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Characteristics of Trypsin-like Protease and Metalloprotease Associated with Mycelium Differentiation of *Streptomyces albidoflavus* SMF301

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Trypsin like protease (TLP) and metalloprotease (MTP) were induced in associated with the mycelium differentiation in *Streptomyces albidoflavus* SMF301. TLP and MTP were purified and characterized from the culture. The molecular mass of TLP and MTP were estimated to be 32 kDa and 18 kDa, respectively. The optimum pH and temperature of TLP were 10 and 40℃. Those of MTP were 8 and 55℃. TLP was stable at alkaline pH (6~9) and unstable above 45℃ and MTP was stable at alkaline pH and unstable above 80℃. Km and Vmax values with benzoyl-arginyl p-nitroanilide of TLP were 139 μM, and 10 nmole of nitroanilide released per min per μg protein, respectively. Km, and Vmax values with a synthetic substrate, leucine p-nitroanilide, of MTP were 58.9 μM, and 3.47 nmol of nitroanilide released per min per μg protein, respectively. TLP was inhibited competitively by leupeptin; the inhibition constant was 0.0031 μM. MTP was inhibited by EDTA, phenonthroline and bestatin.

Key words: *Streptomyces albidoflavus*, trypsin-like protease, metalloprotease, purification, morphological differentiation

Streptomycetes are Gram-positive bacteria with an unusual morphological complexity. Growth of *Streptomyces* on solid media progresses as a sequential formation of substrate mycelium and aerial hyphae (25). The aerial hyphae development was supported at least partially by the utilization of degraded substrate mycelium (15, 25). It has been thought that the aerial mycelium has little access to other sources of nourishment for its growth (4). Little has been learned of the mechanism providing nutrients for aerial mycelium formation. In recent, the role of protease on the differentiation and the supply of nutrient by autolysis has been reported. *Streptomyces exfoliatus* SMF13 produced leupeptin, trypsin like protease and leupeptin inactivating enzyme. Trypsin like protease functions as an essential enzyme involving in cellular turnover metabolism of the mycelial protein in *Streptomyces exfoliatus* SMF13 (12). Streptomycetes are known to produce a variety of extracellular proteases (18). The secretion of extracellular proteolytic enzymes in streptomycetes often coincides temporally with the onset of secondary metabolism or morphological differentiation (2, 7, 8). We isolated a strain of *Streptomyces* producing extracellular proteases and submerged spores (20). The strain was identified as *Streptomyces albidoflavus* by a numerical identification matrix, the TAXON program (20). The physico-chemical properties of spores and the kinetics were studied (14, 21). Since production of extracellular proteases were closely related to morphological differentiation (11), the characteristics of these enzyme was evaluated.

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Materials and Methods

Microorganism and media

The microorganism used in this study was *Streptomyces albidoflavus* SMF13 (20). Stock culture medium consisted of (w/v): 1% glucose, 0.2% peptone, 0.3% yeast extract, 0.1% beef extract and 1.8% agar for solid culture. Seed culture medium contained (w/v): 3.0% glucose, 1.8% soytone, 0.3% peptone and 0.4% $CaCO_3$. Main cul-

ture medium consisted of (w/v): 0.5% glucose, 0.5% Na-caseinate, 0.01% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $CuSO_4 \cdot 5H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$ and 0.0003% $MnCl_2 \cdot 4H_2O$. The initial pH of the media was adjusted to 7.0 before steam sterilization; the phosphate and each of the salts were separately sterilized by membrane filtration (0.2 μm, Millipore).

Strain maintenance and culture conditions

The strain was maintained by transferring to slopes of the stock culture medium each month and storing at 4℃. Spores in YED media were harvested by filtering through cotton. The spore suspension was kept in a refrigerator until use. For surface culture, about 10^3 spores were inoculated evenly on agar plates of main culture medium and incubated at 28℃. For submerged batch culture, the spore suspension was inoculated as a final concentration of 10^6 spores ml^{-1} into 50 ml of seed culture medium contained in a 500 ml baffled flask and cultured at 28℃ for 36 h in a rotary shaking incubator (200 rpm). The seed culture was inoculated (5% as an inoculum size) to 3 litre of the main culture medium contained in a vessel of a jar fermentor (5 litre; Korea Fermentor Co.). The culture temperature was maintained at 28℃ and the initial pH was controlled at 7.0. Agitation and aeration were 200 rpm and 1 v/v/min, respectively.

