Characterization of the leupeptin-inactivating enzyme from *Streptomyces exfoliatus* SMF13 which produces leupeptin

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Leupeptin-inactivating enzyme (LIE) was purified from *Streptomyces exfoliatus* SMF13 by ammonium sulphate fractionation of cell-free culture broth, ultrafiltration, anion-exchange chromatography on DEAE-Sephadex A-50 and gel filtration chromatography on Sephadex G-75. The molecular mass of the purified enzyme was measured as 34 700 Da and the N-terminal amino acid sequence was APTPPDIPLANVPA. Acetyl-leucine, leucine and argininal were identified as the products of leupeptin inactivated by the LIE, indicating that leupeptin is inactivated by hydrolysis of peptide bond between leucine and leucine and between leucine and argininal of leupeptin (acetyl-leucine-leucine-argininal). Synthetic-peptide substrates specificity of LIE showed that LIE has absolute specificity for peptide bonds with leucine in the P1 position, suggesting that LIE is a leucine-specific protease. The optimum pH and temperature were pH 9.0

and 45 °C, respectively. LIE activity was inhibited by metalloprotease inhibitors such as EDTA, EGTA, *o*-phenanthroline and bestatin, but activated by Mg²⁺ and Ca²⁺, suggesting that the enzyme is a metalloprotease. Aerial-mycelium growth and aerial spore formation of *S. exfoliatus* SMF13 were inhibited by the addition of bestatin, an inhibitor of LIE. The inhibition of morphological differentiation was due to the inhibition of trypsin-like protease (TLP) activity, which is essential for aerial-mycelium formation and is inhibited specifically by remaining leupeptin that was not inactivated. These results show that LIEs play a role in controlling the amount of leupeptin during colony development. Therefore, it is suggested that the physiological function of LIE is to inactivate leupeptin when or where TLP activity is required for aerial-mycelium formation.

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 [1]. The aerial hyphae appear to grow at least partially by the utilization of degraded substrate mycelium [2,3], because the aerial mycelium has little access to other sources of nourishment for its growth and development [4].

Streptomycetes produce a variety of extracellular proteases [5] as well as protease inhibitors [6]. Protease inhibitors, which play an important role in inhibiting the proteolytic activity of proteases, are widely distributed in streptomycetes. A typical proteinaceous protease inhibitor, *Streptomyces* subtilisin inhibitor, was found to be distributed widely in streptomycetes, including the strains formerly categorized as *Streptoverticillium* sp. [7]. A typical low-molecular-mass serine/cysteine protease inhibitor, leupeptin, has been isolated from various strains of *Streptomyces* spp. [8–11]. The high frequency of occurrence of protease inhibitors in *Streptomyces* spp. suggests that they might have an important role in some physiological functions.

The secretion of extracellular proteolytic enzymes in streptomycetes often temporally coincides with the onset of secondary metabolism or morphological differentiation (formation of aerial mycelia and spores) [12–14]. The activity of trypsin-like protease (TLP) is especially essential for aerial-mycelium formation of *S. aburaviensis*, *S. albidoflavus* SMF301, *S. coelicolor* A3(2), *S. exfoliatus*, *S. limosus*, *S. microflavus*, *S. roseus*, *S. felleus*, *S. griseus*, *S. lavendulae*, *S. rimosus* and *S. rochei*, because

the aerial-mycelium formation was inhibited under conditions which led to the inhibition of TLP activity or TLP production [15,16].

We have shown that *S. exfoliatus* SMF13 produces leupeptin, leupeptin-inactivating enzyme (LIE) and TLP extracellularly. Production of leupeptin was closely associated with mycelial growth, but leupeptin was inactivated by LIE when mycelium growth reached the stationary phase in submerged cultures, or just before aerial mycelium was formed on surface cultures. TLP activity started to increase as leupeptin activity decreased. The production of TLP was correlated with a decline in biomass of submerged cultures or aerial-mycelium formation on surface cultures. The activity of TLP was specifically inhibited by leupeptin. Autolysis of mycelium after stationary phase in submerged culture was apparently retarded by addition of leupeptin. Furthermore, aerial-mycelium formation on surface cultures was retarded by addition of leupeptin [17]. TLP effectively hydrolysed the mycelial protein extract of *S. exfoliatus* SMF13, but the hydrolytic activity was inhibited by leupeptin. Therefore, it was suggested that the morphological differentiation and production of TLP are co-ordinately 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 [18].

LIE also seems to play a critical role in the differentiation of *S. exfoliatus* SMF13 by controlling the amount of leupeptin that regulates TLP activity. To understand the role of LIE and the inactivation mechanism of leupeptin, LIE was purified and characterized.

