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Two Different Proteases from *Streptomyces hygroscopicus* Are Involved in Transglutaminase Activation

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Transglutaminase (TGase), the only commercial enzyme in the food industry capable of introducing covalent bonds to proteins, is secreted as a zymogen (Pro-TGase) in several *Streptomyces* species. In previous studies, only a metalloprotease has been isolated from *Streptomyces mobaraensis* as an endogenous TGase-activating protease (TAP). In this study, not only an endogenous metalloprotease but also an endogenous serine protease is found to be involved in TGase activation in *Streptomyces hygroscopicus*. In a cell-free system, the TAP inhibitor was first precipitated with cetyltrimethyl ammonium bromide (CTAB) to maintain TAP activity. Subsequently, different types of protease inhibitors were added to identify the TAP involved in TGase activation in *S. hygroscopicus*. TGase activation was inhibited by 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM ethylenediaminetetraacetic acid (EDTA), indicating the involvement of serine protease and metalloprotease in the TGase activation process. Furthermore, the TAP purified from a liquid culture of *S. hygroscopicus* was identified as a serine protease, which is different from the TAP isolated from *S. mobaraensis*. In addition, *Streptomyces* Pro-TGases were found to have a conserved amino acid sequence preceding the N-terminal of TGase, which contained cleavage sites for both serine protease and metalloprotease. These results indicate that endogenous serine and metalloproteases are both involved in TGase activation in *S. hygroscopicus*. To the authors' knowledge, this is the first report that an endogenous serine protease is involved in *Streptomyces* TGase activation.

KEYWORDS: Transglutaminase; *Streptomyces hygroscopicus*; TGase activation; serine protease

INTRODUCTION

Transglutaminase is a family of enzymes that exhibit several catalytic activities: the cross-linking of proteins by forming N^{ε}-(γ-glutamyl) lysine bonds, the incorporation of polyamines into protein, the deamidation of protein-bound glutamines, and the covalent attachment of proteins to long-chain ω-hydroxyl of lipid by ester bond formation (*1*–*4*). It is interesting to find that although TGase was evolved from protease (*5*), it can catalyze a quite different reaction of introducing covalent cross-links between proteins as well as peptides (*6*).

TGase has been found in animals, plants, and microorganisms (*7*). TGases from animals have been classified into at least five types such as plasma, tissue, keratinocyte, epidermis, and prostate types, and are involved in various biological processes (blood clotting, wound healing, keratinization of epidermis, apoptosis, cell differentiation, and cell signaling) (*8*). The physiological functions of TGases from plants and microorganisms remain unclear (*7*).

Among the TGases from different genetic resources, TGase from *Streptomyces* is the only cross-linking enzyme that is currently available for catalyzing covalent bond formation between proteins on a commercial scale. This enzyme has been used for improving the functional properties of various proteins including meat, soy, myosin, globulin, casein, peanut, and whey proteins (*9*). Modification of proteins by TGase has attracted great interest from food scientists. Today, on a commercial scale, production of TGase is mainly carried out by submerged fermentation of *Streptomyces* (*10*).

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TGase from *Streptomyces* is secreted as a zymogen (Pro-TGase) in liquid culture and can be activated by several exogenous proteases, such as bovine trypsin, intestinal chymotrypsin, or dispase from *Bacillus polymyxa* (*11, 12*). Recently, an endogenous TGase-activating metalloprotease (TAMEP) capable of processing Pro-TGase into its mature form was isolated from an agar plate culture of *Streptomyces mobaraensis* (*12*). However, this TAMEP was not successfully isolated from its liquid culture. Therefore, it remains unconfirmed whether TAMEP is involved in TGase activation in liquid culture. Furthermore, it remains also unknown whether metalloprotease is the only protease involved in the TGase activation process.

In previous studies, we isolated a high-yield TGase production strain, *Streptomyces hygroscopicus*, which has potential in TGase production on an industrial scale. Further investigations on *S. hygroscopicus* showed that the activation process of TGase is regulated by a protein of the *Streptomyces* subtilisin inhibitor (SSI) family via inhibition of TGase-activating protease (TAP). Furthermore, this TGase-activating protease inhibitor (TAPI) was found to have surface activity and could be precipitated by cetyltrimethyl ammonium bromide (CTAB) (*13*).