Analysis of mycelial growth and ammonium ion in submerged culture

Mycelia in submerged cultures were harvested aseptically and centrifuged at 10,000×g for 10 min, washed twice with physiological saline solution and once with distilled water. The washed mycelia were collected through vacuum filtration (Whatmann filter paper GF/C), dried at 80℃ for 24 h, and then the dried weight was measured. The concentration of ammonium ions was analyzed by a specific ion analyzer (Model EA940; Orion Research).

Assay of proteases in broth

The culture broth of submerged culture was centrifuged at 10,000×g for 10 min and supernatant was used for assay. Total protease activity was estimated by measuring the concentration of tyrosine liberated from Hammarsten casein (Merck) at 37℃ and pH 7.5 (Tris-HCl buffer, 0.1 M) for 15 min. One unit of casein hydrolytic activity (caseinase) was defined as the amount of enzyme needed for the production of 1 μg of tyrosine equivalent per min from the casein (16). Hydrolytic activity toward synthetic substrate was estimated by measuring the amount of p-nitroanilides liberated from the synthetic substrates, N-Benzoyl-Arg p-nitroanilide (BAPNA) and Leucine p-nitroanilide (LPNA). Enzyme reactions were carried out with 200 μM of aminoacyl p-nitroanilide at 35℃ and pH 7.5 (Tris-HCl buffer 0.1 M). Activity was calculated from the linear part of the curve, using $E_{405} = 9620\ Mol^{-1}\ cm^{-1}$. One unit of hydrolytic activity was defined as the amount of enzyme needed for the production of 1 μM of product (p-nitroanilide) per min (22).

Purification of trypsin like protease and metalloprotease

The culture broth was harvested by centrifugation (1,000×g for 15 min) and extracellular protease was purified as follows; ① Ammonium sulfate fractionation. The culture broth was brought to 45% saturation with ammonium sulfate and the supernatant was brought to 90% saturation with ammonium sulfate, and then centrifuged at 20,000×g for 30 min. ② Chromatography on GPC. The ammonium precipitate was applied to a column of GPC-Sephadex G-75 equilibrated with 0.1 M Tris-HCl and then eluted with same buffer. ③ Chromatography on DEAE-Sephadex. The active fraction was applied to a column of DEAE-Sephadex A-50 equilibrated with 0.1 M Tris-HCl (pH 7.5) and 0.2 M NaCl, and then eluted with the same buffer and a linear gradient of NaCl (0.2 M to 0.7 M). The active fraction was concentrated by ultrafiltration in 0.01 M Tris-HCl (pH 7.5). ④ Chromatography on QAE-Sephadex. The active fraction was applied to a column of QAE-Sephadex A-50 equilibrated with 0.01 M Tris-HCl (pH 7.5), and then eluted with the same buffer and a linear gradient of NaCl (0 M to 0.7 M). The active fraction was concentrated by ultrafiltration in 0.01 M Tris-HCl (pH 7.5).

Molecular mass determination

The molecular mass of the purified protease was estimated by SDS-PAGE. SDS-PAGE was performed using the method of Laemmeli (13) with 12% (w/v) separating gel and 5% (w/v) stacking gel. After electrophoresis, the gel was silver stained.

Substrate gel electrophoresis

The protease activity was visualized in polyacrylamide gels by incorporating 0.1% gelatin into a SDS-PAGE gel (12%). After electrophoresis at 4℃ was complete, the gel was incubated at 35℃ for 3 h in 0.1 M phosphate buffer (pH 7.5) and then stained with Coomassie brilliant blue.

Effect of pH and temperature

The optimum pH towards BAPNA and LPNA was measured over a pH range from 3.0 to 11.0. The effect of pH on enzyme stability was determined by using the

same buffer systems in the pH ranges of 3.0 to 11.0. After preincubation for 90 min at 30℃, the remaining activity was measured. The optimum temperature was determined within a range of 20 to 60℃ at the optimum pH. For evaluation of thermal stability, the enzyme solution was held at various temperatures and the remaining activities was measured as a function of time intervals.

