Trypsin-like protease of *Streptomyces exfoliatus* SMF13, a potential agent in mycelial differentiation

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***Streptomyces exfoliatus* SMF13 sequentially produced leupeptin, leupeptin-inactivating enzyme (LIE) and trypsin-like protease (TLP). TLP was produced upon exhaustion of glucose. Autolysis of mycelium was accompanied by an increase in TLP activity. However, in three *bld* mutants isolated from *S. exfoliatus* SMF13 after UV-mutagenesis, mycelium autolysis did not occur, and neither LIE nor TLP was produced, although leupeptin was produced. Production of both LIE and TLP was restored in a spontaneous *Spo*⁺ revertant of a *bld* mutant. In contrast, two *whi* mutants sequentially produced leupeptin, LIE and TLP. The molecular mass of TLP produced during morphological differentiation was estimated to be 31·8 kDa by SDS-PAGE. The N-terminal amino acid sequence was RVGGTxAAQGNFPFQQLSM. TLP was competitively inhibited by leupeptin; the inhibition constant was 0·015 µM. TLP effectively hydrolysed the mycelial protein extract of *S. exfoliatus* SMF13, but the hydrolytic activity was inhibited by leupeptin. It was concluded that morphological differentiation and production of TLP are coordinately regulated, that TLP may function as an enzyme in the metabolism of mycelial proteins, and that the hydrolytic activity of TLP is regulated by autogenous leupeptin in *S. exfoliatus* SMF13.**

Keywords: *Streptomyces exfoliatus*, trypsin-like protease, leupeptin, regulation, morphological differentiation

INTRODUCTION

Streptomycetes are Gram-positive bacteria with an unusual morphological complexity. The growth of *Streptomyces* on solid media progresses as a sequential formation of substrate mycelium and aerial hyphae (Wildermath, 1970). The aerial hyphae appear to grow, at least partially, by the utilization of degraded substrate mycelium (Mendez *et al.*, 1985; Miguelez *et al.*, 1994), because the aerial mycelium has little access to other sources of nourishment (Chater, 1984). Little has been learned of the mechanism providing nutrients for aerial mycelium formation.

Streptomycetes produce a variety of extracellular

proteases (Peczynska-Czoch & Mordarski, 1988) as well as protease inhibitors (Aoyagi, 1989). The secretion of extracellular proteolytic enzymes in streptomycetes often temporally coincides with the onset of secondary metabolism or morphological differentiation (Ginther, 1979; Gibb & Strohl, 1988; Bascaran *et al.*, 1990).

We (Kim & Lee, 1995) have shown that *Streptomyces exfoliatus* SMF13 sequentially produces leupeptin, leupeptin-inactivating enzyme (LIE) and trypsin-like protease (TLP). Production of leupeptin was closely associated with mycelial growth, but it was inactivated by LIE when mycelial growth reached stationary phase in submerged cultures, or just before aerial mycelium was formed on surface cultures. TLP activity started to increase when leupeptin was nearly inactivated. The production of TLP correlated with a decline in biomass of submerged cultures, or with aerial mycelium formation on surface cultures. The activity of TLP was specifically inhibited by leupeptin. Autolysis of mycelium after stationary phase in submerged cultures was apparently

Abbreviations: BAPNA, *N*-benzoyl-arginine *p*-nitroanilide; BAEE, *N*-benzoyl-L-arginine ethyl ester; LIE, leupeptin-inactivating enzyme; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TLP, trypsin-like protease.

The EMBL accession number for the sequence reported in this paper is P80420.

retarded by addition of leupeptin; furthermore, aerial mycelium formation on surface cultures was retarded by addition of leupeptin (Kim & Lee, 1995).

Various types of mutants of *Streptomyces* altered in the life cycle have been isolated. One type of developmental mutant, designated *bld* due to its 'bald' phenotype, is defective in aerial mycelium formation, and is subsequently impaired in the formation of spores (Champness, 1988). Another type of developmental mutant, designated *whi*, produces white aerial mycelium but fails to form spores (Chater, 1984). Some *bld* mutants of *Streptomyces* are pleiotropically blocked in the biosynthesis of antibiotics and pigments (Chater, 1984; Martin & Demain, 1980).

To elucidate the biological roles of TLP during morphological differentiation of *S. exfoliatus* SMF13, we isolated *bld* mutants and *whi* mutants and characterized their TLP production. We also purified TLP from the culture broth and determined its enzymic and other biochemical properties.

