Production dynamics of extracellular proteases accompanying morphological differentiation of *Streptomyces albidoflavus* SMF301

Sung Gyun Kang, In Seop Kim, Yong Taik Rho and Kye Joon Lee

Author for correspondence: Kye Joon Lee. Tel: +82 2 880 6705. Fax: +82 2 882 9285/888 4911.
e-mail: LKJ12345@alliant.snu.ac.kr

Department of Microbiology, College of Natural Sciences, and Research Centre for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

Three proteases, namely chymotrypsin-like protease (CTP), trypsin-like protease (TLP) and metalloprotease (MTP), were identified in cultures of *Streptomyces albidoflavus* SMF301. The dynamics of protease production were determined and the roles of the proteases in morphological differentiation were deduced to be as follows: CTP is essential for hydrolysing the proteinaceous nitrogen source for mycelium growth; TLP plays a role in the formation of thickened mycelium in submerged culture and of aerial mycelium in solid culture; MTP may participate in the maturation of spores. The unique thickened mycelia in submerged culture are thought to be an intermediate form between mycelium and spores. TLP (molecular mass 32 kDa) and MTP (molecular mass 18 kDa) were purified and their enzymic properties were determined.

Keywords: *Streptomyces albidoflavus*, trypsin-like protease, metalloprotease, submerged spore, morphological differentiation

INTRODUCTION

Aerial spore formation by streptomycetes on solid culture follows aerial mycelium formation, which is stimulated by limiting essential nutrients (Chater, 1984; Hopwood *et al.*, 1970). The growth of aerial mycelium and formation of spores is supported by the utilization of degraded substrate mycelium (Chater, 1989, 1993; Granozzi *et al.*, 1990) because the aerial mycelium has limited access to nutrients (Mendez *et al.*, 1985). Physiological and morphological differentiation in streptomycetes are closely regulated by nitrogen nutrition; extracellular proteases participate in the assimilation of external proteinaceous nitrogen sources (Shapiro, 1989). Although the precise mechanism and role of proteases in growth and differentiation are not well understood, serine protease expressed in stationary-phase cultures of *Streptomyces peucetius* and *Streptomyces lactamdurans* is reported to regulate the turnover metabolism associated with formation of secondary metabolites and morphogenesis (Gibb & Strohl, 1988; Ginther, 1978).

A recent study of the regulation of protease activities during growth and mycelium development concluded that a trypsin-like protease (TLP) was essential for the degradation of mycelial protein in *Streptomyces exfoliatus* SMF13, and that protease production was regulated by an endogenous inhibitor (Kim & Lee, 1995).

Sporulation can take place in submerged cultures of some *Streptomyces* spp. (Kendrick & Ensign, 1983; Daza *et al.*, 1989; Koepsel & Ensign, 1984; Glazebrook *et al.*, 1990), providing greater possibilities for physiological analysis. *Streptomyces albidoflavus* SMF301 produces abundant spores in submerged and solid cultures (Rho *et al.*, 1992). The physical and chemical properties of submerged spores and aerial spores have been compared (Lee & Rho, 1993), and the kinetics of spore formation reported (Rho & Lee, 1994). To study the possible role of proteases in differentiation, the activities of extracellular proteases in relation to morphological differentiation in submerged and solid cultures were characterized and extracellular proteases in the culture broth of *S. albidoflavus* were purified.

METHODS

Micro-organism and media. *Streptomyces albidoflavus* SMF301 (Rho *et al.*, 1992) and bald mutants were used. Bald mutants were derived by UV irradiation and cultivation at high

Abbreviations: BAPNA, *N*-Benzoyl-arginine *p*-nitroanilide; BTPNA, *N*-benzoyl-tyrosine *p*-nitroanilide; CTP, chymotrypsin-like protease; LPNA, leucyl *p*-nitroanilide; MTP, metalloprotease; TLCK, *N* α -*p*-tosyl-L-lysine chloromethyl ketone; TLP, trypsin-like protease; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

temperature (Hara & Beppu, 1982; Hopwood *et al.*, 1985). 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. The seed medium and the main culture medium consisted of (w/v): 0.5% glucose, 0.5% sodium caseinate, 0.01% KH_2PO_4 , 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.001% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.0003% $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. The initial pH of the media was adjusted to 7.0 before steam sterilization. Phosphate and other salts were separately sterilized by membrane filtration (0.2 μm , Millipore).

