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Surfactant Protein of the *Streptomyces* Subtilisin Inhibitor Family Inhibits Transglutaminase Activation in *Streptomyces hygroscopicus*

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Transglutaminase (TGase) is widely used in the food industry for improving protein properties by catalyzing the cross-linking of proteins. In *Streptomyces,* TGase is secreted as a zymogen, and an activation process has been observed in liquid culture. However, the activation mechanism remains unclear. In the present study, the TGase activation process in *Streptomyces hygroscopicus* was investigated by biochemical approaches. In a liquid culture, Pro-TGase was secreted and gradually was converted into active TGase during the growth period; however, in a cell-free system in which cells were removed from the liquid culture, TGase activation stalled unexpectedly. Subsequently, the TGase activation process was found to be inhibited by a TGase-activating protease inhibitor (TAPI). N-Terminal amino acid sequencing and a homology search of the purified TAPI revealed that it is a member of the *Streptomyces* subtilisin inhibitor (SSI) family. Furthermore, it was found that TAPI (0.1 mg/mL) decreased the surface tension of water from 72 to 60 mJ/m^2 within 5 min, suggesting that it possesses surface activity. This is the first report that an SSI member functions as a surfactant protein. On the basis of these findings, a model for TAPI-regulated TGase activation process was proposed. This study provides novel insights into the TGase activation process in *Streptomyces.*

KEYWORDS: Surfactant protein; *Streptomyces* subtilisin inhibitor family; transglutaminase activation; *Streptomyces* differentiation

INTRODUCTION

Streptomyces are filamentous soil bacteria responsible for producing most natural antibiotics used in medicine today (*1*). They adopt unique strategies for growth and propagation to adapt to versatile conditions they may encounter in terrestrial ecosystems. In a hydrophilic environment, they form the substrate mycelium for vegetative growth (*2*). Under nutritional, physicochemical, or biological stresses, they will undergo morphological differentiation. First, hydrophobic aerial hyphae are formed from the substrate mycelium and emerge from the hydrophilic environment to the air. The emergence of aerial hyphae is coupled with programmed cell death of the substrate mycelium. Subsequently, aerial hyphae separate into spores before their own degradation. Reproductive spores are dispersed by wind and insert into a new favorable environment to run another growth cycle beginning with a new substrate mycelium (*3, 4*).

During the life cycle of *Streptomyces*, numerous proteins are secreted to the medium. Among them are hydrolytic enzymes that permit the utilization of proteins, polysaccharides, fats, and other nutrients. During vegetative growth, these enzymes allow the substrate mycelium to utilize high molecular weight biopolymers for nutrition (*5, 6*); while in differentiation, these enzymes, especially proteases and nucleases, contribute to the degradation of the substrate mycelium and aerial hyphae (*3, 6*). Other abundant secreted proteins are morphogenetic surfactant proteins (*7*). They can lower the surface tension to facilitate the formation of aerial mycelium in a hydrophilic environment and also form a hydrophobic shell on the surfaces of aerial mycelia and spores (*4, 7*). Another dominant component of the secreted proteins of *Streptomyces* is a unique transglutaminase (TGase, protein glutamine γ-glutamyltransferase, EC 2.3.2.13) (*8, 9*).

The TGase family of enzymes exhibits several catalytic activities including the cross-linking of proteins by forming $N\epsilon$-(γ-glutamyl) lysine bonds, the incorporation of polyamines into

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protein, the deamidation of protein-bound glutamines, and the covalent attachment of proteins to long-chain ω-hydroxyls of the lipid by ester bond formation (*10–13*). Since the cross-linking activity of TGase can improve the functional properties of various proteins, TGase has been used widely in the food industry. For example, in meat, wheat, and soybean food processing, TGase can be used to improve the texture of these products; in dairy processing, TGase can improve the water-holding capacity of the gel. Currently, TGase is the only cross-linking enzyme that is available on a commercial scale for catalyzing the covalent bond formation between protein molecules, and the modification of proteins by TGase has attracted great interest from food scientists. It is believed that the application of TGase in different food proteins could lead to the development of novel food and processing methodologies (*14, 15*).

TGase has been found in animals, plants, and microorganisms (*16*). TGases from animals have been classified into at least five types (plasma, tissue, keratinocyte, epidermis, and prostate) and are involved in various biological processes such as blood clotting, wound healing, keratinization of epidermis, apoptosis, cell differentiation, and cell signaling (*17*). The physiological functions of TGases from plants and microorganisms are still unclear (*16*). TGase from *Streptomyces mobaraensis* is secreted as a zymogen (Pro-TGase) in liquid cultures and can be activated by several exogenous proteases, such as bovine trypsin, intestinal chymotrypsin, or dispase from *Bacillus polymyxa* (*8, 9*).