METHODS

Micro-organisms and strain maintenance. The parent micro-organism was *S. exfoliatus* SMF13 (Kim *et al.*, 1993; Kim & Lee, 1995). Mutants were obtained by irradiating spores of *S. exfoliatus* SMF13 with UV light (254 nm) to give survival ratios of 0.1–1%. The irradiated spores were then plated on stock culture medium and incubated for 7 d at 28 °C. Colonies were visually screened for *bld* and *whi* mutants (Hopwood *et al.*, 1985). As candidates, flat colonies lacking aerial mycelium formation and fluffy whitish colonies lacking spore formation were subcultured three times. Three stable *bld* mutants (SMF13B1, SMF13B2 and SMF13B3) and two stable *whi* mutants (SMF13W1 and SMF13W2) were finally picked. A spontaneous Spo^+ revertant (SMF13R1) to the wild-type phenotype was obtained from *bld* mutant SMF13B1 after storage for several weeks at 4 °C. Strains were transferred to slopes of stock culture medium each month, and stored at 4 °C.

Media and culture conditions. Stock culture medium (Bennett medium) consisted of (% w/v): glucose, 1; casein hydrolysate, 0.2; yeast extract, 0.1; beef extract, 0.1; and agar, 1.8. Seed culture medium consisted of (% w/v): glucose, 3.0; soytone, 1.8; peptone, 0.3; and CaCO_3 , 0.4. The main culture medium (GCPS medium) consisted of (% w/v): glucose, 0.5; sodium caseinate, 1; KH_2PO_4 , 0.025; K_2HPO_4 , 0.085; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03; NaCl , 0.03; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.001; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.001; and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0003. The phosphate and salts were separately sterilized by filtration (0.2 μm , Millipore membrane). For surface culture, about 10^3 spores were inoculated evenly on GCPS agar medium, or spores from stock culture medium were transferred with sterile toothpicks to GCPS agar medium containing increasing concentrations of glucose, and incubated at 28 °C. For submerged batch cultures, 50 ml seed culture medium in a 500 ml baffled flask was inoculated with a spore suspension to give 10^6 spores ml^{-1} and incubated at 28 °C for 36 h on a rotary shaker (200 r.p.m.). The seed culture was used to inoculate (5%, v/v) 3 l of the GCPS medium contained in a jar fermenter (5 l; Korea Fermentor) maintained at 28 °C; the initial pH was adjusted to 7.0. Agitation and aeration were 200 r.p.m. and $1 \text{ v v}^{-1} \text{ min}^{-1}$, respectively.

Scanning electron microscopy. Colonies developed on agar medium were fixed using the following procedures. Phosphate-

buffered glutaraldehyde solution (8%, v/v; pH 7.4) was poured into holes punched around colonies (Millonig, 1961). Plates were left for 24 h at 4 °C; colonies were cut out to the minimal size from the agar medium and then dried in a sealed plastic box under P_2O_5 at 4 °C. Dried colonies were gold-coated with a Polaron SC502 sputter coater (Fisons) at 15 mA for 1 min under vacuum. The morphology of colonies was observed with a Stereoscan 260 scanning electron microscope (Cambridge).

Analysis of growth, glucose and protein. Mycelium from submerged cultures was harvested aseptically by centrifugation at 12000 *g* for 15 min, washed twice with physiological saline solution and once with distilled water, then collected by vacuum filtration (Whatman filter paper GF/C) and dried at 80 °C for 24 h. The concentration of glucose was measured with dinitrosalicylic acid (Miller, 1959). Protein concentrations were determined by dye binding (Bradford, 1976); bovine serum albumin was used as the standard.

Assay of TLP, leupeptin and LIE. Broth of submerged cultures was centrifuged at 10000 *g* for 10 min and the activities of TLP, leupeptin and LIE in the supernatant were measured. An agar plug (5 × 5 cm) containing a lawn of mycelium was removed from the centre of each plate, or 50 agar plugs (diameter 1.2 cm) containing a colony were removed from each plate, homogenized in 10 ml Tris/HCl buffer (0.1 M, pH 7.5) and centrifuged (10000 *g* for 10 min). The activities of TLP, leupeptin and LIE in the supernatant were measured.

TLP activity was estimated by measuring the amount of *p*-nitroanilide liberated from the *N*-benzoyl-arginine *p*-nitroanilide (BAPNA). Enzyme reactions were carried out with 200 μmol BAPNA at 35 °C and pH 7.5 (Tris/HCl buffer, 0.1 M). Activity was calculated from the linear part of the curve, using $\epsilon_{405} = 9620 \text{ mol}^{-1} \text{ cm}^{-1}$. One unit of TLP activity was defined as the amount of enzyme needed to produce 1 μmol *p*-nitroanilide min^{-1} (Sarath *et al.*, 1989).

The activities of protease inhibitors were calculated as percentage inhibition = $100 \{ (A - B) / A \}$, where *A* is the protease activity without the inhibitor and *B* is the protease activity with the inhibitor (Aoyagi *et al.*, 1969). The concentration of leupeptin in culture broth was calculated from a standard curve prepared with authentic leupeptin and 80 μg papain as the target protease (Kim & Lee, 1995).