Abbreviations used: LIE, leupeptin-inactivating enzyme; TLP, trypsin-like protease; BAPNA, *N*-benzoyl arginine *p*-nitroanilide; CBZ, benzylloxycarbonyl.

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EXPERIMENTAL

Micro-organism and strain maintenance

The micro-organism used was *S. exfoliatus* SMF13 [11,17,19]. The strain was 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): 1 % glucose; 0.2 % casein hydrolysate; 0.1 % yeast extract; 0.1 % beef extract; and 1.8 % agar. Seed culture medium consisted of (w/v): 3 % glucose; 1.8 % soytone; 0.3 % peptone; and 0.4 % CaCO₃. The main culture medium [glucose caseinate phosphate salts (GCPS) medium] consisted of (w/v): 0.5 % glucose; 1 % sodium caseinate; 0.025 % KH₂PO₄; 0.085 % K₂HPO₄; 0.03 % MgSO₄·7H₂O; 0.03 % NaCl; 0.001 % FeSO₄·7H₂O; 0.001 % CuSO₄·5H₂O; 0.001 % CaCl₂·2H₂O; and 0.0003 % MnCl₂·4H₂O. The phosphate and salts were separately sterilized by filtration (0.2 µm, Millipore membrane). For submerged batch cultures, spore suspension was used to inoculate 50 ml of seed culture medium (to 10⁶ spores/ml) in a 500 ml baffled flask, and cultured at 28 °C for 36 h in a rotary shaking incubator (200 r.p.m.). The seed culture was used to inoculate (5 %, v/v) 3 l of the main culture medium contained in a jar fermentor (5 l; Korea Fermentor Co., Incheon, Korea) maintained at 28 °C; the initial pH was adjusted to 7.0. Agitation and aeration were at 200 r.p.m. and 1 vol./min/vol., respectively. For surface culture, spores were transferred with sterile toothpicks on to agar plates of main culture medium, and incubated at 28 °C.

Protein analysis

Protein concentrations were determined by the Bradford method [20]; BSA was used as the standard.

Leupeptin analysis

The concentration of leupeptin was calculated by enzyme inhibition assay using 50 µg of papain and 0.2 mM of *N*-benzoyl-L-arginine ethyl ester as the target protease and papain substrate, respectively. Enzyme reactions were carried out at 25 °C and pH 6.2 (potassium phosphate buffer, 0.1 M). The total volume of the reaction mixture was 2.5 ml. Activity was calculated from the linear part of the curve, using $E_{253} = 1150 \text{ mol} \cdot \text{cm}^{-1}$. The activity of leupeptin was calculated from the following relation; percentage inhibition = 100((A - B)/A), where A is the papain activity without the inhibitor and B is the papain activity with the inhibitor [21]. The concentration of leupeptin was calculated from a standard curve prepared with authentic leupeptin.

LIE analysis

LIE was preincubated at 35 °C and pH 7.5 (Tris/HCl buffer, 0.1 M) for 5 min and then 50 µg of leupeptin was added to the LIE solution. The total volume of the reaction mixture was 1 ml. The reaction mixture was incubated for 10 min at 35 °C, then heated for 5 min at 80 °C for complete inactivation of LIE in the reaction mixture. The remaining amount of leupeptin was assayed. One unit of LIE was defined as the amount of enzyme needed for inactivation of 10 µg of leupeptin/min [17].

TLP analysis

TLP activity was estimated by measuring the amount of *p*-nitroanilide liberated from the *N*-benzoyl arginine *p*-nitroanilide (BAPNA). Enzyme solution was incubated in a volume of 2.5 ml

in 0.1 M Tris/HCl buffer (pH 7.5) at 35 °C for 5 min. Then 30 µl of BAPNA was added to achieve a final concentration of 200 µmol. Activity was calculated from the linear part of the curve, using $E_{405} = 9620 \text{ mol} \cdot \text{cm}^{-1}$. One unit of TLP activity was defined as the amount of enzyme needed to produce 1 µmol of *p*-nitroanilide/min [15].