Recently, an endogenous TGase-activating metalloprotease (TAMEP) capable of processing Pro-TGase into its mature enzyme was isolated from agar plate cultures of *S. mobaraensis* (*8*). This protease was inhibited by a 14 kDa protein (P_{14}), a member of the *Streptomyces* subtilisin inhibitor (SSI) family, which could be isolated from both solid and liquid cultures. However, this endogenous TAMEP could not be identified in liquid cultures even though the TGase activation process could be observed (*8*). Furthermore, it is still uncertain as to whether P_{14} could inhibit TGase activation under physiological conditions. Therefore the mechanism of TGase activation for *Streptomyces*, especially in liquid culture, remains unclear.

To further explore this issue, the activating process of TGase from *Streptomyces hygroscopicus* was investigated in the present study. For the first time, TGase activation was found to be inhibited by a 12 kDa inhibitor (TAPI) in a liquid culture of *Streptomyces*. TAPI is a member of the SSI family and was characterized to exhibit surface activity. This is the first time a SSI family member was found to function as a surfactant protein. Furthermore, a model for TAPI-regulated TGase activation was proposed, providing novel insights into the TGase activation process and a possible role of TAPI in *Streptomyces* differentiation.

MATERIALS AND METHODS

Organisms and Culture Conditions. *S. hygroscopicus* strain WSH03-01 was isolated from soil previously (*18*). Spores were collected after cultivating *S. hygroscopicus* on agar medium at 32 °C for 15 days. Collected spores were washed with water and preserved in 50% glycerol (v/v) at −80 °C. The agar medium (pH 7.0) contained glucose (5 g/L), yeast extract (5 g/L), malt extract (10 g/L), and agar (15 g/L). Spores (4×10^5) were inoculated into a 100 mL medium in a 500 mL flask on a shaker at 30 °C and 200 rpm. The liquid medium (pH 7.0) contained dextrin (20 g/L), peptone (20 g/L), yeast extract (5 g/L), $MgSO_4$ (2 g/L), K_2HPO_4 (2 g/L), KH_2PO_4 (2 g/L), and $CaCl_2$ (1 g/L).

Assay of Pro-TGase and TGase. TGase activity was measured by a colorimetric procedure *10*, in which *N*-CBZ-Gln-Gly (Sigma-Aldrich, Shanghai, China) was used as the substrate. A calibration curve was obtained using L-glutamic acid γ-monohydroxamate (Sigma-Aldrich, Shanghai, China). One unit of transglutaminase activity is defined as the formation of 1 μmol of γ-glutamic acid γ-monohydroxamate per minute at 37 °C. For Pro-TGase, the purified zymogen (0.2 mg/mL, 200 μL) was incubated with 0.1 μg of bovine trypsin (Sigma-Aldrich, Shanghai, China) for 10–120 min at 37 °C, and the activation was terminated by the addition of 0.02 mg of trypsin inhibitor (Sigma-Aldrich, Shanghai, China, type II-S). The newly formed TGase from Pro-TGase was then assayed as described previously. The results are the averages of triplicate assays.

Purification of Pro-TGase and TGase. Pro-TGase and TGase were purified as previously described (*9, 19*). Cells were removed from the culture by centrifugation, and the supernatant was cooled down to 4 °C. Ethanol was added to the supernatant to a final concentration of 70% (v/v). The precipitated proteins were immediately dissolved in 50 mM sodium acetate pH 5.0 (buffer A) and then loaded onto a 25 mL Fractogel EMD SO_3^- column (Merck, Shanghai, China) pre-equilibrated with buffer A. The column was eluted at a flow rate of 1 mL/min using a linear gradient of 0−1 M NaCl in buffer A over 2 h. The fractions containing Pro-TGase were eluted between 0.35 and 0.45 M NaCl. TGase was eluted between 0.45 and 0.55 M NaCl. Fractions of Pro-TGase and TGase were dialyzed extensively against 50 mM Tris-HCl pH 7.0 and stored at 80 °C.

Preparation of the Cell-Removed System. *S. hygroscopicus* was incubated in liquid media at 30 °C for 36 h. Cells were removed by centrifugation at 10 000*g* for 10 min, and the supernatant was filtered by a 0.22 μm membrane to obtain the cell-removed system. This cell-removed system was then incubated under the same conditions as the cell-containing culture for further studies.

Preparation of TGase-Activating Protease. *S. hygroscopicus* was grown in shaking flasks for 36 h. Cells were removed by centrifugation (10 000*g*, 10 min). The supernatant (500 mL) was filtered and treated with CTAB (Sigma-Aldrich, Shanghai, China) to a final concentration of 10 mg/mL. The treated supernatant (pH adjusted to 8.0) was centrifuged (15 000*g*, 30 min) and loaded onto a 25 mL DEAE-sepharose F. F. (GE Healthcare, Shanghai, China) column pre-equilibrated with 50 mM Tris-HCl, pH 8.0 (buffer C) at a flow rate of 1 mL/min. The column was eluted by a linear gradient of 0−1 M NaCl in buffer C over 2 h. Fractions containing the TGase-activating protease (TAP) as identified by its capability of activating purified Pro-TGase were collected between 0.5 and 0.6 M NaCl and dialyzed in buffer B overnight. The dialyzed TAP was then concentrated from 10 to 1 mL (0.028 mg of protein total).