The activity of LIE was determined as follows. Mycelium-free culture broth (1.0 ml) was preincubated with 50 μg leupeptin at 4 °C and pH 7.5 (Tris/HCl buffer, 0.1 M) for 10 min to compensate for the possible interaction between leupeptin and TLP. The preincubated reaction mixture was incubated for 10 min at 35 °C, then heated for 5 min at 80 °C for complete inactivation of any protease and LIE in the reaction mixture. The remaining activity of leupeptin was assayed (*A*). In parallel, the preincubated reaction mixture was heated at 80 °C for 5 min to completely inactivate LIE, then incubated at 35 °C for 10 min. The remaining activity of leupeptin was assayed (*B*). The difference between *A* and *B* was defined as the leupeptin-inactivating activity. One unit of LIE was defined as the amount of enzyme needed for inactivation of 10 μg leupeptin min^{-1} (Kim & Lee, 1995).

Purification of trypsin-like protease. When TLP activity reached its maximum in a batch culture, the broth was harvested by centrifugation (12000 *g* for 15 min). TLP was purified from the cell-free broth by the following procedures at 4 °C. Fractions from the 45–60% ammonium sulfate saturation range were dissolved in distilled water and desalted by ultrafiltration. The desalted solution was adjusted to pH 3.6 with citrate buffer and kept for 30 min. After centrifugation for 20 min at 20000 *g*, the

pellet was discarded. The supernatant solution was desalted by ultrafiltration in 0.05 M Tris/HCl buffer (pH 7.5). The soluble retentate was applied to a column of DEAE-Sephadex A-50 (2.8 × 22 cm) equilibrated with 0.05 M Tris/HCl buffer (pH 7.5), and eluted with a linear gradient of NaCl (0–1 M) in the same buffer. The active fraction was not retained by the resin, and was eluted with the Tris/HCl buffer. It was concentrated by ultrafiltration in 0.005 M Na-phosphate buffer (pH 6.8) and applied to a column of hydroxyapatite (1.5 × 18 cm) equilibrated with 0.005 M Na-phosphate buffer (pH 6.8). After the column had been washed with 0.005 M MgCl₂, the active fraction was eluted with 1 M MgCl₂. It was concentrated and desalted by ultrafiltration in 0.05 M Na-citrate buffer (pH 4.0), then applied to a column of CM-Sephadex (2.8 × 22 cm) equilibrated with 0.05 M Na-citrate buffer (pH 4.0). The column was washed with 3 bed volumes of the same buffer, and TLP was then eluted with a linear gradient of NaCl (0–1 M) in the same buffer. TLP was collected in fractions containing about 0.5 M NaCl.

Molecular mass determination and substrate gel electrophoresis. The molecular mass of the purified TLP was estimated by SDS-PAGE (Laemmli, 1970); the concentration of the running gel was 12% (w/v) and that of the stacking gel was 5% (w/v). After electrophoresis, the gel was stained with Coomassie Blue.

TLP activity was visualized in polyacrylamide gels by incorporating 0.1% gelatin in a 10% (w/v) SDS-PAGE gel. TLP in the sample buffer was not boiled before being applied to the gel.

N-terminal amino acid sequencing. The N-terminal amino acids of TLP were sequenced by a modified phenylthiohydantoin method (Matsudaira, 1987). The similarity of the N-terminal amino acid sequence of TLP to other proteins was determined from the EMBL database using Clustal V software for multiple sequence alignment.

Substrate specificity and synthetic substrate hydrolysis. Hydrolytic activity for bovine albumin (Sigma), egg albumin (Sigma), Hammarsten casein (Merck), collagen (Sigma), elastin (Sigma), haemoglobin (Sigma) and lysozyme (Sigma) was estimated by measuring the concentration of tyrosine liberated from the substrate protein after 15 min reaction at 35 °C and pH 7.5 (Tris/HCl buffer, 0.1 M). One unit of hydrolytic activity was defined as the amount of enzyme needed to produce 1 µg tyrosine equivalent min⁻¹ (Narahashi, 1970).

The kinetic parameters for hydrolysis of aminoacyl *p*-nitroanilides were measured under the optimum reaction conditions; concentrations of the aminoacyl *p*-nitroanilides ranged from 2.5 to 1000 µM (Sarath *et al.*, 1989).

Esterase activity of TLP was estimated by measuring the hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE). Hydrolysis of BAEE was monitored at 253 nm under the same conditions as used for measuring BAPNA hydrolysis. Activity was calculated from the linear part of the curve, using $\epsilon_{253} = 1150 \text{ mol}^{-1} \text{ cm}^{-1}$ (Sarath *et al.*, 1989).