Strain maintenance and culture conditions. Strains were maintained by transfer to slopes of stock culture medium each month, and were stored at 4 °C. For solid cultures, about 10^3 spores harvested from the stock culture agar medium were inoculated evenly on the main agar culture medium and incubated at 28 °C. For submerged batch culture, one loopful of mycelium and spores was used to inoculate 30 ml seed culture medium and incubated at 28 °C for 2 d. The seed culture was used to inoculate 3 l of the main culture medium contained in a jar fermenter (model KF-5L, Korea Fermentor). The culture temperature was maintained at 28 °C and the initial pH was controlled at 7.0. Agitation and aeration were 200 r.p.m. and 1 v $\text{v}^{-1} \text{min}^{-1}$, respectively.

Observation of mycelium morphology and growth. Morphological characteristics were observed with a phase-contrast microscope (Nikon Laphot) and a scanning electron microscope (Stereoscan 260 SEM). 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. 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 a Polaron SC502 sputter coater (Fisons) at 15 mA for 1 min under vacuum conditions. The morphology of colonies was observed with a Stereoscan 260 scanning electron microscope (Cambridge).

To measure mycelium growth, mycelium was harvested by centrifugation (10000 g, 10 min) and washed twice with physiological saline solution and once with distilled water. The washed mycelium was collected by vacuum filtration (Whatman GF/C paper), dried at 80 °C for 24 h, and weighed.

Measurement of submerged spore formation. The culture broth (5 ml) was sonicated for 5 min at 100 W using a sonic dismembrator (model 300, Fisher). The sonicated suspension (0.5 ml) was mixed gently with 0.1 M HCl (4.5 ml) and the mixture was allowed to stand for 5 min before it was diluted in physiological saline and spread on rich agar medium. Colonies were counted after 4 d incubation to give initial spore numbers (Lee & Rho, 1993).

Assay of proteases. The culture broth was centrifuged at 10000 g for 10 min and the supernatant was used for assay. Total protease activity was estimated by measuring the concentration of tyrosine liberated from Hammarsten casein (Merck) at 37 °C 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 to produce 1 μg tyrosine equivalent min^{-1} from the casein (Narahashi, 1970). Hydrolytic activity for synthetic substrates was estimated by measuring the amount of *p*-nitroanilides liberated from various synthetic substrates: *N*-benzoyl-tyrosine *p*-nitroanilide [BTPNA; the specific synthetic substrate of chymotrypsin-like protease (CTP)], *N*-benzoyl-arginine *p*-nitroanilide (BAPNA;

the specific synthetic substrate of TLP) and leucyl *p*-nitroanilide [LPNA; the specific synthetic substrate of metalloprotease (MTP)]. Enzyme reactions were carried out with 200 μM aminoacyl *p*-nitroanilides at 35 °C and pH 7.5 (Tris/HCl buffer, 0.1 M). The activity was calculated from the linear part of the curve, using $\epsilon_{405} = 9620 \text{ mol}^{-1} \text{cm}^{-1}$. One unit of hydrolytic activity was defined as the amount of enzyme needed to produce 1 μmol *p*-nitroanilide min^{-1} (Sarath *et al.*, 1989).

Purification of proteases. The culture broth was harvested by centrifugation (10000 g, 15 min) and fractionated as follows.

(1) **Ammonium sulfate precipitation.** The culture broth was brought to 45% saturation with ammonium sulfate followed by centrifugation (20000 g, 30 min). The supernatant was brought to 90% saturation with ammonium sulfate and then centrifuged at 20000 g for 30 min. The fraction precipitated between 45–90% ammonium sulfate saturation was dissolved in distilled water and desalted by ultrafiltration.