Purification of LIE

When LIE activity reached its maximum in a batch culture, the broth was harvested by centrifugation (12000 *g* for 15 min). LIE was purified from the cell-free broth by the following procedures at 4 °C. Fractions from the 35–70 % ammonium sulphate saturation range were dissolved in distilled water and ultrafiltered through XM50 Diaflo ultrafilter (molecular-mass cut-off, 50000 Da; Amicon, Beverly, U.S.A.). The filtrate was ultrafiltered through YM10 Diaflo ultrafilter (molecular-mass cut-off, 10000 Da). The retentate was solubilized with 0.02 M Tris/HCl buffer (pH 7.5). The protein fraction obtained by ultrafiltration was applied to the column of DEAE-Sephadex A-50 (2.5 × 25 cm) pre-equilibrated with 0.02 M Tris/HCl buffer (pH 7.5) and eluted with the same buffer and a linear gradient of NaCl (0–0.2 M). The flow rate was adjusted to 0.4 ml/min and 6 ml fractions were collected. The active fractions were concentrated and desalted by ultrafiltration (YM10 Diaflo ultrafilter). The active fractions obtained by DEAE-Sephadex A-50 chromatography were applied to a column of Sephadex G-75 superfine (1.5 × 100 cm) pre-equilibrated with 0.02 M Tris/HCl buffer (pH 7.5). The flow rate of buffer was 0.15 ml/min and 6 ml fractions were collected.

Molecular-mass determination

The molecular mass of the purified LIE was estimated by SDS/PAGE [22]; 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. Standard protein markers were phosphorylase *b* (99.6 kDa), BSA (66 kDa), egg albumin (45 kDa), carbonic anhydrase (31.0 kDa), soya bean trypsin-inhibitor (21.5 kDa) and hen egg-white lysozyme (14.4 kDa). The molecular mass of native LIE was determined by analytical gel permeation HPLC with Protein PAK 125 column (Waters, Milford, U.S.A.). Standard protein markers were α-lactoglobulin (18.4 kDa), pepsin (34.7 kDa) and ovalbumin (45 kDa). LIE and protein markers were applied to a Protein PAK 125 column and eluted with 0.1 M sodium phosphate buffer (pH 6.0) at a flow rate of 0.6 ml/min. Void volume was determined by blue-dextran (2000 kDa).

N-terminal amino acid sequencing

The N-terminal amino acids of the LIE were sequenced by a modified phenylthiohydantoin method [24]. The similarity of N-terminal amino acid sequence of LIE to other proteins was determined from the EMBL protein database.

Effect of temperature and pH on enzyme activity

The optimum pH for the hydrolysis of leupeptin was measured using the following buffers at 0.1 M: sodium acetate buffer (pH 4–6), sodium phosphate buffer (pH 6–8), Tris/HCl (pH 7.5–9) and sodium carbonate buffer (pH 9–11). The optimum temperature for the hydrolysis of leupeptin was determined in the range of 20–65 °C at the optimum pH.

Analysis of products of leupeptin inactivated by LIE

10 μg of purified enzyme was incubated with 4 mg of leupeptin in a final volume of 500 μl in 0.05 M Tris/HCl buffer (pH 9.0) at 37 °C for 2 h. The reaction products were subjected to reverse-phase HPLC on a C18 column (Hypersil ODS, 10 μm bead size, 10 $\times$ 250 mm; Hichrom, Reading, U.K.). The column was pre-equilibrated with 10% acetonitrile (Fisher Co., NH, U.S.A.) at a flow rate of 2 ml/min. The products were eluted with a linear gradient of acetonitrile (10–50%). The eluted peaks were detected at 214 nm. Also, reaction products were spotted on 0.2 mm thin layer of silica gel and chromatographed with n-butanol:acetic acid:water (60:15:25, by vol.). Spots were detected by spraying with 50% sulphuric acid in methanol and 0.2% ninhydrin in ethanol [25]. The reaction products were analysed by electron ionization MS using a VG 70-SEQ mass spectrometer in Korea Basic Science Institute.

Effects of inhibitors and influence of metal ions

Protease inhibitors (Sigma) at the concentrations indicated were preincubated with LIE for 5 min at 35 °C and then the residual LIE activity was measured. The influence of divalent metal ions was assayed with the dialysed LIE with 0.02 M Tris/HCl buffer (pH 7.5) for 24 h at 4 °C. LIE was preincubated with 0.5 mM Ba^{2+} , Ca^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} or Zn^{2+} for 5 min and the LIE activity was measured.

Hydrolysis of synthetic substrates and protein substrates

The kinetic parameters for hydrolysis of aminoacyl *p*-nitroanilides were measured at optimum leupeptin-inactivating conditions. Purified enzyme was incubated in a volume of 2.5 ml in 0.1 M Tris/HCl buffer (pH 9.0) at 40 °C for 5 min. Then 30 μl of aminoacyl *p*-nitroanilides were added to achieve final concentrations of 2.5–1000 μM and the initial velocity of substrate hydrolysis was measured by measuring the amount of *p*-nitroanilide liberated, using $E_{405} = 9620 \text{ mol} \cdot \text{cm}^{-1}$ [23]. The K_m and K_{cat} values were calculated from a Lineweaver–Burk reciprocal plot.

