation of plasmid DNAs [11]. C5/L medium (in grams per liter, Glucose, 15.6; Yeast extract, 6.25; $CaCO_3$, 10; NaCl, 6.6 [pH 7.3]) was used for production of SGT. For the purification of SGT, a 7-l Jar fermenter (Korea Fermenter Co.) was used at an aeration rate of 1 vvm and an agitation speed of 250 rpm. The working volume of the fermenter was 5 l. The temperature and pH were controlled at 28°C and 7.0, respectively.

Enzymes and Chemicals

Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs, Inc., and Pronase® (lot No. 055291) was purchased from Calbiochem, Los Angeles, U.S.A.. Other fine chemicals were from Sigma Chemical Co. DEAE-Sepharose, CM-Sepharose, Sephadex G-200 and the Phast system were purchased from Pharmacia (Uppsala, Sweden).

DNA Manipulations

DNA preparation and manipulation was performed by the methods of Maniatis *et al.* [17] for *E. coli* and *Streptomyces* [11]. 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 TAE buffer system [17].

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 Hananhan [8].

Protoplasts of *S. lividans* TK24 were prepared as described by Hong *et al.* [10]. 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 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 as described by Hong *et al.* [10] 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 h, 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.

Quantitation and SDS-PAGE Analysis of Protein

Protein concentration was determined by Bradford's method [5] with 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 [14]. Protein samples were mixed with an appropriate volume of 5× sample buffer and boiled for 3 min before loading onto the gels. After running at 40 mA, the gels were stained with Coomassie Brilliant Blue G-250.

Subcloning of the *sprT* Gene into pWHM3

The overall scheme for the construction of the recombinant plasmid pWHM-T is illustrated in Fig. 1. pG4173, which contains the entire *sprT* gene, was digested with *Eco*RI/*Bgl*II and ptrΔT with *Bgl*II/*Hin*dIII. A 2.2-kb *Eco*RI/

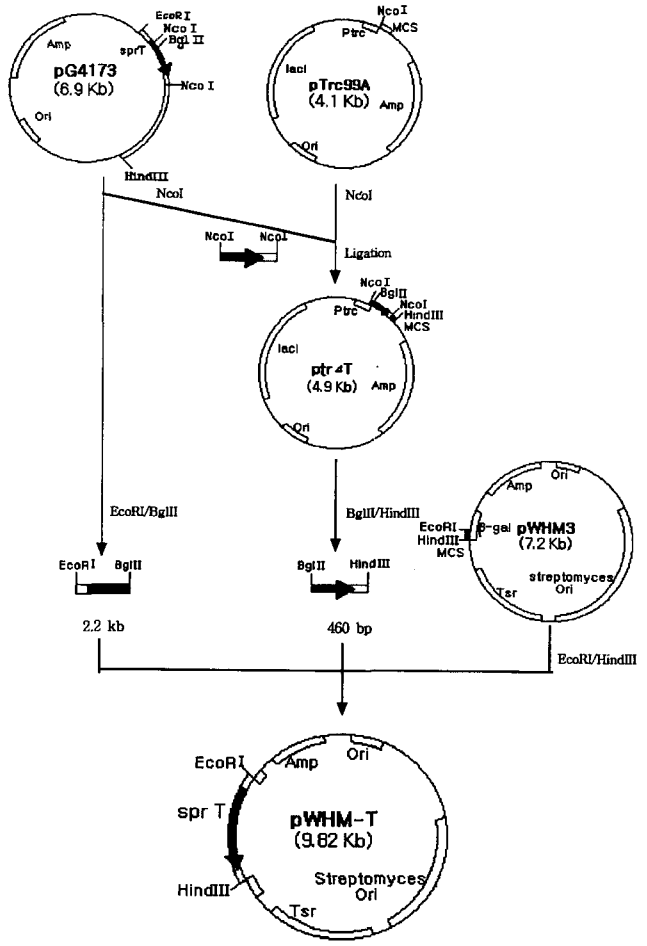

Fig. 1. Construction of expression vector pWHM-T which is derived from pWHM3, an *E. coli–Streptomyces* shuttle vector, with an insert of the entire *sprT* gene at *Eco*RI and *Hin*dIII sites.

