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Transglutaminase from *Streptomyces mobaraensis* is activated by an endogenous metalloprotease

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Streptomyces mobaraensis secretes a Ca^{2+}-independent transglutaminase (TGase) that is activated by removing an N-terminal peptide from a precursor protein during submerged culture in a complex medium [Pasternack, R., Dorsch, S., Otterbach, J. T., Robenek, I. R., Wolf, S. & Fuchsbauer, H.-L. (1998) *Eur. J. Biochem. 257*, 570–576]. However, an activating protease could not be identified, probably because of the presence of a 14-kDa protein (P_{14}) belonging to the *Streptomyces* subtilisin inhibitor family. In contrast, if the microorganism was allowed to grow on a minimal medium, several soluble proteases were extracted, among them the TGase-activating protease (TAMEP). TAMEP was purified by sequential chromatography on DEAE- and Arg-Sepharose and used to determine the cleavage site of TGase. It was clearly shown that the peptide bond between Phe(−4) and Ser(−5) was hydrolyzed, indicating that at least one additional peptidase is necessary to complete TGase processing, even if TAMEP cleavage was sufficient to obtain total activity. Sequence analysis from the N-terminus of TAMEP revealed the close relationship to a zinc *endo*-protease from *S. griseus*. The *S. griseus* protease differs from other members of the M4 protease family, such as thermolysin, in that it may be inhibited by the *Streptomyces* subtilisin inhibitor. P_{14} likewise inhibits TAMEP in approximately equimolar concentrations, suggesting its important role in regulating TGase activity.

Keywords: *Streptomyces mobaraensis*; transglutaminase; transglutaminase activation; transglutaminase activating metalloprotease; *Streptomyces* subtilisin inhibitor.

Streptomycetes are Gram-positive, filamentous soil bacteria that exhibit a complex life-cycle comprising a number of physiologically distinct stages. Above all, vegetative growth generates a ramifying network, called the soil or substrate mycelium [1]. The emergence of aerial mycelium from the surface of colonies is largely undefined, but it is thought that it may be induced by nutrient limitation [2]. In this phase of bacterial development, many vegetative cells die, and accumulated materials such as glycogen, lipids or polyphosphates probably provide the growing aerial hyphae with nutrients [1,3]. Secretion of nucleases and proteases and the degradation of protease-inhibitory molecules may correspondingly support aerial growth by digestion of nucleic acids and proteins [4–6]. Moreover, recent results suggest that hydrolytic enzymes could be crucial in contributing to morphological changes. Characterization of *Streptomyces antibioticus* nucleases, along with an activating protease, has led to the assumption that differentiation in *Streptomyces* mycelium may be a series of strictly regulated events, similar to those that occur in the programmed cell death of eukaryotic cells [4,7]. Eventually, when aerial hyphae growth is complete, spores develop by synchronous insertion of cell walls and cell-wall thickening.

Some *Streptomyces* spp., formerly assigned to the genus *Streptoverticillium* (*Streptoverticillia* are now unified with *Streptomyces* according to Witt & Stackebrandt [8]), secrete large amounts of transglutaminase (TGase, protein glutamine: amine γ-glutamyltransferase, EC 2.3.2.13) in the culture medium [9]. TGases are multifunctional enzymes that are widely distributed among mammals, invertebrates and plants [10–13]. For example, in humans, nine different isoforms of TGase have been identified that may be involved in signal transduction [14], apoptotic death pathways [15], terminal differentiation of epithelia [16], blood coagulation [17] and other intra- and extracellular protein-stabilizing processes [18–20]. Most commonly, proteins are cross-linked by transfer reactions between glutamine- and