To further explore the TGase activation mechanism in *Streptomyces*, which would potentially improve TGase fermentation yield, as well as to shed clues on the physiological function of *Streptomyces* TGase, we explore in this study the TAPs involved in TGase activation in *S. hygroscopicus* liquid culture. We first precipitate TAPI with CTAB from a cell-free system to retain TAP activity. Subsequently, to this CTAB-treated cell-free system were added chemical protease inhibitors that are each known to act against serine, cysteine, metalloproteases, aspartate proteases, or aminopeptidases. TGase activation in this system is inhibited by PMSF and EDTA. Furthermore, a serine TAP that is different from the metalloprotease found in *S. mobaraensis* is purified from *S. hygroscopicus* liquid culture. These results indicate that TGase activation in *S. hygroscopicus* is catalyzed by different proteases, at least a serine protease and a metalloprotease.

MATERIALS AND METHODS

Microorganisms and Culture Conditions. *S. hygroscopicus* strain WSH03-01 was isolated from soil and stored at −80 °C. Spores were collected after cultivation of *S. hygroscopicus* on agar medium at 32 °C for 15 days. Collected spores were washed by water and preserved in water/glycerol (1:1, 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 in 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 TGase. TGase activity was measured by a colorimetric procedure (*1*), in which N-CBZ-Gln-Gly (Sigma, Shanghai, China) was used as the substrate. A calibration curve was obtained using L-glutamic acid γ-monohydroxamate (Sigma). One unit of transglutaminase is defined as that required to generate 1 μmol of γ-glutamic acid γ-monohydroxamate per minute at 37 °C. The results are the averages of triplicate assays.

Preparation of the Cell-free System. *S. hygroscopicus* was incubated in liquid medium at 30 °C for 36 h. Cells were removed by centrifugation at 10000*g* for 10 min, and the supernatant was filtered by a 0.22 μm membrane to obtain a cell-free system. This cell-free system was then incubated at 30 °C for further studies.

Inhibition of TGase Activation in CTAB-Treated Cell-free System. CTAB was added into the cell-free system to a final concentration of 10 mg/mL to precipitate TAPI. Subsequently, different chemicals, including 10 mM ethylene diamine tetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 20 μM (2*S*,3*S*)-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 25 μM pepstatin, and 20 μM bestatin, were respectively added to the system to compare the inhibitory effect on TGase activation. These mixtures were incubated at 30 °C, and samples were taken at different time points followed by the measurement of TGase activity and (or) SDS-PAGE assay.

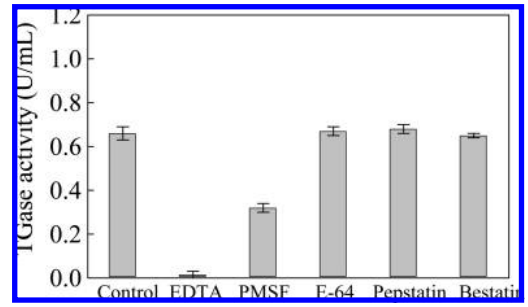

Figure 1. Inhibition of TGase activation in cell-free system by protease inhibitors. Error bars correspond to the standard deviation of three determinations.

Inhibition of TGase Activation Catalyzed by TAP Preparation. To each mixture of 100 μL of Pro-TGase (0.26 mg/mL) and 10 μL of TAP (0.03 mg/mL) preparation were added different chemical inhibitors including 10 mM EDTA, 1 mM PMSF, 20 μM E-64, 25 μM pepstatin, and 20 μM bestatin. Subsequently, these mixtures were incubated at 30 °C for 30 min, followed by analyses of TGase activity and SDS-PAGE. Purification of Pro-TGase and TAP was carried out as described in a previous paper (*13*).

Miscellaneous Methods. SDS-PAGE was carried out with stacking and separating gels of 5 and 12.5% polyacrylamide, respectively (*14*). Electrophoresis gels were stained with Coomassie Brilliant Blue R.