Purification of TAPI (P_{12}). *S. hygroscopicus* was grown in shaking flasks for 36 h. Cells were removed by centrifugation (10 000*g*, 10 min). The supernatant was filtered and loaded onto a 25 mL column filled with Fractogel EMD SO_3^- (Merck, Shanghai, China) pre-equilibrated with buffer A at a flow rate of 1 mL/min. The column was eluted at a flow rate of 1 mL/min using a linear gradient of 0−1 M NaCl in buffer A over 3 h. The fractions containing P_{12} (10 mL) as determined by SDS-PAGE were collected between 0.15 and 0.25 M NaCl and dialyzed in 50 mM Tris-HCl, pH 7.0 (buffer B) overnight.

The dialyzed sample was concentrated from 10 to 1 mL and loaded onto a 15 mL phenyl sepharose 6 F. F. column (GE Healthcare, Shanghai, China) pre-equilibrated with 1.5 M NaCl in buffer B and eluted using a linear gradient of 1.5−0 M NaCl in buffer B at a flow rate of 1 mL/min over 2 h. The elution was continued with buffer B for 20 min and then water for 30 min. Fractions containing P_{12} were eluted by water. Purified P_{12} were collected and stored at −80 °C.

Miscellaneous Methods. Protein concentrations were determined by the Bradford method (*20*) using bovine serum albumin (Sigma-Aldrich, Shanghai, China) as a standard. N-Terminal sequencing was performed by the Shanghai Gene Core Biotechnologies Co., Ltd. SDS-PAGE was carried out by using stacking and separating gels of 5 and 12.5% polyacrylamide, respectively (*21*). Electrophoresis gels were stained with Coomassie Brilliant Blue R. The surface activity of TAPI was determined by using the drop volume method (*22*) in triplicate measurements.

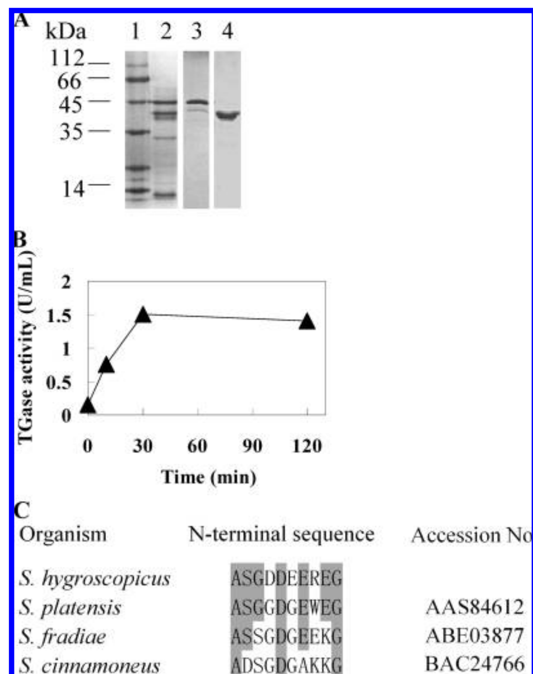

Figure 1. Identification of secreted Pro-TGase from *S. hygroscopicus*. (**A**) Purification of TGase and Pro-TGase. Lane 1, molecular mass markers; lane 2, filtered culture supernatant; lane 3, purified Pro-TGase; and lane 4, purified TGase. (**B**) Activation of Pro-TGase by trypsin. Purified Pro-TGase (0.2 mg/mL, 200 μL) was incubated with bovine trypsin (0.1 mg/mL, 10 μL) and assayed for TGase activity at specified time points. Trypsin digestion was terminated by the addition of 0.02 mg of trypsin inhibitor (Type II-S) before the TGase assay. The results are the averages of triplicate assays. The standard deviations were smaller than 10% of the average values. (**C**) Comparison of the N-terminal sequences of Pro-TGases from *S. hygroscopicus* and other *Streptomyces* species.

RESULTS

TGase from *S. hygroscopicus* Is Secreted as a Zymogen and Is Activated in Liquid Media. Similar to the previously reported Pro-TGase and TGase from *S. mobaraensis* (*9, 19*), both Pro-TGase and TGase of *S. hygroscopicus* were purified to homogeneity, through ethanol precipitation and chromatography, from the 36 h cultured media by monitoring their corresponding activity (**Figure 1A**). The molecular weights of Pro-TGase and TGase were 44 and 40 kDa, respectively (**Figure 1A**). The typical yield for both purified proteins was ~11 mg/L cell culture. As expected, Pro-TGase could be activated by trypsin treatment (**Figure 1B**). The major band in the SDS-PAGE gel of purified Pro-TGase was further analyzed by N-terminal amino acid sequencing. Alignment of this N-terminal sequence and presumed N-terminal sequences of Pro-TGases from other *Streptomyces* species (*23–25*) indicated that they share a significant homology (**Figure 1C**), which further confirmed the identity of the *S. hygroscopicus* Pro-TGase.