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Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; Arg-C, *endo*-protease hydrolyzing Cbz-Pro-Phe-Arg-pNA; Cbz, carbobenzoxy; E-64, (2*S*,3*S*)-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; FA, N-[(3-(2-furyl)]acryloyl; GYM, glucose-yeast-malt medium; P_{14}, TAMEP inhibitor of 14 kDa; Phe-C, *endo*-protease hydrolyzing Suc-Ala-Ala-Pro-Phe-pNA; pNA, *para*-nitroanilide; SGMPII, metalloprotease type II from *Streptomyces griseus*; SSI, *Streptomyces* subtilisin inhibitor; Suc, succinyl; TAMEP, transglutaminase-activating metalloprotease; TGase, transglutaminase.
Proteins and enzymes: *Streptomyces* subtilisin inhibitor from *Streptoverticillium orinoci* (SwissProt entry name SSI_STRON, accession number P80597); *Streptomyces* subtilisin inhibitor from *S. coelicolor* (SwissProt entry name SSI3_STRCO, accession number P29608). Transglutaminase, protein-glutamine: amine γ-glutamyltransferase from *Streptomyces mobaraensis* (EC 2.3.2.13, SwissProt entry name Q8KRJ2, accession number Q8KRJ2); dispase, *Bacillus polymyxa* neutral proteinase (EC 3.4.24.4, no entry); thermolysin, thermophilic-bacterial proteinase from *B. thermoproteolyticus rokko* (EC 3.4.24.27, SwissProt entry name THER_BACTH, accession number P00800); *Streptomyces griseus* metalloproteinase II or neutral proteinase, mycolysin (EC 3.4.24.31, no entry).

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lysine donor proteins, resulting in the formation of N^{ε}-(γ-glutamyl)-L-lysine bridge bonds [21]. However, primary amines, particularly polyamines such as spermine or spermidine [22], can substitute the lysine donor [23]. Moreover, it has been shown that even ester bonds are formed in the cornified cell envelope of the epidermis when the outer protein layer is attached to ω-hydroxyceramides of the lipid membrane [24]. Only in the absence of a suitable nucleophil can TGase-mediated hydrolysis of *endo*-glutamine carboxamide groups take place, thus increasing the negative charge of a protein [25,26]. What role TGase could play in the life-cycle of *Streptomycetes* is as yet unknown, but it seems probable that protein cross-linking could fortify the aerial cell wall and the spore envelope. The idea is supported by labelling experiments of cell-wall proteins in *S. mobaraensis* [27]. Moreover, TGase substrates of the Gram-positive bacterium *Bacillus subtilis* have been characterized to be involved in outer spore assembly and to belong to the α-crystallin family of stress proteins [28]. However, *B. subtilis* does not exhibit the multicellular morphology of *Streptomycetes* and produces an intracellular TGase during a late stage of spore maturation [29]. This is in contrast to the early enzyme secretion by submerged colonies of *S. mobaraensis*, which also implicates a disparate function for the substrate molecules.

The protein structures of the known bacterial TGases are quite different to the mammalian enzymes, in that they lack any sequence homology and have smaller molecular masses [29,30]. TGase from *S. mobaraensis* is secreted as a 42.5-kDa precursor protein, which is processed by the removal of a 45 amino-acid N-terminal peptide [27]. To date, investigations to study the activation procedure have not been carried out, although the activating protease may be important in regulating extracellular TGase activities of the microorganism.

We have recently shown that TGase from *S. mobaraensis* can be activated by various endoproteases, such as bovine trypsin, intestinal chymotrypsin or dispase from *B. polymyxa*, despite leaving di-, tri- or tetrapeptides at the mature N-terminus, respectively [27]. However, we were unable to isolate a TGase-activating protease from submerged cultures grown in a complex medium. In a new approach, we cultured colonies under stress conditions to promote the development of aerial mycelium and the formation of spores. A TGase-activating metalloprotease [TAMEP (a suggested new nomenclature for TGase-activating metalloproteases)] was identified and characterized as belonging to the M4 protease family. Furthermore, the protease was completely inhibited by a 14-kDa protein (P_{14}) produced by colonies grown in both complex and nutrient-deficient media.

Materials and methods

Preparation of protease extracts

Agar plates (92 × 16 mm) containing glucose-yeast-malt medium (GYM) (15 $g{\cdot}L^{-1}$) [glucose (4 $g{\cdot}L^{-1}$), yeast (4 $g{\cdot}L^{-1}$), malt extract (10 $g{\cdot}L^{-1}$) and $CaCO_3$ (2 $g{\cdot}L^{-1}$)], pH 7.2, were inoculated with spores of *S. mobaraensis* (strain 40587; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cultures were maintained at 28 °C for a maximum of 30 days. Well-developed cultures with white, fluffy aerial mycelium were extracted by shaking with 20 mL of 2 mM $CaCl_2$ in 50 mM Tris buffer, pH 7.0, for 24 h at 28 °C. The filtered supernatants were assayed for protease and TGase activity and used in the protease-purification procedures.