The fragment of the *sprT* gene was obtained from pG4173 and ptrΔT as described in Materials and Methods for convenience of entire *sprT* cloning.

*Bgl*II DNA fragment from pG4173 and a 460-bp *Bgl*II/*Hin*dIII DNA fragment from ptrΔT were then subcloned into the *Eco*RI/*Hin*dIII site of pWHM3, a *Streptomyces–E. coli* shuttle vector, by 3-fragment ligation and transformed into *E. coli* DH5α. The resulting recombinant plasmid, pWHM-T, was purified from *E. coli* and used for transformation into *S. lividans* TK24.

Determination of Trypsin Activity

Trypsin activity was measured spectrophotometrically by the release of p-nitroaniline due to the enzymatic hydrolysis of an artificial chromogenic substrate, *N*-α-benzoyl-DL-arginine-p-nitroanilide. The assay reaction mixture, which was composed of 890 μl of 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 was prewarmed for 5 min at 37°C, rapidly mixed with 100 μl of 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 were recorded. One unit of trypsin was defined as the amount of enzyme corresponding to 0.1 increase in absorbance at the above conditions.

Purification of SGT from Pronase

Several column chromatography steps were carried out to purify the SGT from the Pronase. All operations were performed at 4°C, while FPLC operations including Mono-S and Superose-12 column chromatography were performed at room temperatures.

DEAE- and CM-Sepharose column chromatography. The commercially purchased pronase was dissolved in Buffer A (10 mM Na-acetate [pH 5.0], 10% glycerol, 1 mM EDTA) to a final concentration of 0.5 g/ml and then applied on a DEAE-sepharose column (3×20 cm) equilibrated with buffer A. The sample which passed through the DEAE-sepharose column was charged onto a CM-sepharose column (3.5×16 cm) equilibrated with buffer A. The column was washed with the same buffer and then eluted with a stepwise gradient of 50 mM to 500 mM KCl in buffer A at a flow rate of 2.5 ml/min. Fractions were collected in 10 ml volumes. The active fractions were pooled and used for further purification steps.

Sephadex G-200 gel filtration column chromatography. The sample was applied on a Sephadex G-200 column (3.5×60 cm) equilibrated with buffer A and eluted with the same buffer at a flow rate of 7 ml/h. The fractions containing trypsin activity were pooled and used for further purification steps.

Mono-S ion exchange column chromatography. The dialyzed sample was charged onto a Mono-S ion exchange column previously equilibrated with buffer A and eluted at a flow rate of 1 ml/min with a KCl gradient (0 to 1 M) in buffer A. The fractions containing SGT activity were combined and concentrated by Centricon® for the next step.

Superose-12 gel permeation column chromatography. The sample was applied on a Superose-12 column equilibrated with buffer A. Protein was eluted with the same buffer at a flow rate of 0.1 ml/min and the protein elution profile was monitored by measuring absorbance at 280 nm. The trypsin active fractions were detected by assaying the activity.

Mono-S ion exchange column chromatography. The sample was loaded on a Mono-S column equilibrated with buffer A (pH 5.0) and eluted with 10 mM Tris-Cl (pH 8.0) with the Pharmacia FPLC system. Purified SGT was pooled and stored at -70°C.

Purification of SGT from *S. lividans* TK24 Transformant

S. lividans TK24 transformant harboring the pWHM-T recombinant plasmid was grown in the C5/L medium

(5 l) at 28°C for 4 days under aerobic conditions. The culture broth was centrifuged at 6000×g for 15 min to separate cells and supernatant. To the supernatant, ammonium sulfate was added to 25% saturation to precipitate unwanted proteins. After centrifugation, ammonium sulfate was added to the supernatant to reach 55% saturation. The precipitate was collected by centrifugation and dissolved in small quantities of buffer A. The solution was dialyzed against buffer A for 12 hours with several changes of buffer. The purification procedure of SGT from the ammonium sulfate fractionated sample followed the same steps used in the purification from Pronase.