(2) **Gel-permeation chromatography.** The active fraction obtained from the ammonium sulfate fractionation was applied to a column of Sephadex G-75 equilibrated with 0.1 M Tris/HCl (pH 7.5) and then eluted with the same buffer.

(3) **Chromatography on DEAE-Sephadex.** The active fraction from the gel-permeation chromatography 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–0.7 M). The active fraction was concentrated by ultrafiltration in 0.01 M Tris/HCl buffer (pH 7.5).

(4) **Chromatography on QAE-Sephadex.** The active fraction from the DEAE-Sephadex 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–0.7 M). The active fraction was concentrated by ultrafiltration in 0.01 M Tris/HCl (pH 7.5).

Effect of pH and temperature. Optimum pH for the hydrolysis of specific synthetic substrates was measured over a range of pH 3.0–11.0. The effect of pH on enzyme stability was determined by preincubation for 90 min at 30 °C in the same range of pH and the remaining activity was measured at the optimum pH. Optimum temperature was determined within a range of 20–60 °C at the optimum pH. For evaluation of thermal stability, the enzyme solution was held at various temperatures and the remaining activity was measured as a function of reaction time at the optimum temperature.

Substrate specificity and effect of inhibitors. The kinetic parameters for synthetic substrates were measured by the procedures described above. For the determination of kinetic parameters, the concentrations of synthetic substrates were from 2.5 μM to 1000 μM . Protease inhibitors, including leupeptin, antipain, *N* α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), EDTA, amastatin, EGTA, penanthroline and E-64 were preincubated with the protease solution for 5 min. The protease activity remaining was measured. The percentage inhibition was calculated from the following relationship: percentage inhibition = $100\{(A-B)/A\}$, where *A* is protease activity without the inhibitor and *B* is protease activity with the inhibitor (Aoyagi *et al.*, 1969).

Chemicals, reagents and reproducibility. All synthetic substrates and enzyme inhibitors were purchased from Sigma. Experiments were carried out in triplicate, and mean values are given.

RESULTS

Mycelium growth, submerged spore formation, and production of extracellular proteases in batch culture

The substrate specificity and inhibition profile of extracellular proteases in *S. albidoflavus* SMF301 cultures were evaluated (Table 1). Specific synthetic substrates such as BTPNA (for CTP), BAPNA (for TLP), and LPNA (for MTP) were hydrolysed effectively. The inhibition specificity was also evaluated for each protease: BTPNA-hydrolysing activity was inhibited by chymostatin and TPCK; BAPNA-hydrolysing activity was inhibited by leupeptin, TLCK, and antipain; LPNA-hydrolysing activity was inhibited by EDTA, EGTA and amastatin. It was concluded that *S. albidoflavus* SMF301 produces at least three types of protease: the activity on BTPNA is due to a CTP, that on BAPNA is due to a TLP and that on LPNA is due to an MTP (Table 1).

Changes in mycelium growth, submerged spore formation and the activities of CTP, TLP and MTP during batch culture are shown in Fig. 1. Biomass increased as the spores germinated, but new spores were formed from the middle of the exponential growth phase and the biomass declined without an extended stationary phase (Fig. 1a). CTP was the major extracellular protease when sodium caseinate was used as a sole nitrogen source, and mycelium growth was associated with the activity of CTP (Fig. 1b). TLP and MTP started to be produced just before the onset of submerged spore formation. The activity of MTP was high in the decline phase, but that of TLP decreased earlier than MTP (Fig. 1b). Submerged spore formation correlated with the increased activities of TLP and MTP (Fig. 1c).

Effects of external addition of specific protease inhibitors on mycelium growth and submerged spore formation

To assess whether proteases have a role in growth and differentiation, individual protease activities were eliminated from the culture by adding specific inhibitors

Fig. 1. Changes in growth, concentration of submerged spores, CTP, TLP and MTP, and morphology during submerged culture of *S. albidoflavus* SMF301. (a) Growth (○) and submerged spores (●); (b) CTP (▽), TLP (△) and MTP (▲); (c) morphology.