Purification of TAMEP

Filtered protease extract (up to 80 mL) was applied to an 8-mL DEAE Sepharose column (Amersham-Pharmacia) equilibrated with 50 mM Tris/HCl containing 2 mM $CaCl_2$, pH 8.0, at a flow rate of 1.0 $mL{\cdot}min^{-1}$. The column was washed using the same buffer. Chromatography was performed by increasing the NaCl concentration stepwise up to 0.14 M, and 1-mL TAMEP fractions, eluted between 40 mM and 60 mM NaCl, were collected. Fractions able to activate TGase were pooled and stored at −20 °C until required. TAMEP contaminated by traces of an Arg-C endoprotease [*endo*-protease hydrolyzing carbobenzoxy (Cbz)-Pro-Phe-Arg-*para*-nitroanilide (pNA)] was further purified by Arg-Sepharose chromatography. A 10-mL TAMEP pool was loaded at a flow rate of 1.0 $mL{\cdot}min^{-1}$ on a 6-mL Arg-Sepharose 4B column (Amersham-Pharmacia) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 2 mM $CaCl_2$. TAMEP was found in the unbound fraction (elution volume 25–30 mL) causing the distinct second peak clearly separated from the unbound Arg-C-endoprotease eluting first (elution volume of 10–20 mL). Fractions of 1 mL were pooled and stored at −20 °C until required.

N-terminal sequencing

Purified TAMEP was concentrated on a Membrapure PES-10 filter and freeze dried. The residue, dissolved in 0.3 mL of water to give a concentration of 0.8 $mg{\cdot}mL^{-1}$, was separated by SDS/PAGE, electroblotted onto a poly(vinylidene difluoride) membrane (Biorad) and visualized by Coomassie Brilliant Blue R 250 (Sigma) according to the procedures of Laemmli [31], Khyse-Anderson [32] and Matsudaira [33]. The protein at 39 kDa was sequenced with an Applied Biosystems 492 protein sequenator.

Purification of P_{14} from *S. mobaraensis*

S. mobaraensis was cultured in shaking flasks as described previously [27]. Cell aggregates were separated by centrifugation (10 000 ***g***), and the supernatant was filtered. The culture medium was loaded onto a 69-mL column filled with Fractogel EMD SO_3^- (Merck) previously equilibrated with 50 mM acetate buffer, pH 5.0, at a flow rate of 6.5 $mL{\cdot}min^{-1}$. The nonbinding fraction was washed from the column using the same buffer, and elution of the inhibitor was achieved by a linear gradient of 0–1 M NaCl. Active fractions were eluted between 0.25 M and 0.4 M NaCl. Rechromatography at pH 6.0, using a 50 mM phosphate buffer, allowed separation from traces of pro-TGase. The combined fractions, containing the inhibitory peptide, were dialyzed against 50 mM Tris/HCl, pH 7.0, concentrated and frozen at −20 °C. Determination of the N-terminal sequence was performed by Edman analysis, as described above.

Determination of the TAMEP cleavage site

Pro-TGase from *S. mobaraensis* was purified according to the procedure of Pasternack *et al.* [27]. A 90-μL volume of the zymogen (0.37 $mg \cdot mL^{-1}$) was incubated with 120 μL of TAMEP (≈ 0.01 $mg \cdot mL^{-1}$), 5 mM phenylmethanesulfonyl fluoride, 20 μM 4-[(2*S*,3*S*)-3-carboxyoxiran-2-ylcarbonyl-L-leucylamido]butylguanidine (E-64), 25 μM pepstatin and 20 μM bestatin (all inhibitors from Sigma) in 0.1 M Tris/HCl, pH 7.0, containing 2 mM $CaCl_2$ for 30 min at 28 °C. SDS gel electrophoresis of the truncated enzyme was carried out using the borate buffer system of Poduslo [34]. The activated TGase protein was excised and sequenced as outlined above.