Substrate specificity

The kinetic parameters for hydrolysis of BAPNA and LPNA were measured as mentioned above. For the determination of kinetic parameters, concentrations of BAPNA and LPNA were from 2.5 μM to 1000 μM.

Effect of inhibitors

The protease solution with protease inhibitors such as, leupeptin, antipain, tosyl lysyl chloromethyl ketone (TLCK), tosyl phenylalanyl chloromethyl ketone (TPCK), EDTA, E-64 and pepstatin were preincubated for 5 min. The remaining activities of protease were measured. The activity of protease inhibitors was calculated from the following relation: % Inhibition = 100×(A-B)/A, where A is protease activity without the inhibitor and B is protease activity with the inhibitor (1).

Chemicals, reagents and reproducibility

All synthetic substrates and enzyme inhibitors were purchased from Sigma Chemical Co.. All other chemicals were of reagent grade. Each experiment was repeated at least twice and their mean values are given.

Results

Analysis of protease production in the submerged culture

Biomass increased as the spores germinated, but new spores were formed from the middle of exponential growth phase and the biomass declined without an extended stationary phase (Fig. 1). The extracellular protease activity increased as culture time elaspsed, and the ammonium ions accumulated with the activity (Fig. 1). Substrate active staining of culture broth was performed to show the total protease pattern (Fig. 2). As a result, 8 proteases were found in the submerged culture of *Streptomyces albidoflavus* SMF301 (Fig. 2). In order to identify the proteases, the substrate gel after electrophoresis was preincubated with various specific protease inhibitors (Fig. 3 and 4). The two specific bands were disappeared by leupeptin and EDTA. The target bands were considered as a serine protease (trypsin like protease) and a metalloprotease (Fig. 3 and Fig. 4). The two proteases were produced just before the onset of

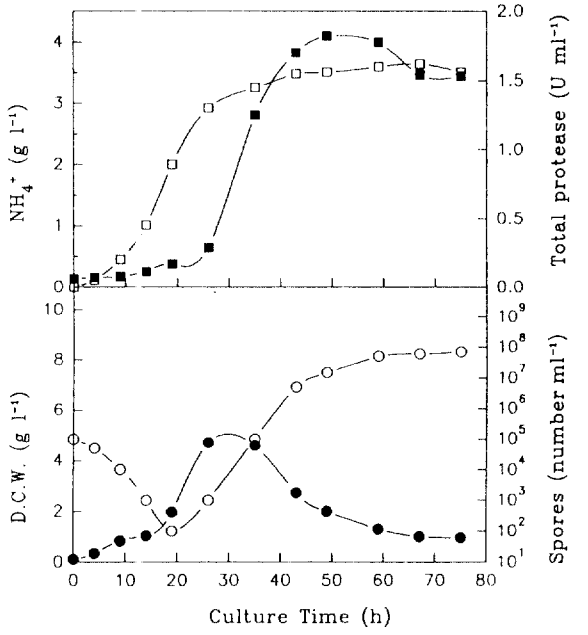

Fig. 1. Changes of ammonium ion (■), total protease activity (□), biomass (●) and spore (○) during the batch culture using Na-caseinate as a sole nitrogen source. (30℃, 1 VVM, 300 RPM, pH 7.0).

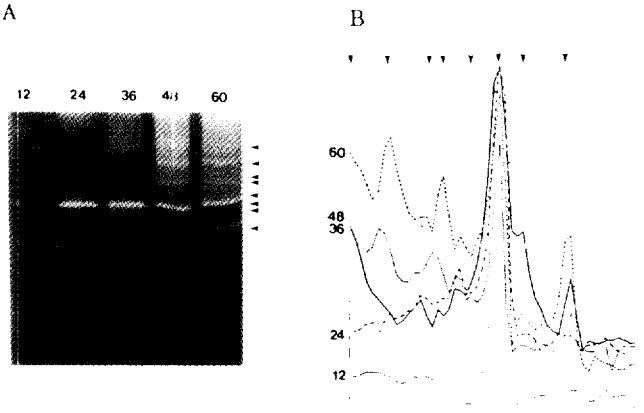

Fig. 2. Analysis of proteases produced during batch culture of *Streptomyces albidoflavus* SMF301 using substrate gel electrophoresis. (A): substrate gel activity staining (B): densitometry.