(TPCK, TLCK, amastatin and EDTA). Spore germination and further mycelium growth were completely inhibited by adding the CTP inhibitor TPCK (0.1 mM) at the beginning of the culture (data not shown). When TPCK was added at 24 h, CTP activity was inhibited

Table 1. Effect of protease inhibitors on hydrolysis of synthetic substrates by broth of *S. albidoflavus* SMF301

The protease activity was mixed with 100 µl cell-free broth from 36 h and 48 h cultures. After preincubation for 5 min, the rate of BAPNA, BTPNA and LPNA hydrolysis was measured. Hydrolytic activity is given as U ml⁻¹ with the degree of inhibition (%) in parentheses.

Substrate	Inhibitor (final concentration)									
	Control	Leupeptin (50 µM)	Antipain (50 µM)	Chymostatin (50 µM)	TLCK (50 µM)	TPCK (50 µM)	E-64 (50 µM)	EDTA (1 mM)	EGTA (1 mM)	Amastatin (1 mM)
BAPNA	1.23	0.01 (100)	0.02 (99)	0.95 (23)	0.01 (100)	0.89 (28)	1.14 (8)	0.98 (21)	0.95 (23)	1.17 (5)
BTPNA	4.32	4.13 (5)	4.12 (5)	2.53 (42)	4.25 (2)	1.62 (43)	4.26 (2)	4.12 (5)	4.25 (2)	4.32 (0)
LPNA	0.23	0.21 (8)	0.23 (0)	0.20 (14)	0.22 (5)	0.24 (0)	0.26 (0)	0.02 (92)	0.01 (96)	0.02 (92)

Fig. 2. Effects of TPCK on growth, submerged spore formation, CTP, TLP and MTP, and changes in morphology during submerged culture of *S. albidoflavus* SMF301. TPCK was added to the culture at the time indicated (↓). (a) Growth (○) and submerged spores (●); (b) CTP (▽), TLP (△) and MTP (▲); (c) morphology.

immediately, whereas the activities of TLP and MTP were not greatly changed. Mycelium growth was also inhibited immediately, but the mycelium differentiated slowly and continuously to give submerged spores (Fig. 2a–c).

When the TLP inhibitor TLCK (0.1 mM) was added to the culture broth at 24 h, submerged spore formation was suppressed, but mycelium growth was not greatly affected (Fig. 3a). The activity of TLP was completely inhibited but CTP and MTP were not affected (Fig. 3b). Mycelial fragmentation or submerged spore formation was rarely observed in the presence of TLCK (Fig. 3c).

When MTP inhibitor (EDTA, 0.1 mM or amastatin, 0.1 mM) was added to the culture broth at 24 h, submerged spore formation was completely suppressed, but mycelium growth was not affected (Fig. 4a). No MTP activity was detected during the 80 h incubation, while the activity of CTP increased late in the culture (Fig. 4b). From microscopic observations, the tips and other parts of the mycelium were thickened, but no submerged spores were detected (Fig. 4c).

Mycelium growth and production of CTP, TLP and MTP

Fig. 3. Effects of TLCK on growth, submerged spore formation, CTP, TLP and MTP, and changes in morphology during submerged culture of *S. albidoflavus* SMF301. TLCK was added to the culture at the time indicated (↓). (a) Growth (○) and submerged spores (●); (b) CTP (▽), TLP (△) and MTP (▲); (c) morphology.

in a submerged culture of a bald mutant are shown in Fig. 5. The mutant grew well but submerged spore formation and mycelium autolysis did not occur (Fig. 5c). The production of CTP was the same as in the parent strain, and was closely related to mycelium growth, but TLP and MTP were not found in the culture supernatant solution (Fig. 5a, b).