Protease assays

Method 1, using pro-TGase. A 20-μL volume of pro-TGase (0.37 $mg \cdot mL^{-1}$) in 50 mM Tris/HCl, pH 7.0, containing 2 mM $CaCl_2$, was incubated with 20 μL of sample and 30 μL of 100 mM Tris/HCl, pH 7.0, containing 2 mM $CaCl_2$, at 28 °C for 30 min. A 20-μL volume of the preparation was used for SDS/PAGE. To determine TGase activity, incubation was continued for 10 min at 37 °C after the addition of 30 mM Cbz-Gln-Gly (Bachem), 0.1 M hydroxylamine and 10 mM glutathione (Sigma) in 100 μL of 0.2 M Tris-acetate, pH 6.0 (150 μL final volume). The reaction was stopped by the addition of 100 μL of a 1 : 1 : 1 mixture of 12% (v/v) HCl, 5% (v/v) $FeCl_3$ and 12% (v/v) trichloroacetic acid, and absorption was measured at 492 nm by using a Genios multifunction-reader (Tecan).

Method 2, using furylacryloylpeptidyl amides. P1′-protease activity was determined according to the method described by Feder [35]. A 10-μL volume of 10 mM N-[(3-(2-furyl)]acryloyl (FA)-Ala-Phe amide ($\Delta\varepsilon_{340} = -0.600$ $mL \cdot \mu mol^{-1}$), FA-Gly-Phe amide ($\Delta\varepsilon_{340} = -0.518$ $mL \cdot \mu mol^{-1}$) or FA-Gly-Leu amide ($\Delta\varepsilon_{340} = -0.359$ $mL \cdot \mu mol^{-1}$) (all peptides from Bachem) were added to 30 μL of protease in 160 μL of 50 mM Tris/HCl buffer, pH 7.2, containing 50 mM Mes, 2 mM $CaCl_2$ and 0.01% Triton-X-100, and the decrease in absorbance (*A*) at 340 nm was measured for 20 min at 37 °C.

Method 3, using p-nitroanilide derivatives. *Endo-* and *exo-*protease activity was determined by monitoring the release of p-nitroaniline at 405 nm for 20 min at 28 °C in 96-well microtitre plates. A 100-μL volume of a protease sample was incubated with 0.4 mM Suc-Ala-Ala-Pro-Phe-pNA, Cbz-Pro-Phe-Arg-pNA (both solutions also contained 5% methanol) and Leu-pNA (all peptides from Bachem) in 100 μL of 50 mM Tris/HCl, pH 7.0, containing 2 mM $CaCl_2$. Activity was calculated using an extinction coefficient of 4643 $mL \cdot mmol^{-1}$. One protease unit was defined as the amount of enzyme needed to produce 1 nmol of p-nitroaniline$\cdot min^{-1}$ under these conditions.

Inhibition of TAMEP by P_{14} from *S. mobaraensis* or other inhibitors

Twenty microlitres of TAMEP (28 $\mu g \cdot mL^{-1}$) and 20 μL of P_{14} (0.5–40 $\mu g \cdot mL^{-1}$) were incubated at 28 °C for 15 min. A 20-μL sample of the mixture was combined with 20 μL of pro-TGase (0.37 $mg \cdot mL^{-1}$) and 30 μL of 0.1 M Tris/HCl, pH 7.0, containing 2 mM $CaCl_2$, and incubation was continued for 30 min. TGase activity was determined as described above, in protease assay 1. Other inhibitors, such as 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), aprotinin, benzamidine, bestatin, chymostatin, EDTA, EGTA, leupeptin, pepstatin, *o*-phenanthroline, phosphoramidon, and phenylmethanesulfonyl fluoride (Sigma and ICN Biomedicals) were also used. Thermolysin from *B. thermoproteolyticus rokko* and dispase from *B. polymyxa* for comparative measurements were from Sigma and Roche Diagnostics, respectively.

Preparation of polyclonal antibodies against P_{14}

Eight-hundred micrograms of P_{14}, purified as described above, was heated for 10 min in SDS buffer, pH 6.8, concentrated by SDS/PAGE and stained with Coomassie Brilliant Blue. After several washes with water (for at least 10 min), the protein at 14 kDa was excised and used for immunization. Rabbit immunization was performed by Eurogentec. The antibodies were purified on Biorad DEAE-Affi-Gel Blue columns, according to the manufacturer's protocol.

Other analytical procedures

Protein concentrations were estimated using a bicinchoninic acid protein assay kit using bovine serum albumin as standard, according to the manufacturer's (Pierce) instructions.