N-terminal Sequencing of SGT

The purified SGT was separated on SDS-polyacrylamide gels and transferred onto nitrocellulose paper by electrotransfer of gel at 0.65 mA/cm^2 for 2 h in transfer buffer (0.1% Tris, 1.44% gycine, 20% methanol). The N-terminal analysis of blotted protein was performed at the Korea Basic Science Institute.

Isoelectric Focusing and Stability of SGT

The isoelectric point (pI) of SGT was performed through the Pharmacia Phast system and was estimated by comparison with standard proteins. To study the enzymatic properties of the SGT, the activity of the purified SGT from the transformant and Pronase toward BAPNA was measured at various pHs ranging from 3 to 11.5 at 37°C. To evaluate the optimum temperature for enzyme reaction, the enzyme activity was measured at varying temperatures ranging from 4°C to 90°C at pH 8.0.

Results

Purification of SGT from Pronase

The SGT was purified from the Pronase powder through various purification steps as described in Materials and Methods. The elution profiles of each chromatographic step were omitted. When the sample was applied on Mono-S column and eluted with KCl solution, other fractions which did not contain the SGT showed stronger activity than that of SGT-containing fractions toward the artificial substrate, BAPNA (data not shown). It was calculated that more than 60% of the total activity in Pronase powder should be non-SGT associated activity. Therefore, the real content of SGT in Pronase will be lower than the amount estimated from the BAPNA assay.

The active fraction of each purification step was analyzed by SDS-PAGE (Fig. 2). The purification yield is summarized in Table 2. The molecular weight of the purified SGT was estimated to be about 28 kDa in SDS-PAGE, which was larger than the size calculated from the amino acid sequence. The purified SGT was degraded during storage and minor smaller protein bands were detected in SDS-PAGE (Fig. 2, lane 6). The final recovery yield of SGT was 2.6%, with specific activity increased by 25 fold (Table 2).

Expression of the *sprT* Gene in *S. lividans* TK24

The transformant of *S. lividans* TK24 haboring pWHM-T was cultured in C5/L medium designed for production of SGT. When the enzyme activity was measured with BAPNA, the activity of the transformant was 5 times higher than that of *S. griseus* (Fig. 3). Most of the enzyme activity was present in culture broth, but very little was present in cell-extract. The transformant was

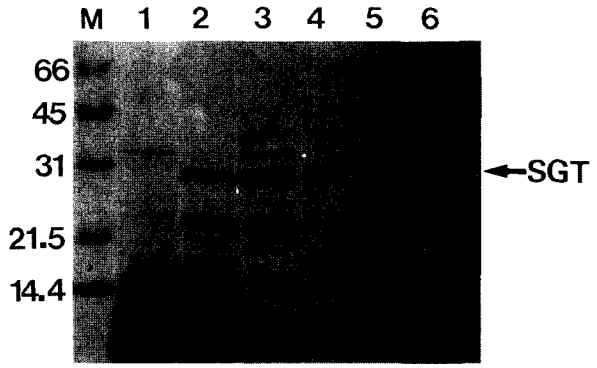

Fig. 2. Analysis of purified SGT from Pronase by SDS-polyacrylamide gel electrophoresis.

Samples during purification were separated on 15% gel and stained with Coomassie Brilliant Blue. Lane M, molecular weight standards; lane 1, Pronase solution; lane 2, CM-Sepharose eluate; lane 3, Sephadex G-200 eluate; lane 4, Mono-S eluate with KCl; lane 5, Superose-12 eluate; lane 6, Mono-S eluate by pH difference. The SGT protein is depicted by a thick arrow.

Table 2. Summary of purification steps of SGT from pronase.