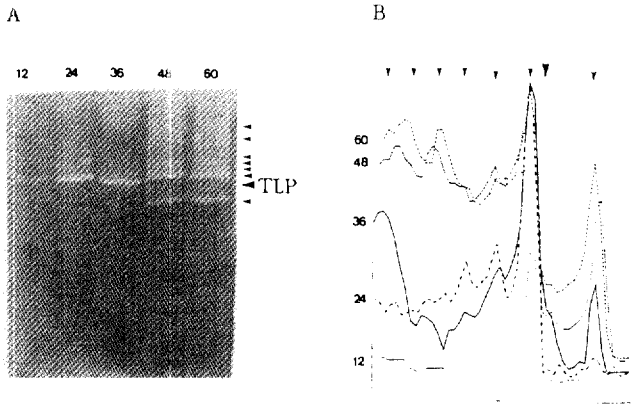

Fig. 3. Identification of proteases inhibited by leupeptin or TLCK. (A): substrate gel activity staining (B): densitometry.

submerged spore formation. The activity of metalloprotease was still high in the decline phase, but that of serine protease decreased earlier than the metalloprotease (Fig. 2).

Purification of trypsin-like protease and metalloprotease

The results of the purification of TLP and MTP from the cell free culture broth are summarized in Table 1. TLP was purified 37.2 fold with a recovery of 6.9% and MTP was 42.8 fold with a recovery of 8.9%. The purified enzyme was shown on SDS-PAGE (Fig. 5), and the molecular mass of TLP and MTP were 32 kDa and 18 kDa.

Optimum reaction conditions and stability of the purified TLP and MTP

The optimum pH and temperature of TLP were 10 and 40℃, respectively (Fig. 6A and Fig. 7A). TLP was stable in both neutral and alkaline pH, over 70% of the activity remaining compared to the original activity by preincubation at 30℃ for 90 min in buffers. However, the protease was unstable at acidic pH and a pH higher than 10 (data not shown). TLP was also unstable at temperatures above 45℃. More than 70% of the activity was lost by treatment at 50℃ for 10 min (data not shown).

The optimum pH and temperature of MTP for the hydrolysis of a synthetic substrate (LPNA) were 8 and 55℃, respectively (Fig. 6B and Fig. 7B). The enzyme was stable in both neutral and alkaline pH, over 70% of the activity remaining compared to the original activity by preincubation at 40℃ for 90 min in buffers. However,

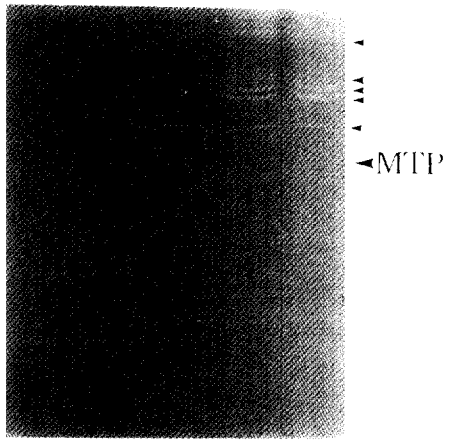

Fig. 4. Identification of proteases inhibited by EDTA.

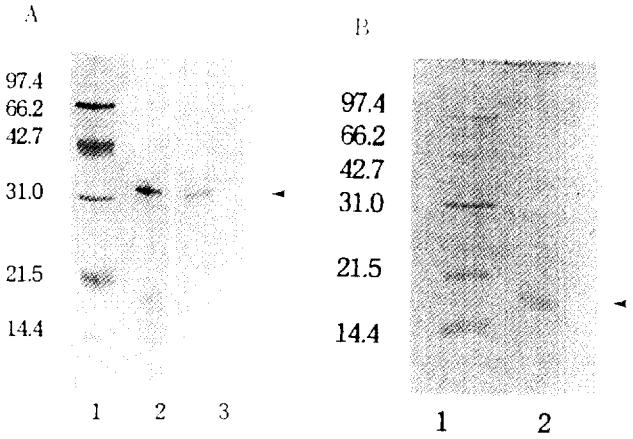

Fig. 5. SDS-polyacrylamide gel electrophoresis of TLP (A) and MTP (B) purified from *Streptomyces albidoflavus* SMF301. (A) Lanes: 1, molecular weight standards; 2, partially purified TLP; 3, purified TLP. (B) Lanes: 1, molecular weight standards; 2, purified MTP.