PAGE was performed in the presence of SDS, according to the method of Laemmli [31] and Poduslo [34], using the Biorad Mini-Protean II apparatus. Proteins separated by SDS/PAGE were transferred onto nitrocellulose with a Semi-Dry Transblot unit (Biorad) (90 min at 20 V) using the buffer system of Towbin *et al.* [36]. IEF was carried out with the LKB 2117 Multiphor II model (Amersham-Pharmacia) using Servalyt Precote acrylamide gels (Serva), following the manufacturer's instructions. Marker mixtures from Sigma (M-2789 for capillary electrophoresis), Biorad (161-0305, low range) and Serva (39211, IEF markers 3–10) were used to estimate molecular mass and isoelectric point.

Electrophoresis gels were stained with $AgNO_3$, according to Blum *et al.* [37], or with Coomassie Brilliant Blue R [0.25% (v/v) in 9.2% (v/v) acetic acid and 45.4% (v/v) ethanol]. Immunostaining was carried out as described by Pasternack *et al.* [27]. Protease activity on IEF gels was visualized by overlaying an agarose gel (15 $mg \cdot mL^{-1}$) containing 0.2 $mg \cdot mL^{-1}$ α_S-casein and 2 mM $CaCl_2$ in 50 mM Tris/HCl, pH 8.0, and by staining with Coomassie Blue.

Results

Proteases secreted by *S. mobaraensis* on a nutrition-deficient medium

S. mobaraensis usually forms exospores on solid agar containing only glucose, yeast and malt extracts, and calcium carbonate. Formation of aerial mycelium is

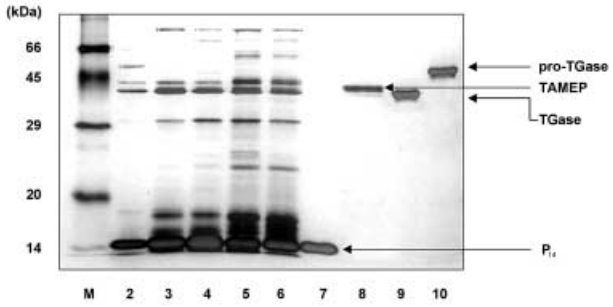

Fig. 1. Silver-stained protein profiles of *Streptomyces mobaraensis* colonies grown on glucose-yeast-malt (GYM) agar. Plate cultures were extracted with 2 mM $CaCl_2$ in 50 mM Tris/HCl, pH 7.0, at 28 °C for 24 h, and supernatants were filtered before application. Lanes 2–6, extracts of 1-, 3-, 7-, 16- and 28-day cultures; lane 7, transglutaminase-activating metalloprotease (TAMEP) inhibitor of 14 kDa (P_{14}) (0.6 μg) purified from liquid culture; lane 8, purified TAMEP (0.2 μg); lane 9, mature transglutaminase (TGase) (0.5 μg); and lane 10, pro-TGase (0.5 μg).

commonly observed by culturing the microorganism at 28–30 °C for not more than 3 days. If these cultures were extracted at pH 7.0 for about 1 day using a Ca^{2+}-containing Tris buffer, extracts capable of activating TGase were obtained. Activation was not observed if extracts from fresh culture medium were used. As seen in Fig. 1, pro-TGase was only present in extracts from 1-day-old colonies, while the processed enzyme could also be found in senescent cultures. A small band above mature TGase shows the activating protease, TAMEP. The major protein at about 14 kDa was stained by antibodies raised against P_{14} from liquid cultures (results not shown), an inhibitor of TAMEP, as shown below. The result suggests the occurrence of a modified, less active or inactive P_{14} species in the GYM extracts.