Purification step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Yield (%)	Purification folds
Pronase solution	681.5	1710207	2509	100.0	1.0
CM-Sepharose	49.5	538350	10876	31.5	4.3
Sephadex G-200	30.9	375972	12167	22.0	4.8
Mono-S (KCl elution)	9.0	117855	13095	6.9	5.2
Superose 12 HR 10/30	2.7	52962	19616	3.1	7.8
Mono-S (pH elution)	0.72	45124	62672	2.6	25.0

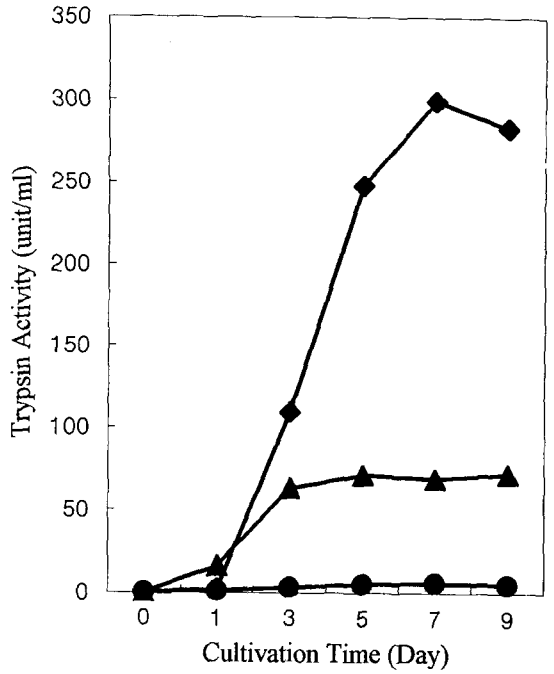

Fig. 3. Production of trypsin-like protease by *S. griseus* ATCC10137 and *S. lividans* TK24 transformants cultivated in C5/L medium.

Each strain was cultured in 100 ml of C5/L broth in a 500 ml of baffled flask at 28°C with vigorous shaking at 250 rpm. 5 ml of culture broth was sampled everyday and used for measurement of trypsin activity after centrifugation as described in Material and Methods. Because of the insoluble ingredient in C5/L medium, the enzyme activity was expressed in unit/ml of culture broth. ●—●, *S. lividans* TK24 carrying pWHM3; ■—■, *S. lividans* TK24 harboring pWHM-T; ▲—▲, *S. griseus* ATCC10137.

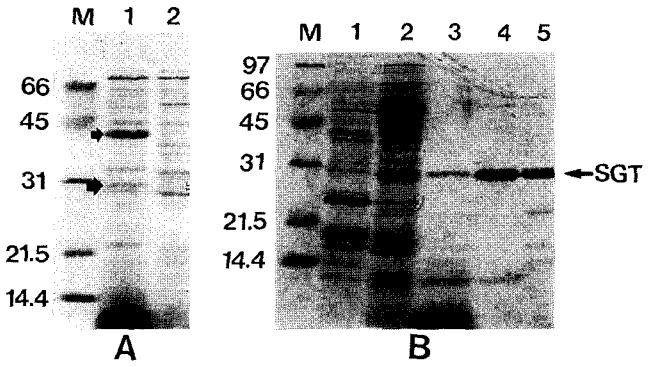

Fig. 4. Detection of expressed protein (A) and purification of SGT (B) from *S. lividans* harboring plasmid pWHM-T.

A. The extracellular protein in culture broth of *S. lividans* was precipitated with trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis. Lane M, molecular weight standards; lane 1, sample from *S. lividans* TK24 carrying pWHM-T; lane 2, sample from *S. lividans* TK24 carrying pWHM3 as control. The SGT protein with a molecular weight of 28 kDa is indicated by a large arrow and the larger protein of 42 kDa is represented by a small arrow in lane 1. **B.** Samples during each purification step were separated on 15% gel and stained with Coomassie Brilliant Blue. Lane M, molecular weight standards; lane 1, total extracellular protein; lane 2, ammonium sulfate fractionation; lane 3, CM-Sepharose eluate; lane 4, Mono-S eluate with KCl; lane 5, Superose-12 eluate. The SGT protein is depicted by a thick arrow.

cultured in R2YE medium and the relationship between cell growth and SGT activity was monitored at various times. In R2YE medium containing thiostrepton, SGT production started in the stationary phase of cell growth (data not shown).