Table 1. Purification of TLP and MTP from *Streptomyces albidoflavus* SMF301

(A) TLP

Purification Step	Protein (mg)	Activity (units)	Specific activity (units mg protein^{-1})	Purification fold	Yield (%)
Culture broth	720.0	856.0	1.18	1.0	100.0
$(NH_4)_2SO_4$ fractionation	359.0	514.0	1.40	1.2	60.2
GPC-75	96.0	264.0	2.75	2.3	31.0
DEAE-Sephadex	6.6	140.0	21.20	17.9	16.3
QAE-Sephadex	1.4	59.6	44.10	37.2	6.9

(B) MTP

Purification Step	Protein (mg)	Activity (units)	Specific activity (units mg protein^{-1})	Purification fold	Yield (%)
Culture broth	720	61490	85	1.0	100
Ammonium sulfate fractionation	359	36894	103	1.2	60
GPC-75	95	19062	199	2.3	31
DEAE-Sephadex	4.8	8184	1705	19.9	13
AE-Sephadex	1.5	5477	3651	42.8	9

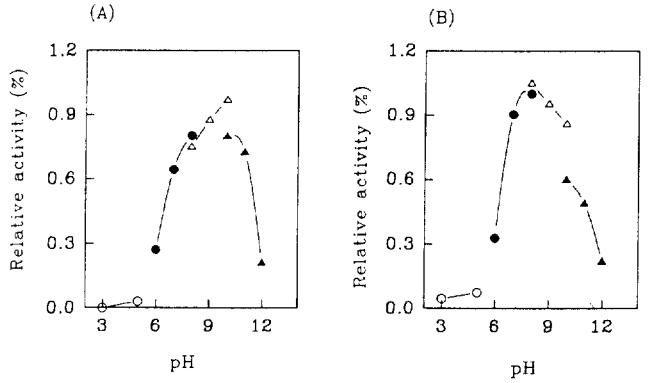

Fig. 6. Effect of pH on the activity of TLP (A) and MTP (B). (○), 0.1 M acetate buffer; (●), 0.1 M phosphate buffer; (△), Tris-HCl buffer; (▲), Carbonate buffer.

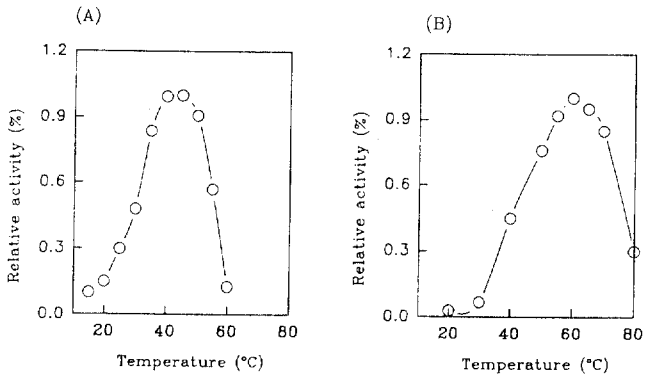

Fig. 7. Effect of temperature on the activity of TLP (A) and MTP (B).

Table 2. Hydrolysis of protein substrate by TLP and MTP by *Streptomyces albidoflavus* SMF301

Substrate	Wavelength	TLP (Units μg^{-1})	MTP (Units μg^{-1})
Albumin (bovine)	280	0.18	17.5
Albumin (egg)	280	0.45	ND
Azocasein	440	0.42	ND
Hammarsten casein	280	7.78	331.
Collagen	280	ND*	ND
Elastin	280	0.04	17
Elastin Congo red	495	0.05	38.9
Haemoglobin	280	7.9	75

ND, not detected.

the protease was unstable at acidic pH and a pH higher than 10. The protease was also unstable at temperatures above 80℃ (data not shown).

Substrate specificity and kinetic parameters

TLP obtained from *S. albidoflavus* SMF301 hydrolyzed a broad range of native proteins such as bovine serum albumin, egg albumin, Hammarsten casein, and haemoglobin (Table 2). The activity against casein and haemoglobin was greater than that against albumin, and elastin. MTP also hydrolyzed a broad range of native proteins such as bovine serum albumin, Hammarsten casein, elastin, and haemoglobin (Table 2).