Effects of external addition of specific protease inhibitors on substrate mycelium, aerial mycelium and aerial spore formation

On agar medium and in the absence of protease inhibitors, *S. albidoflavus* SMF301 grew with the sequential formation of substrate mycelium, aerial mycelium and aerial spores (Fig. 6a). However, substrate mycelium growth was completely inhibited by the CTP inhibitor; addition of TPCK (0.1 mM) to the centre of an agar plate gave a clear zone (data not shown). When TLP inhibitor (TLCK, 0.1 mM) was added in this way, aerial mycelium growth and further aerial spore formation in the region appeared to be inhibited (Fig. 6b). However, substrate mycelium

Fig. 4. Effects of EDTA on growth, submerged spore formation, CTP, TLP and MTP, and changes in morphology during submerged culture of *S. albidoflavus* SMF301. EDTA was added to the culture at the time indicated (↓). (a) Growth (○) and submerged spores (●); (b) CTP (▽), TLP (△) and MTP (▲); (c) morphology.

Fig. 5. Changes in growth, concentration of submerged spores, CTP, TLP and MTP, and morphology in submerged culture of a bald mutant of *S. albidoflavus* SMF301. (a) Growth (○) and submerged spores (●); (b) CTP (▽), TLP (△) and MTP (▲); (c) morphology.

growth was not inhibited, suggesting that TLP played a role in mycelium differentiation but not in the substrate mycelium growth. Substrate mycelium growth as well as aerial mycelium growth were not inhibited at 0.1 mM by the MTP inhibitors EDTA or amastatin, whereas aerial spore formation was completely inhibited (Fig. 6c). The results suggested that MTP might participate in the formation of spores from aerial mycelium.

Purification and characterization of proteases

Since TLP and MTP might be involved in the morphological differentiation of *S. albidoflavus* SMF301, the proteases were purified from the culture broth (Table 2a, b). Molecular masses of the purified TLP and MTP were estimated by SDS-PAGE to be 32 kDa and 18 kDa, respectively (Fig. 7a, b).

The purified TLP was stable from pH 6 to 9 but the optimum pH for the hydrolysis of a synthetic substrate (BAPNA) was pH 9. The optimum temperature was 40 °C; TLP was unstable at temperatures above 45 °C

(Table 3). The K_m and V_{max} values for BAPNA under optimum conditions were 139 μ M and 10 nmol min⁻¹, respectively. TLP was competitively inhibited by leupeptin and the inhibition constant of leupeptin (K_i) was 0.0031 μ M (Table 3). The activity of TLP was not affected by Ca²⁺, Mg²⁺ and Mn²⁺ at a concentration of 5 mM, but Hg²⁺ brought about a decrease in the activity (Table 4).

The optimum pH for hydrolysis of a synthetic substrate (LPNA) by MTP was pH 8 and the optimum temperature was 55 °C. The enzyme was stable in the range of pH 6–9, and below 55 °C. The K_m and V_{max} values for LPNA were 58.9 μ M and 3.47 nmol min⁻¹, respectively (Table 3). The activity of MTP was stimulated by Co²⁺ and Ca²⁺ at a concentration of 5 mM, but Mn²⁺, Mg²⁺ and Zn²⁺ did not affect the activity, and Hg²⁺ decreased the activity (Table 4).

Fig. 6. Morphological characteristics of *S. albidoflavus* SMF301 grown on solid culture with protease inhibitors. (a) No addition of protease inhibitors; (b) addition of TLP inhibitor (0.1 mM TLCK); (c) addition of MTP inhibitor (0.1 mM EDTA). Lanes: 1, scanning electron micrographs; 2, photographs of solid cultures. Scale-bars indicate 10 μ m, 10 μ m and 20 μ m in (a), (b) and (c) (lane 1), respectively.

Table 2. Purification of TLP and MTP from *S. albidoflavus* SMF301

Purification step	Protein (mg)	Activity (U)	Specific activity [U (mg protein ⁻¹)]	Purification (-fold)	Yield (%)
(a) Purification of TLP					
Culture broth	720	856	1.2	1.0	100
Ammonium sulfate fractionation	359	514	1.4	1.2	60.2
GPC-75	96	264	2.8	2.3	31.0
DEAE-Sephadex	6.6	140	21.2	17.9	16.3
QAE-Sephadex	1.4	59.6	44.1	37.2	6.9
(b) Purification of MTP					
Culture broth	720	61 490	85	1.0	100
Ammonium sulfate fractionation	359	36 894	103	1.2	60
GPC-75	96	19 062	199	2.3	31
DEAE-Sephadex	4.8	8184	1705	19.9	13
QAE-Sephadex	1.5	5477	3651	42.8	9

Fig. 7. SDS-PAGE of purified TLP and MTP. (a) Lanes: 1, standard protein markers; 2, partially purified TLP; 3, purified TLP. (b) Lanes: 1, standard protein markers; 2, purified MTP. Standard protein markers are: 99.6 kDa, phosphorylase *b*; 66 kDa, BSA; 45 kDa, egg albumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 kDa, hen egg white lysozyme). Arrows point to the purified TLP and MTP.