To determine as to whether there is a dynamic process of Pro-TGase activation during the culture, the relative levels of Pro-TGase and TGase in the liquid culture were monitored as a function of time (**Figure 2**). The SDS-PAGE of the culture supernatant showed that Pro-TGase appeared at about 24 h and reached the maximum concentration at about 36 h. Subsequently, with the level of Pro-TGase decreasing, the level of mature TGase increased gradually (**Figure 2A**), accompanied by a significant increase in TGase activity (**Figure 2B**). The TGase activity reached the highest value of 2.4 U/mL at about

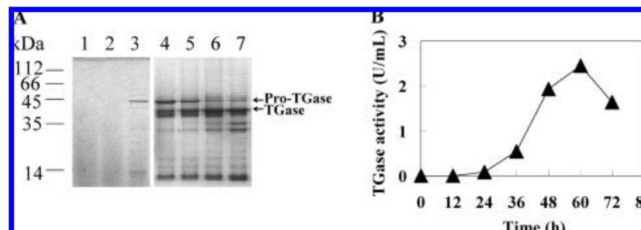

Figure 2. Dynamic process of TGase secretion and activation in liquid cultures of *S. hygroscopicus*. (**A**) The time course of protein profiles of the culture supernatant. Lanes 1–7, culture supernatants after 0, 12, 24, 36, 48, 60, and 72 h of cell growth. (**B**) Time course of TGase activity in the medium. The results are the averages of triplicate assays. The standard deviations were lower than 10% of the values.

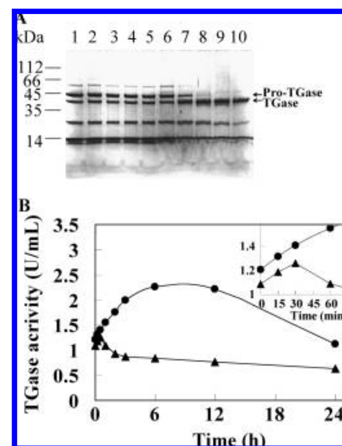

Figure 3. Silencing of TGase activation in a cell-removed system was reversed by CTAB. (**A**) Kinetic profiles of Pro-TGase and TGase levels in the cell-removed culture system. Lanes 1–5, 36 h cell-removed system analyzed after 0, 3, 6, 12, and 24 h of further incubation, respectively, and lanes 6–10, 36 h CTAB treated cell-removed system analyzed after 0, 3, 6, 12, and 24 h of incubation, respectively. (**B**) Time course of TGase activity in the cell-removed system. TGase activities were determined for the cell-removed system (▲) and the CTAB treated cell-removed system (●) after 0, 3, 6, 12, and 24 h of further incubation. Inset: TGase activity of samples taken at 0, 15, 30, and 60 min. The results are the averages of triplicate assays. The standard deviations were smaller than 10% of the average values.

60 h. These results suggested that TGase from *S. hygroscopicus* was secreted as a zymogen and was subsequently activated by an as-yet unknown TAP during the culture process.

TAP Is Located in the Cell-Free Fraction and Is Inhibited by TAPI. To determine the location of TAP (i.e., to determine as to whether it was secreted on the cell surface or in the liquid media), TGase activation was monitored for a cell-free system in which cells were removed after 36 h of growth. Both Pro-TGase level and TGase activity in the 36 h cell-free fraction remained steady after a slight increase of TGase activity during the first 30 min (**Figure 3**). This phenomenon represented silencing of TGase activation, which was in sharp contrast to the cell-containing condition (**Figure 2**). Such a silencing effect would lead to the assumption that TAP exists on the cell surface and was removed along with the cells. However, the addition of fragments of cell walls or cell membranes did not activate the enzyme in the previously stated silenced sample (data not shown).

The true cause behind this silencing phenomenon was later revealed, accidentally, by the addition of CTAB to this cell-removed system, which resulted in protein precipitation. Analy-