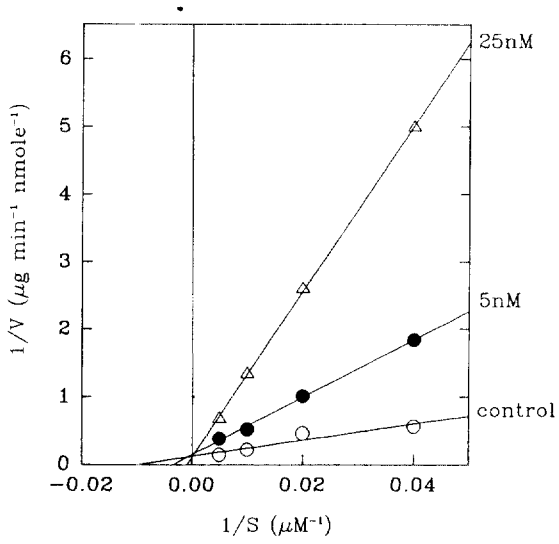

Fig. 8. Interaction kinetics between TLP and leupeptin. Lineweaver-Burk plot of BAPNA concentration against the rate of hydrolysis of trypsin like protease in the absence and presence of leupeptin.

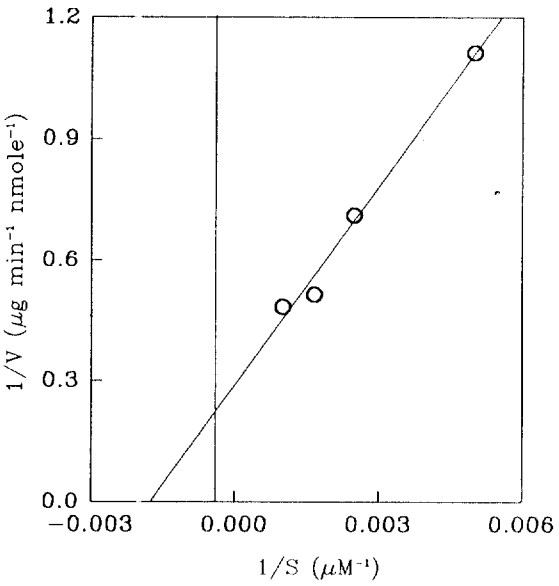

Fig. 9. Lineweaver-Burk plot of LPNA concentration against the rate of hydrolysis by metalloprotease.

TLP could hydrolyze the arginine site but could not hydrolyze chymotrypsin substrates such as N-benzoyl-Tyr p-nitroanilide and N-succinyl-Gly-Gly-Phe p-nitroanilide. The kinetic parameter (Km and Vmax) of BAPNA were 139 μM^{-1}, 0.0061 nmole min^{-1} μg^{-1} (Fig. 8).

MTP could hydrolyze the leucine site but could not hydrolyze other substrates such as N-benzoyl-Tyr p-ni-

Table 3. Effects of protease inhibitors in the activity of TLP and MTP

Inhibitor	Class of inhibitor	Concentration	Inhibition of TLP (%)	Inhibition of MTP (%)
No addition			ND	ND
Leupeptin	Serine/Cysteine	50 μM	100	ND
Antipain	Serine/Cysteine	50 μM	99	ND
TLCK	Trypsin-like serine	50 μM	100	6
Chymostatin	Serine/Cysteine	50 μM	39	ND
TPCK	Chymotrypsin-like serine	50 μM	14	ND
EDTA	Metallo	1 mM	20	100
Phenonthroline	Metallo	1 mM	10	100
Bestatine	Metallo	50 μM	ND	70
E-64	Cysteine	10 μM	2	ND
Pepstatin	Aspartic	10 μM	11	ND

ND; not detected.