Table 3. Chemical and enzymic properties of purified TLP and MTP

Properties	TLP	MTP
Molecular mass	32 kDa	18 kDa
Optimum pH	9	8
Stable pH range	6–9	6–9
Optimum temperature (°C)	40	55
Stable temperature range (°C)	4–40	4–55
K_m	139 μ M	58.9 μ M
V_{max}	10 nmol min ⁻¹	3.47 nmol min ⁻¹
K_i of leupeptin	0.0031 μ M	

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 ·2H ₂ O	110	290
CoCl ₂ ·6H ₂ O	87	450
CuSO ₄ ·7H ₂ O	90	95
HgCl ₂	15	30
MgSO ₄ ·7H ₂ O	95	92
MnCl ₂ ·2H ₂ O	97	90
ZnSO ₄ ·7H ₂ O	83	104

DISCUSSION

Morphological differentiation in *S. albidoflavus* SMF301 is accompanied by the sequential production of CTP, TLP and MTP. CTP production appeared to be closely linked to mycelial growth, and a specific inhibitor of CTP inhibited mycelial growth in solid and submerged cultures

containing protein as the sole nitrogen source. Since CTP was produced only in cultures containing protein as a sole nitrogen source, and was not detectable in culture broths containing amino acids or inorganic nitrogen sources (data not shown), this enzyme may be essential for hydrolysing proteinaceous nitrogen sources for mycelium growth. A similar role for CTP was demonstrated in *S. exfoliatus* SMF13 (Kim & Lee, 1995).

Aerial mycelium formation after substrate mycelium growth was inhibited by a TLP inhibitor, and aerial spore formation was inhibited by an MTP inhibitor. Since a bald mutant of *S. albidoflavus* SMF301 was defective in MTP and TLP production (data not shown), it is possible that TLP participates in the formation of aerial mycelium, and MTP participates in the formation of aerial spores. Such a role for TLP might be related to its putative role in utilization of substrate mycelium protein for the growth of aerial mycelium in *S. exfoliatus* KIS13 (Kim & Lee, 1995).

Growth and morphological differentiation of *S. albidoflavus* SMF301 in submerged culture depended on the culture conditions. Spores germinated and elongated to form mycelium when nutrients were abundant, but the mycelium differentiated to form spores when some nutrients were limited (Lee & Rho, 1993; Rho & Lee, 1994). Submerged spore formation was accompanied by the production of TLP and MTP, and was inhibited by the addition of inhibitors of TLP and MTP. Unusual thickened mycelium resistant to acid and ultrasonication (data not shown) formed when MTP inhibitor was added. This thickening was similar to that occurring in submerged or aerial spores (Lee & Rho, 1993). The defective production of TLP and MTP in submerged cultures of a bald mutant suggest the possibility that the thickened mycelium is an intermediate between vegetative mycelium and mature spores. TLP might play a role in the formation of thickened mycelium or branching, and MTP might participate in the formation of submerged spores from the thickened mycelium. The temporal relationship between

Fig. 8. Morphological differentiation of *S. albidoflavus* SMF13 and production of CTP, TLP and MTP in submerged and solid cultures. (a) Morphological changes in submerged culture (Lee & Rho, 1993); (b) morphological changes in solid culture (Wildermath, 1970); (c) phases of protease production.

the production of CTP, TLP and MTP, and morphological differentiation of *S. albidoflavus* SMF301 is depicted in Fig. 8. Future work is directed at the genetic analysis of protease production to evaluate its function during differentiation.

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