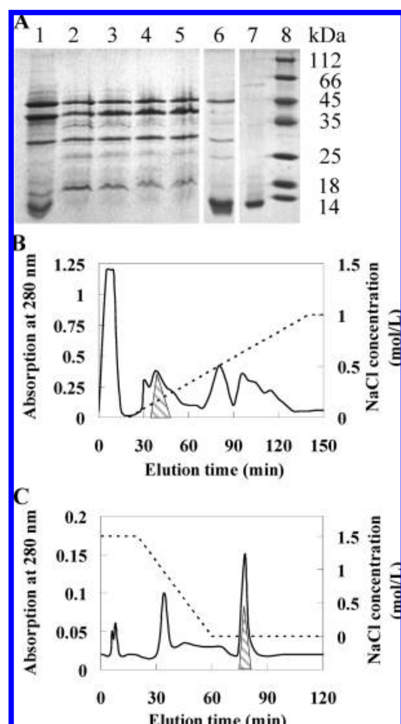

Figure 4. Purification of a potential TAPI - P_{12}. (**A**) SDS-PAGE analysis of CTAB treated cell-removed supernatant and of the purification procedures of P_{12}. Lane 1, cell-removed supernatant of the 36 h culture; lanes 2–5, supernatants of the 36 h cell-free fraction treated with 0.2, 1, 5, or 10 mg/mL CTAB, respectively; lane 6, P_{12}-containing fractions after a Fractogel EMD SO_3^- column; lane 7, P_{12}-containing fractions after a phenyl sepharose 6 F. F. column; and lane 8, molecular mass markers. (**B**) Chromatogram of P_{12} purification by a Fractogel EMD SO_3^- column. Solid line: UV absorbance of proteins andbroken line: NaCl gradient. Fractions containing P_{12} are hatched (**C**) Chromatogram of P_{12} purification by a phenyl sepharose 6 F. F. column. Solid line: UV absorbance of proteins and broken line: NaCl gradient. Fractions containing P_{12} were eluted by water (shown hatched).

sis of the CTAB treated supernatant showed that the TGase activation process resumed (**Figure 3**) and that the rate of activation was even higher than that in the cell-containing liquid culture. This surprising finding suggested that TAP exists in the liquid media and is inhibited in the cell-removed system. The inhibitor(s) for TAP (TAPI) can be precipitated by CTAB.

Identification of TAPI. In an effort to identify the potential TAPI, both CTAB treated and untreated 36 h cell-removed supernatants were further analyzed by SDS-PAGE (**Figure 4A**). The major difference before and after CTAB treatment was a 12 kDa protein (hereafter referred to as P_{12}), which disappeared in the CTAB treated sample, suggesting that P_{12} might be an inhibitor of TAP. By monitoring the molecular mass of 12 kDa as judged by SDS-PAGE, P_{12} was purified from the liquid culture through ethanol precipitation, ion-exchange (**Figure 4B**), and hydrophobic interaction chromatography (**Figure 4C**). The final product was homogeneous as determined by SDS-PAGE (**Figure 4A**), and the yield was ~6 mg/L culture. It is worth mentioning that, during the process of purification, P_{12} was strongly adsorbed to the hydrophobic interaction column and could be eluted only by water (**Figure 4C**).

To determine as to whether P_{12} is an inhibitor of TAP, efforts were first made to obtain the TAP from the culture supernatant. The 36 h culture supernatant was treated with CTAB to remove P_{12} before further purification by weak anion-exchange chromatography (**Figure 5A**). The TAP-rich fraction as judged by the ability to activate Pro-TGase was used for inhibition experiments with P_{12}. As shown in **Figure 5B**, Pro-TGase was readily converted to TGase by the TAP-rich preparation, and the TGase activity was clearly detected (1.12 U/mL). However, the activation of Pro-TGase was completely blocked by P_{12} as no 40 kDa band (TGase) was observed, and no TGase activity was detected in the presence of P_{12}. Furthermore, to determine as to whether purified P_{12} could be precipitated by CTAB, different concentrations of CTAB (0.4, 2, and 10 mg/mL) were added to purified P_{12}, upon which protein precipitation occurred, and the SDS-PAGE of the supernatant confirmed the disappearance of P_{12} (**Figure 5B**). Therefore, P_{12} was confirmed to function as a TAPI in the liquid culture and will be referred to in this article as TAPI.

TAPI Is a Surfactant Protein and Belongs to the SSI Family. The observation that TAPI was strongly adsorbed to the hydrophobic interaction column and could be eluted only by water suggested that it at least has a partially hydrophobic surface. This hydrophobic nature, combined with its water solubility, led us to hypothesize that TAPI may possess surface activity. To test this hypothesis, TAPI (0.1 mg/mL) was tested for surface activity using the drop volume method (*22*). The surface tension of water decreased within 5 min from 72 ± 1 to 60 ± 1 mJ/m^2 in the presence of TAPI, indicating that TAPI can function as a surfactant protein.

To further confirm the surfactant property of TAPI, the effect of agitation on the TGase activation process in the cell-removed system was determined. Assuming that the surface activity of TAPI would lead it to be distributed mainly at the air–liquid interface in static conditions, mixing by agitation would allow more TAP molecules to be bound to TAPI and thus limit TGase activation. Indeed, as shown in **Figure 6**, the level of TGase activation was reduced by agitation as compared to the static conditions, suggesting that the distribution of TAPI in solution is not homogeneous and supporting its function as a surfactant protein.

To reveal the molecular identity of TAPI, the protein band corresponding to TAPI in the SDS-PAGE gel was subjected to N-terminal amino acid sequencing. A homology analysis showed that TAPI belongs to the SSI family, whose members are strong inhibitors of subtilisin (**Figure 7**) (*26*).