Table 4. Effect of metal ions on the activity of TLP and MTP

Metal salts	Relative activity (%) of TLP	Relative activity (%) of MTP
None	100	100
$CaCl_2 \cdot 2H_2O$	110	290
$CoCl_2 \cdot 6H_2O$	87	450
$CuSO_4 \cdot 7H_2O$	90	95
$HgCl_2$	15	30
$MgSO_4 \cdot 7H_2O$	95	92
$MnCl_2 \cdot 2H_2O$	97	90
$ZnSO_4 \cdot 7H_2O$	83	104

troanilide, N-benzoyl-Arginine p-nitroanilide and N-succinyl-Gly-Gly-Phe p-nitroanilide. Therefore, the protease was thought to be a leucine amino peptidase. The kinetic parameter (Km and Vmax) of LPNA were 58.9 μM^{-1}, 3.47 nmole min^{-1} μg^{-1} (Fig. 9).

Inhibitor specificity and inhibition kinetics

The activity of TLP was completely inhibited by 50 μM antipain or leupeptin (serine/cysteine protease inhibitor) and by 50 μM TLCK (trypsin specific inhibitor). However, the activity of TLP was inhibited less than 15% by 50 μM E-64 (cysteine protease inhibitor), pepstatin (aspartic protease inhibitor), TPCK (chymotrypsin specific inhibitor) and 1 mM EDTA (metallo-protease inhibitor) (Table 3). TLP was competitively inhibited by leupeptin, the inhibition constant being 0.00615 μM (Fig. 8).

The activity of MTP was completely inhibited by 50 μM EDTA or EGTA (metallo protease) and by 50 μM bestatin. However, the activity of MTP was inhibited less than 15% by 50 μM E-64 (cysteine protease inhibitor), pepstatin (aspartic protease inhibitor), TPCK (chymotrypsin specific inhibitor) and 1 mM EDTA (metallo-protease inhibitor) (Table 3). The activity of MTP was stimulated by Co^{2+} and Ca^{2+} at a concentration of 5 mM, but Mn^{2+}, Mg^{2+} and Zn^{2+} did not affect the activity, and Hg^{2+} decreased the activity (Table 4).

Discussion

In this study, we have examined the physicochemical and enzymatic properties of purified protease which might be associated with spore formation in *Streptomyces albidoflavus* SMF301. The results on substrate and inhibitor specificities support the conclusion that enzymes can be classified as members of trypsin like protease and the metalloprotease (leucine endopeptidase).

The molecular mass of TLP from *S. albidoflavus* SMF 301 was higher than those of other TLPs reported from *Streptomyces* spp.: 19 kDa for *S. griseus* (23), 19 kDa for *S. moderatus* (3), 21.4 kDa for *S. erythreus* (10), 22~25 kDa for *S. paromomycinus* (5), 28 kDa for *S. rimosus* (19), and 31.5 kDa for *Streptomyces* 771 (17). TLP from *S. albidoflavus* SMF301 showed similar pH optimum (7.5) and thermal instability to those of other TLPs from *Streptomyces*.

The Km (0.139 mM) of TLP from *S. albidoflavus* SMF 301 for BAPNA was within the range of previously reported values: 0.476 mM for *S. moderatus* (3), 0.029 mM for *S. paromomycinus* (5), 0.101 mM for *S. griseus* (9), 0.328 mM for *S. rimosus* (19), and 0.039 mM for *S. erythreus* (27). Those values are much lower than that of bovine trypsin (1.49 mM) (27).

MTP activity was inhibited by EDTA and reactivated by Co^{2+} in the course of hydrolysis of LPNA. The enzyme was quite stable at 70°C. The enzyme was found to be inhibited at relatively high concentration of chymostatin. However, thermolysin and other metalloprotease were also inhibited with 100 μM chymostatin. The inactivation effect could be explained partially by the hydrophobic nature of chymostatin (24).

The molecular weight of this protease is somewhat lower than those for other metalloendopeptidases such as thermolysin, M, 34,600, *Bacillus subtilis* neutral pro-

tease, M_r 33,800, (26) and *Pseudomonas aeruginosa* elastase, M_r 32,900, (6). *Streptomyces albidoflavus* SMF301 produced 8 extracellular proteases in the batch culture using Na-caseinate as a sole nitrogen source. Trypsin like protease and MTP started to be produced at 24 h and the production time of TLP and MTP were associated with the submerged spore formation.

The proteases might be involved in submerged spore formation and aerial spore formation although the molecular mechanism is not clear yet. It is the first step to elucidate the role of protease on differentiation in *Streptomyces*. The molecular work will be followed to show the role clearly.

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