Degradation of mycelium protein. The mycelium harvested from the decline phase culture broth of *S. exfoliatus* SMF13 was washed three times with Tris/HCl buffer (pH 7.5, 0.1 M), disrupted by sonication (100 W, 5 min) at 4 °C, then centrifuged at 20000 *g* for 30 min. The supernatant was used as the mycelium protein extract. TLP (5 µg) dissolved in 200 µl Tris/HCl buffer (pH 7.5, 0.1 M) was preincubated at 35 °C for 5 min with or without 10 µg leupeptin. Then 2.8 mg mycelium protein dissolved in 500 µl Tris/HCl buffer was added, and the

reaction mixture was incubated at 35 °C. The hydrolysis of mycelium proteins was measured by SDS-PAGE as a function of time.

Analysis of fermentation kinetic parameters. Specific growth rate (μ), specific mycelium degradation rate (k_d), specific glucose uptake rate (q_s), specific leupeptin production rate (q_{Lpt}) and specific LIE production rate (q_{LIE}) in batch cultures were analysed as follows (Pirt, 1975):

$$\mu = \ln(x_2/x_1)/(t_2 - t_1)$$

and

$$k_d = -\ln(x_2/x_1)/(t_2 - t_1),$$

where x and t are dry cell weight (g l⁻¹) and time (h), respectively;

$$q_s = (ds/dt)/x,$$

where ds/dt are changes in concentration of glucose during infinitesimal time (g l⁻¹ h⁻¹);

$$q_{\text{Lpt}} = (d_{\text{Lpt}}/dt)/x,$$

where d_{Lpt}/dt is the increase in the concentration of leupeptin during infinitesimal time (g l⁻¹ h⁻¹);

$$q_{\text{LIE}} = (d_{\text{LIE}}/dt)/x,$$

where d_{LIE}/dt is the increase in the activity of LIE during infinitesimal time (U l⁻¹ h⁻¹).

RESULTS

Isolation and morphological characterization of *bld* and *whi* mutants from *S. exfoliatus* SMF13

Three *bld* and *whi* mutants were isolated from 10000 colonies of UV-treated spores of *S. exfoliatus* SMF13. The surface of the parent strain growing on Bennett agar or GCPS agar was tough and leathery. However, the surface of *bld* mutants was flat after 14 d growth and that of *whi* mutants was fluffy and whitish.

Scanning electron micrographs showed that the surface of colonies of the parent strain on GCPS agar consisted primarily of long rectiflexible spore chains, and that the surface of spores was smooth (Fig. 1a). However, the colony surface of *bld* mutants consisted of substrate hyphae embedded in agar medium, and lacked aerial mycelium and spores (Fig. 1b). The colony surface of *whi* mutants consisted of mycelia projecting upwards into the air (Fig. 1c).

The parent strain produced brown pigment in association with aerial mycelium formation; however, none of the *bld* mutants formed soluble pigments until 14 d growth. A spontaneous revertant to the wild-type phenotype obtained from *bld* mutant SMF13B1 during storage for several weeks at 4 °C sporulated like the parent strain and also regained pigment production.

Physiological characteristics of *bld* and *whi* mutants and the Spo⁺ revertant

In the surface culture of the parent strain (Fig. 2a), leupeptin was produced in association with the growth of substrate mycelium but it was inactivated by LIE,

Fig. 1. Scanning electron micrographs of *S. exfoliatus* SMF13 (a), *bld* mutant SMF13B1 (b) and *whi* mutant SMF13W1 (c) on GCPS agar after 10 d incubation, and *S. exfoliatus* SMF13 (d) in GCPS agar supplemented with 2% glucose after 10 d incubation. Bars, 2 μ m.

production of which began before aerial mycelium formed. Formation of aerial mycelium coincided with increases in the activities of LIE and TLP. However, in *bld* mutant SMF13B1 (Fig. 2b), leupeptin produced during the growth of substrate mycelium persisted throughout the culture, and neither LIE nor TLP was produced. *Bald* mutants SMF13B2 and SMF13B3 showed the same phenotype as SMF13B1. In *whi* mutant SMF13W1 (Fig. 2c), leupeptin produced during substrate mycelium growth was inactivated by LIE, although the rates of leupeptin inactivation and of LIE production were lower than in the parent strain. When leupeptin was nearly inactivated, TLP activity emerged and aerial mycelium growth started. White mutant SMF13W2 showed the same phenotype as SMF13W1. The *Spo*⁺ revertant SMF13R1 regained LIE and TLP production and showed the same pattern of morphological differentiation and production of LIE and TLP as the parent strain (Fig. 2d).

In a submerged batch culture of the parent strain (Fig. 3a), leupeptin produced during mycelium growth started to be degraded, in conjunction with the production of LIE, when glucose was nearly depleted and the mycelium growth rate declined. Moreover, TLP activity started to rise when leupeptin was nearly inactivated, coincident with a decrease in biomass concentration. In *bld* mutant SMF13B1 (Fig. 3b), the rate of leupeptin inactivation was much lower, and LIE and TLP activities were not

detected. Moreover, mycelium autolysis was negligible. SMF13B2 and SMF13B3 showed the same phenotype as SMF13B1. In *whi* mutant SMF13W1 (Fig. 3c), mycelium autolysis as well as production of LIE and TLP was retarded compared to the parent strain. SMF13W2 showed the same phenotype as SMF13W1, whereas *Spo*⁺ revertant SMF13R1 showed the same pattern of growth and production of LIE and TLP as the parent strain (data not shown).