DISCUSSION

Previous studies on *S. mobaraensis* TGase have shown that TGase was secreted as a zymogen and was activated under liquid culture conditions (*8, 9*). Although an endogenous TAP has been identified in solid cultures, no endogenous TAP could be isolated in liquid cultures (*8*). In the present study, the existence of TAP as well as its inhibitor TAPI was confirmed in liquid cultures based on the following findings: (i) both the precursor and the mature forms of TGase can be isolated and purified from liquid cultures of *S. hygroscopicus* (**Figure 1**); (ii) a dynamic activation process was observed during cell growth (**Figure 2**); (iii) a TAP-rich preparation that readily catalyzed the conversion of Pro-TGase to TGase was obtained (**Figure 5**); (iv) the silenced TGase activation process in a cell-free system could be resumed by the removal of a 12 kDa protein P_{12} (**Figure 3**); and (v) purified P_{12} inhibited the activation of Pro-TGase in vitro (**Figure 5B**).

It is notable that TAP catalyzed TGase activation occurred despite the presence of TAPI in the liquid culture, which suggests that not all TAP molecules were bound and inhibited by TAPI. The finding that TAPI possesses a surface activity

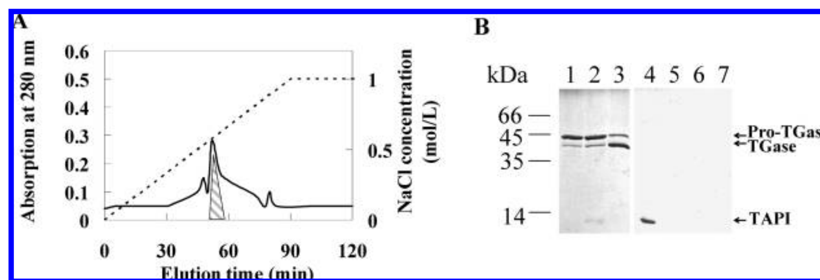

Figure 5. TAP preparation and its inhibition by P_{12}. (**A**) Preparation of a TAP-rich fraction using DEAE-Sepharose F. F. column. Solid line: UV absorption of proteins and broken line: NaCl gradient. Fractions containing TAP are shown as hatched. (**B**) Inhibition of TAP by P_{12}. Lane 1, purified Pro-TGase; lane 2, incubation mixture of purified Pro-TGase (2 μg), TAP-rich fraction (0.2 μg), and purified P_{12} (0.4 μg) at 30 °C for 30 min; lane 3, incubation mixture of purified Pro-TGase (2 μg) and TAP-rich fraction (0.2 μg); lane 4, purified P_{12}; and lanes 5–7, supernatants of purified P_{12} treated with CTAB at concentrations of 0.4, 2, and 10 mg/mL, respectively.

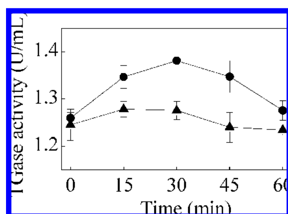

Figure 6. Agitation effects on the TGase activation process in the cell-removed system. TGase activities were determined for the cell-removed system in static (●) and in agitating (▲) conditions (30 s shaking on a shaker every 5 min). Error bars correspond to the standard deviation of three determinations.

Name	Organism	N-terminal sequence	Accession No.
P_{12}	*S. hygroscopicus*	GLYAATTALV	
PSN	*S. antifibrinolyticus*	GLYAP–SALV	P01007
SIL1	*S. cacaoi*	SLYAP–SAVV	P29606
SIL7	*S. ambofaciens*	SLHAP–SALV	AAB36208

Figure 7. Alignment of the N-terminal sequence of P_{12} from *S. hygroscopicus* with those of the SSI family of proteins.

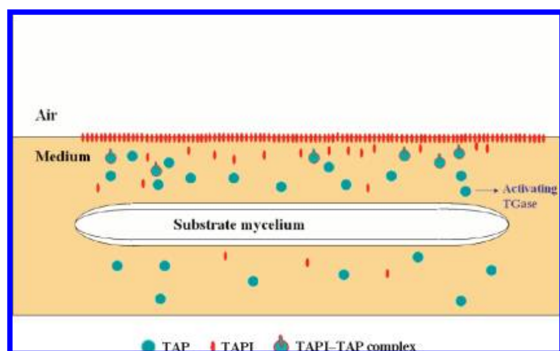

Figure 8. Model for the TAPI-regulated TGase activation process in liquid culture.

here provided a reasonable answer to this unusual phenomenon. It is very likely that TAPI molecules are distributed mostly at the air–liquid interface, which allows sufficient free TAP molecules to exist in the submerged liquid and to perform its function. In fact, this possibility is supported by our observation that TGase activation was reduced by agitation as compared to the static conditions in the cell-removed system (**Figure 6**).