Hydrolysis activity towards dipeptide and tripeptide substrates was measured. Purified enzyme (2 μg) was combined with 4 mM of peptide substrates in a final volume of 200 μl in 0.02 M Tris/HCl buffer (pH 9.0). The reaction products removed at 0, 1 and 2 h were spotted on a 0.2 mm thick layer of silica gel and chromatographed with n-butanol:acetic acid:water (60:15:25, by vol.). Spots were detected by spraying with 0.2% ninhydrin in ethanol and Rydon–Smith reagent [25]. Carboxypeptidase activity was measured by using an enzyme-coupled colorimetric assay of *N*-CBZ (benzyloxycarbonyl)-leucine-alanine hydrolysis as described by Fujita et al. [26] and modified by DiSanto and Logan [27].

Hydrolysis activity for BSA, egg albumin, collagen, elastin, haemoglobin, lysozyme (all from Sigma), Hammarsten casein (Merck) and TLP purified from the culture broth of *S. exfoliatus* SMF13 [18] was estimated by measuring the concentration of tyrosine liberated from the substrate protein after 15 min reaction at 40 °C and pH 7.5 (0.1 M Tris/HCl buffer) as described by Kim and Lee [18] and Narahashi [28]. Hydrolysis activity for TLP was also determined by TLP activity analysis after the reaction.

Effect of LIE inhibitor on the morphological differentiation of *S. exfoliatus* SMF13

Bestatin was dissolved in distilled water and sterilized by filtration (0.2 μm , Millipore). Various amounts (0.1, 0.2 or 0.4 mg) of

bestatin were added aseptically to the top of colonies developed on agar pieces in a 96-well plate just before the onset of aerial-mycelium formation. After 5 day culture the morphological differentiation of each colony was observed and the number of colonies which differentiated to aerial mycelium and further sporulated are expressed as a percentage. Fifty agar pieces, each containing a colony, were pooled, homogenized in 10 ml Tris/HCl buffer (0.1 M, pH 7.5) and centrifuged (10000 *g* for 10 min). Concentration of leupeptin and activity of LIE and TLP in the supernatant were measured before or after dialysis against 0.02 M Tris/HCl buffer (pH 7.5).

Scanning electron microscopy

Colonies developed on agar medium were fixed using the following procedures: 8% phosphate-buffered glutaraldehyde solution (pH 7.4) was poured on to the top of the agar pieces [29]. 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 Revco box under P_2O_5 at 4 °C. Dried colonies were gold-coated with Polaron SC502 Sputter Coater (Fisons, Loughborough, U.K.) at 15 mA for 1 min under vacuum. The morphology of colonies were observed with a Streoscan 260 scanning electron microscope (Cambridge Ltd., U.K.).

RESULTS

Purification of LIE and N-terminal amino acid sequence

LIE was purified 64.2-fold with a recovery of 12.5% (Table 1). The purified enzyme showed a single band by SDS/PAGE (Figure 1), and the molecular mass of the denatured LIE was estimated to be 34.7 kDa. The molecular mass of the native LIE was estimated by analytical gel permeation HPLC with Protein PAK 125 column to be 33.5 kDa, indicating that LIE is a monomer. The N-terminal amino acid sequence of the purified LIE was determined to be APTPPDIPLANVPA. This sequence was used to search for similar sequences in the EMBL protein database and showed no significant similarity to any enzyme or proteins.

Optimum reaction conditions of LIE and analysis of products of leupeptin degraded by LIE

The optimum pH and temperature for the inactivation of leupeptin were 9.0 and 40 °C, respectively. LIE was stable at temperatures below 50 °C and unstable at temperatures above 60 °C. More than 35% of the activity was lost by treatment at 60 °C for 30 min. The products of leupeptin inactivated by LIE were analysed with HPLC (Figure 2). The chromatogram of leupeptin showed the typical three peaks with retention times of 28.7, 30 and 33 min, as has been reported for the three isomers [8]. However, the chromatogram of reaction products showed two different major peaks. Also the reaction products were analysed with silica–TLC. The chromatogram of authentic leupeptin showed the typical three spots with the R_f values of 0.61, 0.72 and 0.75. However, the chromatogram of reaction products showed three different R_f values of 0.15, 0.35 and 0.50 (results not shown). Three different spots were extracted with butanol and further analysed with HPLC. The retention times of the spots of R_f 0.15 and R_f 0.35 were identical to those of peaks 1 and 2, respectively. The spot of R_f 0.5 did not show any significant peak under these HPLC conditions. When leucine was chromatographed on a thin layer of silica gel with the above conditions, the R_f value was 0.5. This silica–TLC result showed that the spot of R_f 0.5 was leucine. The mass spectra of peak 1 and peak 2 showed $\text{M}+\text{H}^+$ ions at m/z 159 and m/z 174,

Table 1 Purification of LIE from *S. exfoliatus* SMF13

For experimental details, see the text. The values are from 3 l of culture supernatant. Fractions were assayed using leupeptin as the substrate. One unit of LIE was defined as the amount of enzyme needed for inactivation of 10 μ g of leupeptin/min.