Analysis of kinetic parameters for batch cultures showed very similar specific growth rates (μ), specific glucose uptake rates (q_s) and specific leupeptin production rates (q_{lpt}) in the parent strain, *bld* mutants, *whi* mutants and *Spo*⁺ revertant (Table 1). However, the specific mycelium degradation rates (k_d) in the parent strain and *Spo*⁺ revertant were much higher than those in *whi* and *bld* mutants.

Prevention of aerial mycelium formation and TLP production in the parent strain by glucose

Addition of 2% glucose in the GCPS agar used for surface culture of the parent strain prevented the formation of both aerial mycelium and brown pigment. Scanning electron micrographs of colonies showed substrate mycelium without aerial mycelium and spores (Fig. 1d). Production of leupeptin increased with increasing

Fig. 2. Relationship between morphological differentiation of mycelium and production of leupeptin (■), LIE (△) and TLP (□) in surface cultures of *S. exfoliatus* SMF13 (a), *bld* mutant SMF13B1 (b), *whi* mutant SMF13W1 (c) and *Spo*⁺ revertant SMF13R1 (d). Cultures were grown on GCPS agar.

concentrations of glucose (Table 2). However, production of LIE and TLP decreased, and was completely absent at glucose concentrations above 2%.

Purification of trypsin-like protease, and determination of the N-terminal amino acid sequence

TLP was purified 47.2-fold with a recovery of 10.2% (Table 3). The purified enzyme showed a single band by SDS-PAGE (Fig. 4a), and the molecular mass of the denatured protease was estimated to be 31.8 kDa. A zymogram of the purified protease showed a single band of activity, which was completely inhibited by treatment with leupeptin obtained from the same strain (Fig. 4b).

The N-terminal amino acid sequence of the purified TLP was determined to be RVGGT_xAAQGNFPFQQ_xLSM. The sequence showed 73%, 68% and 57% similarity to the N-terminal amino acid sequence of TLPs from *Streptomyces griseus*, *Streptomyces fradiae* and *Streptomyces glaucescens*, respectively (Fig. 5).

Optimum reaction conditions and substrate specificity of the purified TLP

The optimum pH and temperature for the hydrolysis of Hammarsten casein and a synthetic substrate (BAPNA) with TLP were 7.5 and 35 °C, respectively. Over 70% of the original activity was present after preincubation at

Fig. 3. Changes in the concentrations of biomass (●), glucose (○), leupeptin (■), LIE (△) and TLP (□) in submerged batch cultures of *S. exfoliatus* SMF13 (a), *bld* mutant SMF13B1 (b) and *whi* mutant SMF13W1 (c). Cultures were grown in GCPS medium.

Table 1. Kinetic parameters for batch cultures of *S. exfoliatus* SMF13, three *bld* mutants (SMF13B1, SMF13B2 and SMF13B3), two *whi* mutants (SMF13W1 and SMF13W2) and Spo⁺ revertant SMF13R1

Kinetic parameters	SMF13	SMF13B1	SMF13B2	SMF13B3	SMF13W1	SMF13W2	SMF13R1
Specific growth rate (μ ; h ⁻¹)*	0.068	0.066	0.067	0.069	0.066	0.068	0.068
Specific glucose uptake rate (q_s ; g g ⁻¹ h ⁻¹)*	0.062	0.065	0.064	0.063	0.064	0.065	0.065
Specific leupeptin production rate (q_{lpt} ; g g ⁻¹ h ⁻¹)*	0.014	0.014	0.015	0.014	0.015	0.015	0.015
Specific mycelium degradation rate (k_d ; h ⁻¹)†	0.0185	0.0021	0.0023	0.0022	0.0087	0.0085	0.0184

* Maximum values calculated from data obtained during exponential growth.

† Mean values calculated from data obtained during death phase.

30 °C for 90 min in buffers between pH 3 and pH 8. However, the protease was unstable at a pH higher than 9, and at temperatures above 40 °C. More than 70 % of the activity was lost by treatment at 40 °C for 10 min, and the activity was completely lost by treatment at 50 °C for 10 min.

TLP from *S. exfoliatus* SMF13 hydrolysed a broad range of native proteins such as bovine serum albumin, egg albumin, Hammarsten casein, collagen, haemoglobin and lysozyme (Table 4). The activity against casein and haemoglobin was greater than that against albumin, collagen and lysozyme. However, TLP obtained from *S. exfoliatus* SMF13 could not hydrolyse elastin. The K_m

and V_{max} values obtained with Hammarsten casein as a substrate were 0.61 mg ml⁻¹ and 449.2 tyrosine equivalents min⁻¹ μ M⁻¹, respectively.