RESULTS AND DISCUSSION

Previously it was found that TAP activity in *S. hygroscopicus* was inhibited by an inhibitor (TAPI), and this inhibitor could be precipitated by CTAB (*13*). To identify the possible proteases involved in the TGase activation process, first CTAB was added to the cell-free system to retain TAP activity. Subsequently, 10 mM EDTA, 1 mM PMSF, 20 μM E-64, 25 μM pepstatin, and 20 μM bestatin were added to this CTAB-treated cell-free system, respectively. These chemicals are known to act against metalloproteases, serine, cysteine, and aspartate proteases, and aminopeptidases, respectively. After incubation for 3 h, TGase activation was not inhibited by E-64, pepstatin, and bestatin but was inhibited by EDTA and PMSF (**Figure 1**). Compared with the control without protease inhibitor, TGase activation was entirely inhibited by EDTA and partially inhibited by PMSF. To eliminate the possibility that the concentration of PMSF was too low, PMSF at concentrations of 5 and 10 mM was added to the CTAB-treated cell-free system. Similar inhibitive tendencies were observed as compared with the addition of 1 mM PMSF (data not shown), which indicated that a PMSF concentration of 1 mM was sufficient.

To further explore the inhibition of TGase activation by EDTA and PMSF, the protein profile and enzyme activity of CTAB-treated cell-free system supernatant, containing EDTA (10 mM) or PMSF (1 mM), were analyzed with time. Because PMSF has a half-life of about 30 min in water solution, 1 mmol of PMSF was added to 1 mL of CTAB-treated cell-free system every 3 h. As shown in **Figure 2**, TGase activation was entirely inhibited by EDTA and was partially inhibited by PMSF, indicating that serine protease and metalloprotease may be involved in TGase activation.

Furthermore, the TAP purified from *S. hygroscopicus* liquid culture, which was obtained per our previous paper (*13*), was identified as serine protease. To the mixtures of 100 μL of Pro-TGase (0.26 mg/mL) and 10 μL of TAP (0.03 mg/mL) were

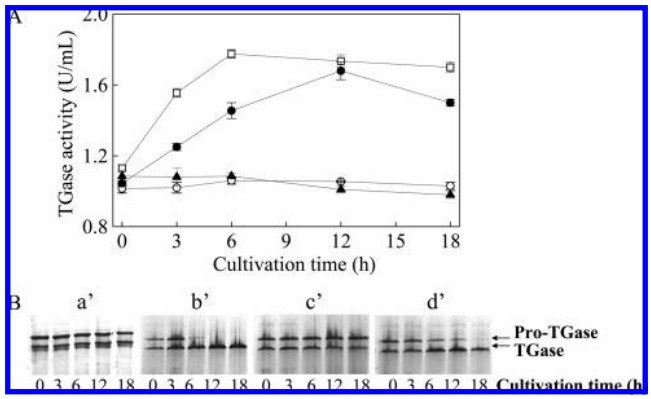

Figure 2. Effects of PMSF and EDTA on TGase activation in CTAB-treated cell-free system. (**A**) TGase activity assay. Compared with the control without CTAB (○), the control with CTAB (□) showed an obvious increase of TGase activity, which represented a process of TGase activation, whereas this activation process was entirely inhibited by EDTA (▲) and partially inhibited by PMSF (●). Error bars correspond to the standard deviation of three determinations. (**B**) SDS-PAGE analysis: (**a′**) control without CTAB; (**b′**) control with CTAB; (**c′**) system with CTAB and EDTA; (**d′**) system with CTAB and PMSF. Compared with the controls (with or without CTAB), TGase activation in the cell-free system was partially inhibited by PMSF and entirely inhibited by EDTA.

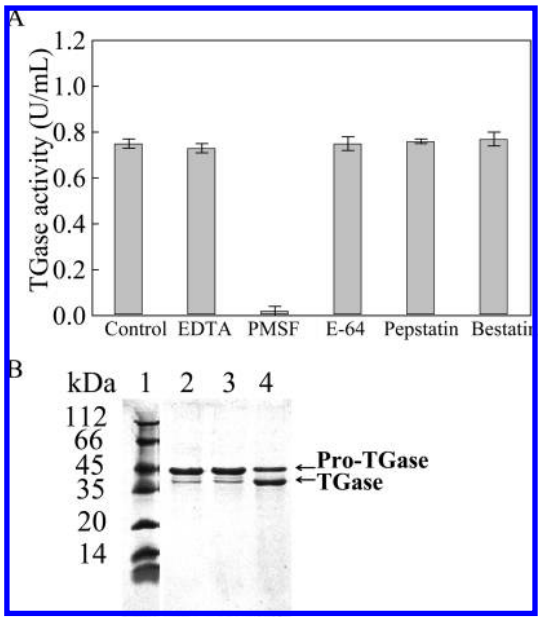

Figure 3. Inhibition of TAP by different protease inhibitors: (**A**) TGase activity assay. Error bars correspond to the standard deviation of three determinations; (**B**) SDS-PAGE analysis for determination of the protease type of TAP (lane 1, molecular mass markers; lane 2, Pro-TGase; lane 3, PMSF incubated with TAP and Pro-TGase; lane 4, TAP incubated with Pro-TGase). Conversion from Pro-TGase to TGase catalyzed by TAP was inhibited by PMSF.