Procedure	Total proteins (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free supernatant	1530	780	0.51	1	100
Ammonium sulphate fractionation	225	585	2.6	5.1	75
Ultrafiltration	24.4	374	15.3	30	47.9
DEAE-Sephadex A-50	8.03	172.4	21.47	42.1	22.1
Sephadex G-75	2.97	97.2	32.72	64.2	12.5

Figure 1 SDS/PAGE of LIE purified from *S. exfoliatus* SMF13

The purified LIE preparation was revealed by Coomassie Blue colouration after SDS/PAGE [12% (w/v) gel]. Lane 1, molecular-mass standards; lane 2, purified LIE.

respectively. From the mass fragmentation patterns, peak 1 and peak 2 were identified as argininal and acetyl-leucine, respectively. The above results are summarized in Table 2. From these results, it was concluded that leupeptin was cleaved to acetyl-leucine, leucine and argininal by LIE (Figure 3).

Hydrolysis of synthetic peptide substrates and protein substrates by LIE

The catalytic activity of LIE towards a number of synthetic aminoacyl *p*-nitroanilides was measured (Table 3). Aminoacyl *p*-nitroanilides bearing leucine at the P1 site were hydrolysed, but those bearing tyrosine, arginine, glutamate, proline, phenyl-alanine, valine and alanine were not hydrolysed. The K_{cat}/K_m values for Leu *p*-nitroanilide was very much higher than those for pGlu-Phe-Leu *p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide, suggesting that free leucine on the N-terminal side of the scissile bond is favoured over peptidyl-leucine. The enzyme showed similar specificity towards dipeptide and tripeptide substrates. Compounds bearing leucine at the N-terminal position were hydrolysed, but those bearing arginine, glycine, methionine, proline and serine were not hydrolysed (Table 4). From the above results it was concluded that the enzyme has a strict preference for leucine at the P1 site. LIE did not liberate *N*-CBZ from *N*-CBZ-Leu-Ala, a typical substrate for leucine carboxypeptidase.

LIE could not hydrolyse native proteins such as BSA, egg albumin, Hammarsten casein, collagen, elastin, haemoglobin and lysozyme. LIE also could not hydrolyse TLP purified from

Figure 2 C18 HPLC chromatogram of authentic leupeptin (A) and products of leupeptin inactivated by LIE (B)

Authentic leupeptin and products of leupeptin inactivated by LIE were subjected to reverse-phase HPLC on a C18 column (Hypersil ODS, 10 μ m bead size, 10 $\times$ 250 mm) and were eluted with a linear gradient of acetonitrile (10–50%) at a flow rate of 2 ml/min. The eluted peaks were detected at 214 nm. Peak 1 and peak 2 were identified as argininal and acetyl-leucine, respectively.

the culture broth of *S. exfoliatus* SMF13. LIE did not influence the hydrolytic activity of TLP towards protein substrates, Hammarsten casein and haemoglobin as well as a synthetic substrate, BAPNA, when LIE was incubated with TLP.

Inhibitor specificity and metal-ion effect

The effect of protease inhibitors on the hydrolytic activity of LIE towards leupeptin and Leu *p*-nitroanilides as the substrates was

Table 2 Identification of leupeptin and products of leupeptin inactivated by LIE

For experimental details, see the text. Products of leupeptin inactivated by LIE were analysed by electron ionization MS using VG 70-SEQ mass spectrometer in the Korean Basic Science Institute. The numbers in parentheses are the percent relative intensities. M⁺, molecular ion.

Retention time on C18 HPLC (min)	R _F value on silica gel TLC	Mass spectral fragmentation (<i>m/z</i>)	Identification
28.7, 30, 33	0.61, 0.72, 0.75		Leupeptin
10 (peak 1)	0.15	159(M + H ⁺ , 6) 142(6) 101(18) 100(100) 59(72) 58(44)	Argininal
23 (peak 2)	0.35	174(M + ⁺ , 100) 173(M ⁺ , 38) 130(5) 128(43) 86(75)	Acetyl-leucine
	0.50		Leucine

Figure 3 Cleavage sites of leupeptin by LIE

The cleavage sites are indicated by arrows.