Kinetic parameters for the hydrolysis of a number of synthetic aminoacyl *p*-nitroanilides by TLP are given in Table 5. The enzyme could hydrolyse various synthetic substrates for trypsin (aminoacyl *p*-nitroanilides containing arginine at the cleavage site). However, it could not hydrolyse chymotrypsin substrates such as *N*-benzoyl-Tyr *p*-nitroanilide and *N*-succinyl-Gly-Gly-Phe *p*-nitroanilide. Moreover, TLP did not hydrolyse the synthetic elastase substrate *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide.

Table 2. Effect of glucose on aerial mycelium formation and production of leupeptin, LIE and TLP in surface cultures of *S. exfoliatus* SMF13

Spores from stock culture medium were used to inoculate GCPS agar containing increasing concentrations of glucose. Fifty agar plugs, each containing a colony, were pooled and analysed after 7 d culture. Concentrations of leupeptin, LIE and TLP were measured as described in Methods. ND, Not detected.

Glucose (%)	Aerial mycelium (%)	Leupeptin (μg per colony)	LIE (mU per colony)	TLP (mU per colony)
0.0	100	8.5	23.4	9.8
1.0	60	60.8	8.3	4.5
2.0	0	256.2	0.4	ND
3.0	0	258.4	0.3	ND

Inhibitor specificity and inhibition kinetics

The effect of protease inhibitors was determined using BAPNA as the substrate for purified TLP. Activity was completely inhibited by 50 μM antipain or leupeptin (serine/cysteine protease inhibitor) and by 50 μM *N*- α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (trypsin-specific inhibitor). However, it was inhibited less than 15% by 50 μM E-64 (cysteine protease inhibitor), pepstatin (aspartic protease inhibitor), L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone (chymotrypsin-specific inhibitor) and 1 mM EDTA (metallo-protease inhibitor) (data not shown). TLP was competitively inhibited by leupeptin, the inhibition constant being 0.015 μM . The enzyme was also competitively inhibited by antipain, soybean trypsin inhibitor and TLCK; the inhibition constants were 0.534, 0.029 and 3.411 μM , respectively (data not shown).

TLP of *S. exfoliatus* SMF13 was not affected at a concentration of 10 mM during a 1 h treatment by agents such as cysteine, dithiothreitol, glutathione and mercaptoethanol that break disulfide bonds in protein molecules. A 2 h treatment with 10 mM mercaptoethanol reduced enzyme activity by only 15%. The enzyme was also unaffected by iodoacetic acid, which modifies -SH groups in proteins. Like other bacterial trypsins, and unlike

Fig. 4. SDS-PAGE (a) and substrate gel electrophoresis (b) of TLP purified from *S. exfoliatus* SMF13. (a) Lanes: 1, molecular mass standards; 2, purified TLP. (b) Zymogram of TLP obtained by 10% SDS-PAGE containing 0.1% gelatin. After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100 for 2 h to remove the SDS. The gel was incubated at 35 °C for 4 h in 0.05 M Tris/HCl buffer (pH 7.5) without (lane 1) or with (lane 2) 50 μM leupeptin. After incubation, the gel was stained with Coomassie Blue.

mammalian trypsins, TLP from *S. exfoliatus* SMF13 was not activated by calcium ions (Keil, 1971). Mg^{2+} and Mn^{2+} did not affect the protease activity at a concentration of 5 mM, but Cu^{2+} , Hg^{2+} and Zn^{2+} decreased the activity by more than 50%.

Degradation of mycelium protein by TLP

Protein extracted from *S. exfoliatus* SMF13 mycelium was effectively hydrolysed by TLP (Fig. 6a), but the hydrolysis was completely inhibited by leupeptin (Fig. 6b). Although protease activities were apparently present in the mycelium, autodigestion of the mycelium extract was very

Table 3. Purification of TLP from *S. exfoliatus* SMF13

Purification step	Protein (mg)	Activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Culture broth	1524.0	1555	1.02	1.0	100.0
(NH ₄) ₂ SO ₄ fractionation	179.7	990	5.51	5.4	63.7
pH adjustment to 3.6	75.0	780	10.4	10.2	50.2
DEAE-Sephadex	19.9	323	16.2	15.9	20.8
Hydroxyapatite	6.47	227	35.1	34.4	14.6
CM-Sephadex	3.29	159	48.1	47.2	10.2

SET	R	V	G	G	T	x	A	A	Q	G	N	F	P	F	Q	Q	x	L	S	M
SGT	V	V	G	G	T	R	A	A	Q	G	E	F	P	F	M	V	R	L	S	M
SFT	V	V	G	G	T	R	A	A	Q	G	E	F	P	W	M	V	H	L	S	M
SGLT	I	G	G	K	P	A	A	Q	N	E	F	P	F	M	V	H	L	S	M	