aded the different inhibitors mentioned before, respectively, followed by TGase activity assay. As shown in **Figure 3A**, TAP was inhibited by PMSF, which suggested this protease purified from *S. hygroscopicus* liquid culture was a serine protease. Subsequently, SDS-PAGE analysis ensured this estimation (**Figure 3B**).

Previously, an endogenous metalloprotease that activated Pro-TGase was found in *Streptomyces*. In this study, an endogenous serine protease, which activated Pro-TGase, was also detected. Additionally, serine protease and metalloprotease activities were

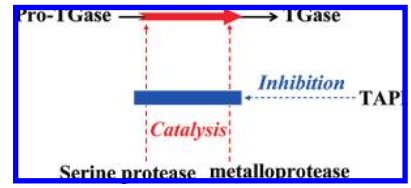

Figure 4. Regulation of TGase activation in *S. hygroscopicus*.

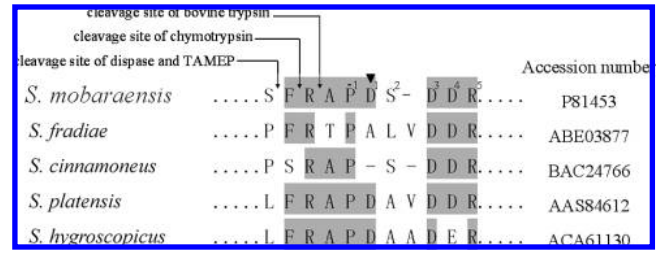

Figure 5. Comparison of the amino acid sequences of Pro-TGase from *S. mobaraensis* and other *Streptomyces* species. The N-terminal amino acid of mature TGase is indicated by▼, and the cleavage sites of different proteases are marked with arrowheads.

found to be involved in TGase activation in a cell-free system of *S. hygroscopicus* liquid culture broth, after TAPI was precipitated by CTAB (**Figure 2**). Putting these together, TGase activation in *Streptomyces* seems to be a multiprotease-involved process, where metalloprotease and serine protease may be related to this process, and these proteases were inhibited by TAPI (**Figure 4**).

This hypothesis was further supported by the following finding that the amino acid sequence of *Streptomyces* Pro-TGase contained conserved multiprotease cleavage sites. As shown in **Figure 5**, compared with the amino acid sequence of *S. mobaraensis* Pro-TGase, the small segment before the N-terminal of mature TGase in *Streptomyces* exhibits a high degree of conservation, where several cleavage sites of metalloprotease and serine protease exist. These protease cleavage sites allow Pro-TGase to be activated by a variety of proteases.

Previous research with *Streptomyces* suggests that metalloprotease, serine protease, and SSI protein are involved in differentiation by the regulation of A-factor, a microbial hormone that triggers secondary metabolism and cell differentiation (*15, 16*). Regulation of TGase activation by metalloprotease, serine protease, and SSI protein suggests that TGase may also be involved in the differentiation of *Streptomyces*. TGase may cross-link proteins on the cell surface against degradation of the environmental proteases during differentiation due to its cross-linking activity. Such activation of Pro-TGase, which was catalyzed by a variety of proteases, may endow *Streptomyces* with an ability to respond rapidly to insult from environmental proteases. Further studies are needed to support these hypotheses.

In summary, serine protease and metalloprotease were found to be involved in the TGase activation in *S. hygroscopicus*. To our knowledge, this is the first report of an endogenous serine protease involved in *Streptomyces* TGase activation.

ABBREVIATIONS USED

TGase, transglutaminase; TAP, TGase-activating protease; TAMEP, a metalloprotease capable of activating TGase; TAPI, TGase-activating protease inhibitor; P_{12}, a 12 kDa protein; SSI, *Streptomyces* subtilisin inhibitor; CTAB, cetyltrimethyl ammonium bromide; EDTA, ethylenediaminetetraacetic acid; E-64, (2*S*,3*S*)-epoxysuccinyl-$_L$-leucylamido-(4-guanidino)butane; PMSF, phenylmethanesulfonyl fluoride.

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