The extracellular protein of the *S. lividans* TK24 transformant was analyzed on 15% SDS-PAGE, and the SGT protein which has a molecular weight of 28 kDa was confirmed (Fig. 4A, lane 1). Besides the SGT, another protein with a molecular weight of 42 kDa was produced in a large amount. This protein is thought to have been a premature form of SGT. On the basis of the densitometric analysis of the gel, the SGT protein expressed in *S. lividans* TK24 was estimated to be 14% of the total extracellular protein.

Purification of SGT from *S. lividans* TK24 Transformant

S. lividans TK24 transformants harboring pWHM-T were grown at 28°C for 5 days in 5 l of C5/L medium containing thiostrepton. SGT was purified to homogeneity *via* a series of purification steps as described.

The elution profile in each step was omitted too. The active fraction of each purification step was analyzed by SDS-PAGE (Fig. 4B), and some degraded proteins of SGT were detected from the purified SGT (Fig. 4B, lane 5). The overall yield of the purification procedure is summarized in Table 3. The final recovery yield of SGT was 6.3%, with specific activity increased by 221 fold.

Table 3. Summary of purification steps of SGT from *S. lividans* TK24 carrying pWHM-T.

Purification step	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Yield (%)	Purification folds
Supernatant	4701.0	664877	141	100.0	1.0
25%-55% Ammonium sulfate	508.0	276489	544	41.6	3.9
CM-Sepharose	8.9	97485	10953	14.7	77.7
Mono-S (KCl elution)	2.8	55135	19691	8.3	139.7
Superose 12 HR 10/30	1.35	42059	31155	6.3	

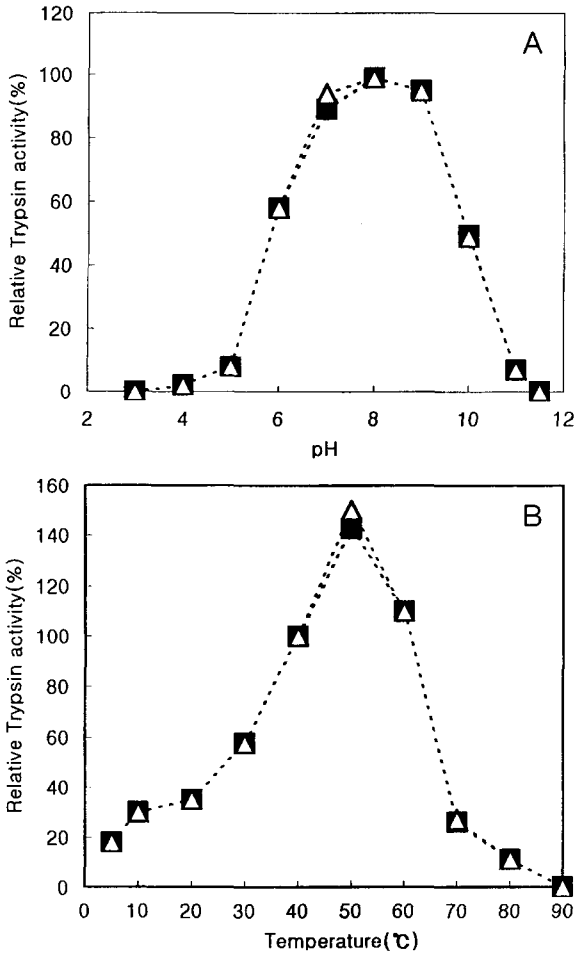

Fig. 5. Effects of pH and temperature on the SGT activity.
The enzyme activity of the SGT purified from Pronase and transformant was measured with the artificial substrate, BAPNA, at various pHs ranging from 3 to 11.5 at 37°C (A) and at varying temperatures ranging from 4°C to 90°C at pH 8.0 (B). ■---■, SGT from *S. lividans*; △---△, SGT from Pronase.