On the basis of the previous analyses, a model for the TAPI-regulated TGase activation process in *Streptomyces* liquid culture was proposed (**Figure 8**): (i) both TAP and TAPI are secreted to the extracellular environment; (ii) in static conditions, TAPI, as a surfactant protein, is mainly distributed at the air–liquid interface, whereas in shaking flask cultures, agitation will allow some TAPI molecules to be submerged; and (iii) TAP, which is secreted continuously, maintains a sufficient level in the flask cultures due to the unique distribution pattern of TAPI and is able to perform its function to activate Pro-TGase; however, in the cell-removed system described in the Results, TAP secretion is discontinued, and TAP molecules are depleted by binding to TAPI under agitation, leading to the silencing of Pro-TGase activation.

The TAPI-regulated TGase activation model here is different from the previously proposed one (*8*). In the previous proposal, it was suggested that TAP was inhibited by its inhibitor when secreted and would not exhibit its activity until the inhibitor was degraded by a second protease (*8, 27*); however, no second protease has been isolated so far. In addition, this proposal could not explain as to why TAP was unable to be isolated from liquid cultures even though the TGase activation process was present. According to the current model, it is evident that all TAP molecules will eventually be bound and inhibited by TAPI during a regular purification process from a liquid culture, which is similar to what happened in our cell-removed system (**Figure 3**). Only when TAPI is removed in advance, for example, by using precipitation by CTAB in the present study, could the active form of TAP be obtained.

A N-terminal sequencing and homology search showed that TAPI belongs to the SSI family (**Figure 7**). Proteins in this family are known to be strong inhibitors of subtilisin and are widely distributed among *Streptomyces*. Furthermore, TAPI was characterized as a surfactant protein. As far as we know, this is the first description of a SSI protein functioning as a surfactant. Previous studies suggested that SSI proteins are related to the differentiation of *Streptomyces*, but no reasonable explanation has been provided so far (*28, 29*). In the present study, it is suggested that TAPI may contribute to the differentiation by regulating TGase activation. In a hydrophilic substrate environment, the surfactant TAPI would allow TGase to be activated, and the active TGase would be involved in the programmed cell death of substrate mycelia, which is similar to the TGase from mammal tissue (*17*); while in the hydrophobic environment of surface colonies, TGase remains inactive and has no effect on the growth of aerial hypha, thus ensuing spore formation. Alternatively, the surface activity of TAPI may contribute to the differentiation by lowering the surface tension to facilitate the formation of aerial mycelium similar to other morphogenetic surfactant proteins (*4, 7*). Further information is needed to support these hypotheses.

In summary, the TGase activation in liquid cultures of *Streptomyces* was found to be inhibited by TAPI, a SSI protein. More importantly, TAPI was found to possess a surface activity, representing a novel function for the SSI family of proteins. A

model for the surfactant TAPI-regulated TGase activation process in a liquid environment was proposed. These findings provide new insight into the TGase activation process in *Streptomyces*.

ABBREVIATIONS USED

P_{12}, a 12 kDa protein; SSI, *Streptomyces* subtilisin inhibitor; TAP, TGase-activating protease; TAPI, TGase-activating protease inhibitor; TGase, transglutaminase; MW, molecular weight; CTAB, cetyltrimethyl ammonium bromide.

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LITERATURE CITED

(1) Bentley, S. D.; Chater, K. F.; Cerdeno-Tarraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C. H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Neil, S.; Rabbinowitsch, E.; Rajandream, M. A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood, D. A. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature (London, U.K.)* **2002**, *417*, 141–147.

(2) ElliotM. A.; Karoonuthaisir, N.; Huang, J.; Bibb, M. J.; Cohen, S. N.; Kao, C. M.; Buttner, M. J. The chaplins: A family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genes Dev.* **2003**, *17*, 1727–1740.

(3) Miguélez, E. M.; Hardisson, C.; Manzanal, M. B. Hyphal death during colony development in *Streptomyces antibioticus*: Morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. *J. Cell. Biol.* **1999**, *145*, 515–525.

(4) Claessen, D.; Rink, R.; De Jong, W.; Siebring, J.; De Vreugd, P.; Boersma, F. G. H.; Dijkhuizen, L.; Wösten, H. A. B. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. *Genes Dev.* **2003**, *17*, 1714–1726.

(5) Rao, M. B.; Tanksale, A. M.; Ghatge, M. S.; Deshpande, V. V. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 597–635.

(6) Nicieza, R. G.; Huergo, J.; Connolly, B. A.; Sanchez., J., Purification, characterization, and role of nucleases and serine proteases in *Streptomyces* differentiation. J. Biol. Chem. 1999, *274*, 20366–20375.

(7) Willey, J. M.; Willems, A.; Kodani, S.; Nodwell, J. R. Morphogenetic surfactants and their role in the formation of aerial hyphae in *Streptomyces coelicolor*. *Mol. Microbiol.* **2006**, *59*, 731–742.

(8) Zotzel, J.; Keller, P.; Fuchsbauer, H.-L. Transglutaminase from *Streptomyces mobaraensis* is activated by an endogenous metalloprotease. *Eur. J. Biochem.* **2003**, *270*, 3214–3222.