Fig. 5. Comparison of the N-terminal amino acid sequence of TLP purified from *S. exfoliatus* SMF13 with those of other *Streptomyces* TLPs. SET, *S. exfoliatus* SMF13 TLP; SGT, *S. griseus* TLP (Olafson *et al.*, 1975); SFT, *S. fradiae* TLP (N. Kikuchi and others, personal communication); SGLT, *S. glaucescens* TLP (G. Hintermann, personal communication). Identical amino acids are boxed; x denotes an amino acid of uncertain identity.

Table 4. Hydrolysis of protein substrates by TLP purified from *S. exfoliatus* SMF13

The experimental conditions are given in Methods. After the reaction was complete, absorbance of trichloroacetic-acid-soluble material was measured at the wavelength specified. For albumin, Hammarsten casein, collagen, elastin, haemoglobin and lysozyme, 1 unit of activity was defined as the amount of TLP needed to produce 1 µg tyrosine equivalent min⁻¹. For azocasein, 1 unit of activity was defined as the amount of TLP needed to produce an absorbance increase of 0.001 min⁻¹ cm⁻¹.

Substrate	Wavelength (nm)	U (µg protein) ⁻¹
Azocasein	440	3.23
Hammarsten casein	280	1.45
Haemoglobin	280	1.31
Albumin (egg)	280	0.50
Albumin (bovine)	280	0.44
Collagen	280	0.39
Lysozyme	280	0.17
Elastin	280	0.00
Elastin Congo Red	495	0.00

slow (Fig. 6c). Chymotrypsin-like activity on *N*-benzoyl-Tyr *p*-nitroanilide [specific activity 0.4556 mU (µg protein)⁻¹] was higher than trypsin-like activity on BAPNA [specific activity 0.008 mU (µg protein)⁻¹] in the mycelium protein.

DISCUSSION

Morphological differentiation in *Streptomyces* often coincides with the biosynthesis of pigment, antibiotics and certain extracellular enzymes (Babcock & Kendrick, 1988; Chater, 1984; Martin & Demain, 1980). *bld* mutants isolated from *Streptomyces coelicolor* and *S. griseus* were pleiotropically blocked in the biosynthesis of pigment and antibiotics (Babcock & Kendrick, 1988; Champness, 1988; Daza *et al.*, 1990; Merrick, 1976). All the *bld* mutants obtained from *S. exfoliatus* SMF13 during this study were pleiotropically blocked in the biosynthesis of

LIE and TLP, as well as in pigment production. In contrast, the Spo⁺ revertant SMF13R1 regained the ability to produce pigment, LIE and TLP, and showed the same pattern of growth and production of LIE and TLP as the parent strain. This correlation between the *bld* phenotype, loss of LIE and TLP activities and loss of pigment production indicated that the phenomena could be controlled by the same mechanism. Because LIE and TLP were produced only in the strains producing aerial mycelium, such as the parent strain and *whi* mutants, these enzymes may have a role in aerial mycelium formation, but not in spore formation.

The physiological and morphological characteristics of the *bld* mutants were very similar to those reported for the parent strain grown with excess glucose (Kim & Lee, 1995). Here also, morphological differentiation was completely repressed under conditions that prevented LIE and TLP synthesis. These results suggest a linkage between morphological differentiation and the production of LIE and TLP.

Extracellular proteases with the characteristics of TLP have been reported in many species of *Streptomyces* (Peczynska-Czoch & Mordarski, 1988). That the activity is associated with differentiation is plausible, because the production of TLP in *Streptomyces* spp. coincides with the onset of aerial mycelium formation (Ginther, 1979; Gibb & Strohl, 1988; Bascaran *et al.*, 1990). Especially in *S. exfoliatus* SMF13, aerial mycelium formation was retarded by the external addition of leupeptin, and it resumed after inactivation of leupeptin by LIE (Kim & Lee, 1995). Addition of TLCK, a specific inhibitor of TLP, to the top surface of colonies inhibited aerial mycelium formation in many streptomycetes, including *S. aburaviensis*, *S. coelicolor*, *S. limosus*, *S. microflavus*, *S. roseus*, *S. albidoflavus*, *S. lavendulae*, *S. rochei*, *S. griseus*, *S. phaeochromogenes*, *S. felleus* and *S. rimosus*, and in *S. exfoliatus* SMF13 the inhibition persisted for at least 5 d (unpublished results). The long duration of TLCK inhibition could be attributed to complete inactivation of TLP by TLCK, which is stable under the conditions used.