Characterization of SGT

There was no difference between the isoelectric points of the SGT, estimated to be below 4.65, purified from Pronase and the *S. lividans* transformant (data not shown). The pH and temperature for maximum activity of SGT were pH 8.0 and 50°C, respectively (Fig. 5). The amino acid sequence of SGT purified from the *S. lividans* TK24 transformant was determined as Val-Val-Gly-Gly-Thr, which was identical to the sequence purified from Pronase [20].

Discussion

SGT is a bacterial serine proteinase that is more similar to mammalian proteinase such as trypsin than to other bacterial proteinases such as *S. griseus* protease A and B [24, 25]. The *sprT* gene had previously been cloned from a *S. griseus* genomic library [13]. The base composition of the *sprT* coding region is 72.4% G+C. When the *sprT* gene was linked and introduced to pTrc99A expression vector in *E. coli* [1], we could not detect the expressed protein (data not shown). The main reason for this may have been due to the extremely biased usage of synonymous codons with 96.5% of *sprT* codons possessing G or C in the third position or a large number of rare codons in the coding sequence of the *sprT* gene [4, 16].

The low content of SGT in Pronase made it difficult to purify SGT. However, the subcloning of *sprT* into pWHM3 has resulted in the overexpression of SGT in *S. lividans*, which made it convenient to purify SGT. With the same purification procedure, the specific activity after the final purification step increased 221 fold in the case of overexpressed sample, but only 25 fold in the case of Pronase. This fact emphasizes the importance of the starting material in the purification process. The molecular weight of SGT was estimated to be about 28 kDa in SDS-PAGE, which was larger than the calculated value. Another expressed protein with a molecular weight of 42 kDa was also detected. This larger protein is thought to have been a premature form of SGT. From the nucleotide sequence, it was expected that the premature form of SGT has additional 36 amino acids for processing during secretion. If the larger protein of 42 kDa is indeed a premature form of SGT, however, the translational start codon should be the 250th GTG coding for valine in the reported nucleotide sequence and the SGT protein will have an additional 130 amino acids [13]. Most of extracellular proteases have been found to be synthesized as precursor forms, which are then processed to the mature forms of the proteases [30]. The precursor form of SGT has not been discovered. Therefore, Western blot analysis should be performed to determine whether this larger protein is another form of SGT.

When the trypsin activity of Pronase was measured using BAPNA as a substrate, a significant level of non-SGT associated trypsin activity was found. This result indicates that the real trypsin activity of SGT should be lower than that measured in Pronase and *S. griseus*. Therefore, it can be concluded that the transformant of *S. lividans* harboring pWHM-T produces more than two times the SGT calculated by BAPNA assay.

The N-terminal sequence of SGT purified from the transformant was the same as that of SGT in Pronase, which is the mature form. Therefore, it is expected that the N-terminal processing mechanisms of SGT in recombinant *S. lividans* TK24 and *S. griseus* will be similar. Measurements of enzyme activity in a range of pHs and temperatures indicated that SGT is heat-stable and alkaline-resistant.

SGT production was increased in the stationary phase of cell growth. *Streptomyces* produces many extracellular enzymes as well as secondary metabolites when the logarithmic growth ceases, and many complex regulatory cascades are involved in the control of morphological and physiological differentiation. Recently, it was reported that trypsin-like proteases play very important roles in the differentiation of the substrate mycelium to the aerial one, spore formation in *S. albogriseolus* [18], metabolism of the mycelial proteins at the late stationary phase of submerged cultures, and during aerial mycelium formation on surface cultures in *Streptomyces exfoliatus* [12]. When we obtained the transformant of *S. lividans* with pWHM-T, we found that the shape of the colony was severely changed into a volcanoform and only the outer area of the volcano could sporulate with white colors, in contrast to the smooth colony with gray colored spores of *S. lividans* TK24 (data not shown). This result implies that the expression of *sprT* can influence morphological differentiation even in heterologous hosts. Therefore, we plan to continue our study focussing on the real intracellular function of proteinase, especially on its morphological and physiological differentiation.

Acknowledgments

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