Table 3 Synthetic aminoacyl *p*-nitroanilide specificity of LIE

Concentration of the aminoacyl *p*-nitroanilides ranged from 2.5 to 1000 μ M. The kinetic parameters were calculated from the data obtained from reactions at concentrations of aminoacyl *p*-nitroanilides in the linear response range. *N*-Benzoyl-Arg *p*-nitroanilide, *N*-benzoyl-Ile-Gly-Gly-Arg *p*-nitroanilide, *N*-benzoyl-Tyr *p*-nitroanilide, Glu *p*-nitroanilide, Gly-Pro *p*-nitroanilide, *N*-glutaryl-Phe *p*-nitroanilide, *N*-succinyl-Gly-Gly-Phe *p*-nitroanilide, *N*-succinyl-Ala-Ala-Val *p*-nitroanilide and *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide were not hydrolysed.

Synthetic aminoacyl <i>p</i> -nitroanilides	K _m (μ M)	K _{cat} (s ⁻¹)	K _{cat} /K _m (s ⁻¹ · μ M ⁻¹)
Leu <i>p</i> -nitroanilide	1.5	243	162
pGlu-Phe-Leu <i>p</i> -nitroanilide	33	12.2	0.37
<i>N</i> -Succinyl-Ala-Ala-Pro-Leu <i>p</i> -nitroanilide	102	2.7	0.03

measured (Table 5). The activity of LIE was inhibited by EDTA, EGTA and *o*-phenanthroline, which are metalloprotease inhibitors, but not by phosphoramidon which inhibits some metalloendopeptidases. It was also inhibited by bestatin which is an inhibitor of metalloaminopeptidase. However, the activity of LIE was not inhibited by trypsin-like serine-protease inhibitor, chymotrypsin-like serine-protease inhibitor, antipain (trypsin-like and many cysteine proteases inhibitor), E-64 (cysteine proteases inhibitor) and pepstatin (aspartic proteases inhibitor).

The effect of various metal ions on the activity of LIE is shown in Table 6. Addition of Ca²⁺ and Mg²⁺ stimulated the activity of the enzyme, while the addition of Hg²⁺, Ni²⁺ and Zn²⁺ inhibited the activity significantly.

Effect of addition of bestatin on aerial-mycelium formation in surface culture of *S. exfoliatus* SMF13

The effect of external addition of bestatin, an inhibitor of LIE, on the morphological differentiation of *S. exfoliatus* SMF13 in surface culture was measured. When bestatin (0.2 mg/colony)

Table 4 Hydrolysis of dipeptide and tripeptide substrates by LIE

Hydrolysis activity towards dipeptide and tripeptide substrates was measured. Purified enzyme (2 μ g) was combined with 4 mM of peptide substrates in a final volume of 200 μ l in 0.02 M Tris/HCl buffer (pH 9.0). Aliquots removed at 0, 1 and 2 h were spotted on a 0.2 mm thin layer of silica gel and chromatographed with *n*-butanol:acetic acid:water (60:15:25). Hydrolysed products were identified using authentic amino acids or peptides as the standard after colour development as described in the text. ++, Completely hydrolysed within 2 h; +, partially hydrolysed within 2 h; -, not hydrolysed.

Synthetic peptides	Degree of hydrolysis	Hydrolysed products
Leu-Leu	++	Leu
Leu-Arg	++	Leu, Arg
Leu-Asn	++	Leu, Asn
Leu-Asp	++	Leu, Asp
Leu-Ala	+	Leu, Ala
Leu- β -Ala	-	
Arg-Ser	-	
Arg-Asp	-	
Gly-Leu	-	
Met-Arg	-	
Pro-Gly	-	
Ser-Met	-	
Leu-Leu-Leu	++	Leu
Leu-Leu-Tyr	+	Leu, Tyr
Leu-Ser-Phe	+	Leu, Ser-Phe
Leu-Gly-Gly	+	Leu, Gly-Gly

Table 5 Effect of protease inhibitors on the hydrolysis activity of LIE towards leupeptin or Leu *p*-nitroanilide as the substrates

Enzyme (2 μ g) was preincubated with different inhibitors for 5 min before the addition of substrates. Activity is expressed by the percentage of inhibition. The abbreviation used are: TLCK, *N*- α -*p*-tosyl-L-lysyl chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane.

Inhibitor	Concentration (mM)	Inhibition (%)	
		Leupeptin	Leu <i>p</i> -nitroanilide
<i>o</i> -Phenanthroline	0.1	100 $\pm$ 2	100 $\pm$ 2
Bestatin	0.1	87 $\pm$ 4	90 $\pm$ 3
EDTA	2.0	72 $\pm$ 5	70 $\pm$ 4
EGTA	2.0	73 $\pm$ 4	72 $\pm$ 7
Phosphoramidon	0.1	0	0
Leupeptin	0.1	0	35 $\pm$ 8
Antipain	0.1	0	0
Phenylmethylsulphonyl fluoride	1.0	0	0
TLCK	0.1	0	0
TPCK	0.1	0	0
E-64	0.1	0	0
Pepstatin	0.1	0	0

Table 6 Effect of divalent cations on LIE activity

Dialysed enzyme (2 µg) was preincubated with different cations for 5 min and then LIE activity was measured. Activity is expressed as a percentage of the control (100% activity). Control assays were performed with distilled water instead of cations.