The N-terminal amino acid sequence of TLP from *S. exfoliatus* is very similar to the N-terminal sequences of TLPs from *S. griseus*, *S. fradiae* and *S. glaucescens*. In its pH optimum and thermal instability, the *S. exfoliatus* SMF13 enzyme also resembles TLPs from other *Streptomyces*. However, TLP from *S. exfoliatus* SMF13 was stable at pH 4.5, whereas the activity of other *Streptomyces* TLPs was lost at this pH. The *K_m* (0.16 mM) of TLP from *S. exfoliatus* SMF13 for BAPNA was within the range of previously reported *Streptomyces* spp. values: 0.476 mM for *S. moderatus* (Chandrasekaran & Dhar, 1987), 0.029 mM for *S. paromomycinus* (Chauvet *et al.*, 1976), 0.101 mM for *S. griseus* (Hatanaka *et al.*, 1985), 0.328 mM for *S. rimosus* (Renko *et al.*, 1989) and 0.039 mM for *S. erythraeus* (Yoshida *et al.*, 1971). The *K_m* (0.0137 mM) of TLP from *S. exfoliatus* SMF13 for BAEE was also within the range of other *Streptomyces* TLPs: 0.152 mM for *S. moderatus* (Chandrasekaran & Dhar, 1987), 0.005 mM for *S. griseus* (Hatanaka *et al.*, 1985), 0.015 mM for *S. fradiae* (Moriwara & Tsuzuki, 1968), 0.182 mM for *Streptomyces*

Table 5. Kinetic parameters for the hydrolysis of some peptidyl *p*-nitroanilides

Concentrations of the aminoacyl *p*-nitroanilides ranged from 2.5 to 1000 μM . The kinetic parameters were calculated from data obtained from reactions at concentrations of aminoacyl *p*-nitroanilides in the linear response range; NA, nitroanilide.

Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)
<i>N</i> -Benzoyl-Val-Gly-Arg <i>p</i> -NA	35.5	181	5.11
<i>N</i> -Benzoyl-Pro-Phe-Arg <i>p</i> -NA	87.5	159	1.82
<i>N</i> -Benzoyl-Val-Leu-Arg <i>p</i> -NA	162	145	0.89
<i>N</i> -Benzoyl-Phe-Val-Arg <i>p</i> -NA	553	477	0.86
<i>N</i> -Benzoyl-Ile-Glu-Gly-Arg <i>p</i> -NA	1560	494	0.32
<i>N</i> -Benzoyl-Arg <i>p</i> -NA	160	27	0.17
<i>N</i> -Benzoyl-Tyr <i>p</i> -NA	—	—	—
<i>N</i> -Succinyl-Gly-Gly-Phe <i>p</i> -NA	—	—	—
<i>N</i> -Succinyl-Ala-Ala-Ala <i>p</i> -NA	—	—	—
<i>N</i> -Succinyl-Ala-Ala-Val <i>p</i> -NA	—	—	—
<i>N</i> -Succinyl-Tyr-Leu-Val <i>p</i> -NA	—	—	—

Fig. 6. Degradation of a mycelial protein extract of *S. exfoliatus* SMF13 by purified TLP. The enzyme (5 μg) dissolved in 200 μl Tris/HCl buffer (pH 7.5, 0.1 M) was preincubated at 35 $^{\circ}\text{C}$ for 5 min without (a) or with (b) 10 μg leupeptin. Mycelium protein extract (2.8 mg) dissolved in 500 μl Tris/HCl buffer was added and the reaction mixture was incubated at 35 $^{\circ}\text{C}$. Hydrolysis was measured by SDS-PAGE of the mycelial protein extract as a function of time. As a control, autodigestion of a mycelial protein extract was measured (c).

771 (Palubinskas *et al.*, 1984) and 0.018 mM for *S. erythraeus* (Yoshida *et al.*, 1971).

Our results are consistent with TLP and its autogenous inhibitor leupeptin having morphogenetic roles in *S. exfoliatus* SMF13. Mycelium protein extract was hydrolysed by TLP obtained from *S. exfoliatus* SMF13, and leupeptin clearly inhibited this hydrolysis. Moreover, the three *bld* mutants obtained by UV-mutagenesis were pleiotropically blocked in the biosynthesis of TLP, and mycelium autolysis in submerged cultures of these mutants was negligible compared with that in the parent strain. Since autoradiographic studies of colony development in *Streptomyces* have shown that substrate mycelium is metabolized to support aerial mycelium growth

(Mendez *et al.*, 1985; Miguelez *et al.*, 1994), we suggest that TLP digests substrate mycelium proteins in surface cultures (or non-growing mycelium proteins in submerged cultures) when the cultures are faced with nutrient limitation. Amino acids or oligopeptides liberated from the proteins by TLP support the growth of aerial mycelium on surface cultures or tip growth in submerged cultures; leupeptin protects growing substrate mycelium at the colony margins or growing tip mycelium from hydrolysis by TLP produced in growth-limited mycelium.

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