(9) Pasternack, R.; Dorsch, S.; Otterbach, J. T.; Robenek, I. R.; Wolf, S.; Fuchsbauer, H.-L. Bacterial pro-transglutaminase from *Streptoverticillium mobaraense* purification, characterization, and sequence of the zymogen. *Eur. J. Biochem.* **1998**, *257*, 570–576.

(10) Grossowicz, N.; Wainfan, E.; Borek, E.; Waelsch, H. The enzymatic formation of hydroxamic acids from glutamine and asparagine. *J. Biol. Chem.* **1950**, *187*, 111–125.

(11) Bergamini, C. M.; Signorini, M.; Barbato, R.; Menabo, R.; Di-Lisa, F.; Gorza, L.; Beninati, S. Transglutaminase catalyzed polymerization of troponin in vitro. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 201–206.

(12) Pasternack, R.; Laurent, H. P.; Ruth, T.; Kaiser, A.; Schon, N.; Fuchsbauer, H. L. A fluorescent substrate of transglutaminase for detection and characterization of glutamine acceptor compounds. *Anal. Biochem.* **1997**, *249*, 54–60.

(13) Nemes, Z.; Marekov, L. N.; Fésüs, L.; Steinert, P. M. A novel function for transglutaminase 1: Attachment of long-chain ω-hydroxyceramides to involucrin by ester bond formation. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8402–8407.

(14) Yokoyama, K.; Nio, N.; Kikuchi, Y. Properties and applications of microbial transglutaminase. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 447–454.

(15) Zhu, Y.; Rinzema, A.; Tramper, J.; Bol, J. Microbial transglutaminase: A review of its production and application in food processing. *Appl. Microbiol. Biotechnol.* **1995**, *44*, 277–282.

(16) Beninati, S.; Piacentini, M. The transglutaminase family: An overview. Minireview article. *Amino Acids* **2004**, *26*, 367–372.

(17) Griffin, M.; Casadio, R.; Bergamini, C. M. Transglutaminases: Nature's biological glues. *Biochem. J.* **2002**, *368*, 377–396.

(18) Cui, L.; Du, G.; Zhang, D.; Liu, H.; Chen, J. Purification and characterization of transglutaminase from a newly isolated *Streptomyces hygroscopicus*. *Food Chem.* **2007**, *105*, 612–618.

(19) Gerber, U.; Jucknischke, U.; Putzien, S.; Fuchsbauer, H.-L. A rapid and simple method for the purification of transglutaminase from *Streptoverticillium mobaraense*. *Biochem. J.* **1994**, *299*, 825–829.

(20) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(21) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Press: Cold Spring Harbor, NY, 2001.

(22) Pu, B; Chen, D. A study of the measurement of surface and interfacial tension by the maximum liquid drop volume method. *J. Colloid Interface Sci.* **2001**, *235*, 273–277.

(23) Lin, Y.-S.; Chao, M.-L.; Liu, C.-H.; Tseng, M.; Chu, W.-S. Cloning of the gene coding for transglutaminase from *Streptomyces platensis* and its expression in *Streptomyces lividans*. *Process Biochem.* **2006**, *41*, 519–524.

(24) Liu, X. Q.; Yang, X. Q.; Xie, F. H.; Song, L. Y.; Zhang, G. Q.; Qian, S. J. On-column refolding and purification of transglutaminase from *Streptomyces fradiae* expressed as inclusion bodies in *Escherichia coli*. *Protein Expression Purif.* **2007**, *51*, 179–186.

(25) Taguchi, S.; Arakawa, K.; Yokoyama, K.; Takehana, S.; Takagi, H.; Momose, H. Overexpression and purification of microbial pro-transglutaminase from *Streptomyces cinnamoneum* and in vitro processing by S*treptomyces albogriseolus* proteases. *J. Biosci. Bioeng.* **2002**, *94*, 478–481.

(26) Terabe, M.; Kojima, S.; Taguchi, S.; Momose, H.; Miura, K.-I. New subtilisin−trypsin inhibitors produced by *Streptomyces*: Primary structures and their relationship to other proteinase inhibitors from *Streptomyces*. *Biochim. Biophys. Acta* **1996**, *1292*, 233–240.

(27) Zotzel, J.; Pasternack, R.; Pelzer, C.; Ziegert, D.; Mainusch, M.; Fuchsbauer, H.-L. Activated transglutaminase from *Streptomyces mobaraensis* is processed by a tripeptidyl aminopeptidase in the final step. *Eur. J. Biochem.* **2003**, *270*, 4149–4155.

(28) Hirano, S.; Kato, J.-Y.; Ohnishi, Y.; Horinouchi, S. Control of the *Streptomyces* subtilisin inhibitor gene by AdpA in the A-factor regulatory cascade in *Streptomyces griseus*. *J. Bacteriol.* **2006**, *188*, 6207–6216.

(29) Suzuki, M.; Taguchi, S.; Yamada, S.; Kojima, S.; Miura, K; Momose, H. A novel member of the subtilisin-like protease family from *Streptomyces albogriseolus*. *J. Bacteriol.* **1997**, *179*, 430–438.

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