Cations	Concentration (mM)	Activity (% of control)
Control		100
BaCl ₂	0.5	98 ± 3
CaCl ₂	0.5	236 ± 8
CoCl ₂	0.5	35 ± 2
CuCl ₂	0.5	24 ± 4
FeCl ₂	0.5	62 ± 6
HgCl ₂	0.5	2 ± 0.1
NiCl ₂	0.5	2 ± 0.3
MgCl ₂	0.5	128 ± 9
MnCl ₂	0.5	26 ± 5
ZnCl ₂	0.5	0

Figure 4 Scanning electron micrographs of *S. exfoliatus* SMF13 grown on GCPS agar medium with bestatin (A) or GCPS agar medium alone (B)

Bestatin was dissolved in distilled water in a concentration of 0.2 mg/30 µl and 0.2 mg of bestatin was added aseptically to the top surface of colonies developed on agar pieces just before the onset of aerial-mycelial formation (20 h cultures). The morphology of colonies were observed after 5 days of culture. (A) Shows typical substrate-mycelial growth and (B) shows typical aerial-mycelial growth and sporulation. Bars, 2 µm.

was added externally to the top surface of a colony before the onset of aerial-mycelial formation (after 20 h of culture), a significant retardation of aerial-mycelial formation was

observed. Scanning electron micrographs of 5 day old cultures showed that the surface consisted of substrate mycelial growth without aerial mycelia and spores (Figure 4A), although the control cultures without addition of bestatin consisted primarily of long rectiflexible spore chains (Figure 4B).

The inhibition of aerial-mycelium formation and further aerial-spore formation was closely related to the concentration of bestatin added externally. Activities of LIE and TLP in GCPS agar medium decreased with increasing concentrations of bestatin. However, concentration of leupeptin increased (Table 7). Activities of LIE and TLP in GCPS agar medium supplemented with 0.4 mg bestatin increased from 0.2 mU/colony and 0 mU/colony to 8.2 mU/colony and 4.1 mU/colony, respectively, after dialysis. This increase in activities of LIE and TLP was due to removal of low-molecular-mass inhibitors, bestatin and leupeptin by the dialysis. These results indicated that the increase in leupeptin concentration was clearly due to inhibition of LIE activity by bestatin and the decrease in TLP activity was due to the increase of leupeptin concentration in GCPS agar medium added with bestatin.

DISCUSSION

The major finding of this study was that there is a extracellular enzyme which inactivates leupeptin, a serine/cysteine protease inhibitor, in a leupeptin-producing bacteria. Another significant finding was that LIE plays a significant role in regulation of morphological differentiation in a leupeptin-producing *S. exfoliatus* SMF13. In the present study we purified and characterized LIE from *S. exfoliatus* SMF13, which degrades leupeptin (acetyl-leucine-leucine-argininal) into three components, acetyl-leucine, leucine and argininal. This is the first detailed report of the presence of an extracellular enzyme which inactivates an extracellular protease inhibitor in an inhibitor-producing strain.

The synthetic-peptide-substrate specificity and inhibitor specificity of LIE have shown that this enzyme is a type of metalloprotease that has a strict preference for leucine at the P1 site. LIE hydrolysed synthetic-peptide substrates bearing leucine at the P1 site, but could not hydrolyse those bearing tyrosine, arginine, glutamate, proline, phenylalanine, valine, glycine, alanine, methionine or serine at this site. This high substrate specificity of LIE suggests that this enzyme plays a significant physiological role rather than a nutritional role for hydrolysis of protein substrates in *S. exfoliatus* SMF13. LIE could not hydrolyse protein substrates such as BSA, egg albumin, Hammarsten casein, collagen, elastin, haemoglobin and lysozyme. Previous reports showed that chymotrypsin-like pro-

Table 7 Effect of bestatin on aerial-mycelium formation, concentration of leupeptin and activity of LIE and TLP in surface cultures of *S. exfoliatus* SMF13

Various amounts (0.1, 0.2 or 0.4 mg) of bestatin were added aseptically to the top of colonies developed on GCPS agar pieces in 96-well plate just before the onset of aerial-mycelial formation. After 5 day culture the morphological differentiation of each colony was observed and the number of colonies which differentiated to aerial mycelium and further sporulated are expressed as a percentage. Fifty agar pieces, each containing a colony, were pooled, homogenized in 10 ml Tris/HCl buffer (0.1 M, pH 7.5) and centrifuged (10000 g for 10 min), after 5 day culture. The concentration of leupeptin in the supernatant was measured and activities of LIE and TLP in the supernatant were measured before or after dialysis against Tris/HCl buffer (0.02 M, pH 7.5). n.d., Not determined.

Bestatin added (mg/colony)	Aerial mycelium (%)	Leupeptin (µg/colony)	LIE before dialysis (mU/colony)	LIE after dialysis (mU/colony)	TLP before dialysis (mU/colony)	TLP after dialysis (mU/colony)
0	100	8.3	22.5	20.3	9.7	11.5
0.1	18	19.2	7.3	n.d.	3.6	n.d.
0.2	6	50.7	4.2	n.d.	2.3	n.d.
0.4	0	80.5	0.2	8.2	0	4.1

tease is essential for the hydrolysis of proteinaceous nitrogen sources for mycelium growth in *S. exfoliatus* SMF13 [17].

LIE did not liberate *N*-CBZ from *N*-CBZ-Leu-Ala, a leucine carboxypeptidase substrate, indicating that this enzyme does not have carboxypeptidase activity. Kinetic analysis of synthetic-peptide-substrate hydrolysis has shown that free leucine on the N-terminal side of the scissile bond is favoured over peptidyl-leucine. These results suggest that LIE belongs to the leucine aminopeptidases. There have been some reports about leucine aminopeptidases in several species of *Streptomyces*, however, the biological functions have never been discussed. Leucine aminopeptidase from *S. lividans* 1326 showed high specificity for the leucine residue at the P1 site [30]. However leucine aminopeptidases from *S. rimosus* showed broad substrate specificity not only for leucine at the P1 site but also for methionine, phenylalanine, tyrosine, tryptophan, histidine and valine [31]. The K_m (1.5 μ M) of LIE from *S. exfoliatus* SMF13 for Leu *p*-nitroanilide was very low compared with previously reported leucine aminopeptidase values from *Streptomyces* spp. of 300 μ M for *S. lividans* [30] and 250 μ M for *S. rimosus* [31].

A property shared by most leucine aminopeptidases from *Streptomyces* spp. is their dependence on divalent metal ions for activity. LIE was inactivated by metal-chelating agents and metalloaminopeptidase inhibitors. Addition of Ca^{2+} and Mg^{2+} stimulated the activity of LIE, whereas the addition of Hg^{2+} , Ni^{2+} and Zn^{2+} inhibited the activity significantly. Stimulation by Ca^{2+} is also shown for enzymes from *S. rimosus* [31], *S. fradiae* [32], *S. peptidofaciens* [33] and *S. griseus* [34]. Inhibition by Hg^{2+} , Ni^{2+} and Zn^{2+} could be due to the ability of these metal ions to bind free SH groups in the cysteine residue. These data suggest that LIE is a thiol-sensitive metalloprotease.

The filamentous growth habit of streptomycetes permits three different types of developmental stage, substrate mycelium, aerial mycelium and spore, within the one colony. Usually, aerial-mycelium formation and sporulation start from the central part of the colony where nutrient limitation is most likely to be encountered, although substrate mycelium continues to extend at the colony margins. The activity of leupeptin was high in the region of actively growing substrate mycelium, whereas the activity of LIE and TLP was high in the region where aerial mycelium or spores occurred. Morphological differentiation was completely repressed under glucose- or casamino-acid-excess conditions that prevented LIE and TLP synthesis. However, production of LIE and TLP started sequentially in relation with morphological differentiation under nutrient-limitation conditions [35]. In three *blb* (the bald phenotype) mutants isolated from *S. exfoliatus* SMF13 after UV mutagenesis, neither LIE nor TLP was produced, although leupeptin was produced. TLP functions as an enzyme in the metabolism of mycelial proteins for scavenging amino acids to support the growth of aerial mycelium [18]. The inhibition of leupeptin inactivation by inhibiting LIE activity with the addition of LIE inhibitor resulted in the retardation of aerial-mycelium formation, which was due to inhibition of TLP activity by remaining leupeptin that was not inactivated. The results suggested the possible regulatory role of LIE in morphological differentiation of *S. exfoliatus* SMF13; LIE inactivates leupeptin when or where TLP activity is necessary.

In summary, we have reported here the purification and characterization of LIE from the culture supernatant of *S.*

exfoliatus SMF13. It is an extracellular, leucine-specific protease. LIE is clearly a novel enzyme, based on the substrate specificity and partial N-terminal sequence. The role of LIE in regulating the activities of leupeptin and TLP provides an insight into the morphological differentiation of *Streptomyces* spp.

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