Several chromophoric peptides derived from the cleavage site of bacterial TGase were used to precisely define the enzyme involved in TGase activation (Fig. 2), and *exo*- and *endo*-proteases could be determined. Release of p-nitroaniline from the N-protected peptides Cbz-Pro-Phe-Arg-pNA and Suc-Ala-Ala-Pro-Phe-pNA, suggested the expression of trypsin-like (Arg-C) and chymotrypsin-like (Phe-C: *endo*-protease hydrolyzing Suc-Ala-Ala-Pro-Phe-pNA) proteases widely distributed among *Streptomyces* spp. (see refs 38 and 39 for examples), whereas hydrolysis of Leu-pNA indicated the additional presence of an aminopeptidase. However, if a plate supernatant was treated with a mixture of 5 mM phenylmethanesulfonyl fluoride, 20 μM E-64, 25 μM pepstatin and 20 μM bestatin, inhibitory molecules known to act against serine, cysteine and aspartate proteases or aminopeptidases, respectively, proteolysis of the synthetic compounds were no longer detectable but cleavage of TGase still occurred. In a further attempt, the same inhibitory mixture with the addition of 15 mM EDTA could now completely suppress the extract-mediated activation of TGase, indicating that the activity of the endogenous protease is dependent on cations forming complexes with EDTA.

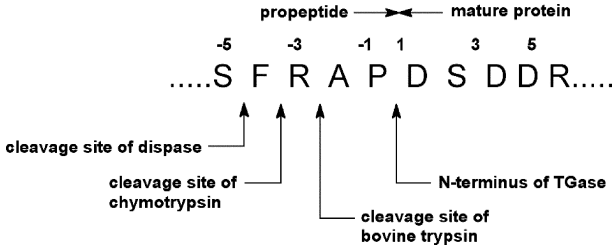

Fig. 2. Amino acids at the cleavage site of transglutaminase (TGase) from *Streptomyces mobaraensis*. The peptide bond between the activation peptide and the mature enzyme, as well as the cleavage sites of bovine trypsin, chymotrypsin and dispase from *Bacillus polymyxa* described in ref. 27, are indicated by arrows.

Purification of the TGase-activating protease

The TGase-activating protease was purified from plate supernatants by chromatography on DEAE Sepharose (results not shown). In a typical experiment, 80 mL of extract was separated in the presence of Ca^{2+} at pH 8.0 on an 8-mL column. High absorption of the unbound fractions may have been a result of the brownish-coloured extract rather than to substantial protein content. The TGase-activating protease was eluted by a stepwise NaCl gradient at concentrations of 40–60 mM and characterized by its ability to activate TGase (Fig. 3). Complete processing occurred if aliquots of TAMEP, eluted after 155–170 min, were used (Fig. 3A, lines 3–6). It can also be seen that residues of dimer pro-TGase (that can also be stained by TGase-specific antibodies), indicated by faint bands at

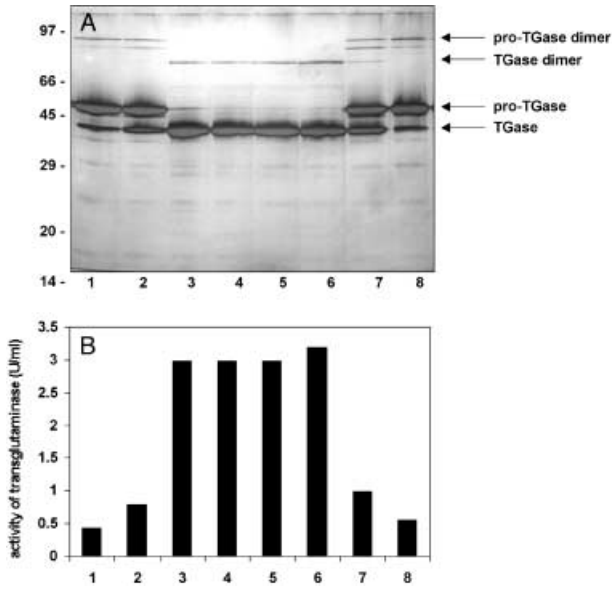

Fig. 3. Identification of the transglutaminase (TGase)-activating protease. (A) Silver-stained SDS polyacrylamide gel of 30 μL of pro-TGase (0.37 $mg \cdot mL^{-1}$) processed by 20 μL of the DEAE Sepharose fractions eluted after 140, 145, 155, 160, 165, 170, 175 and 180 min, corresponding to NaCl concentrations of 40–60 mM (lanes 1–8); (B) TGase activity.

≈ 85 kDa, disappeared completely if the zymogen was incubated with the main fractions. A new protein of ≈ 76 kDa was found instead, and it seems evident that this is a dimer of mature TGase formed by the action of the *endo*-protease. A third band situated between both TGase dimers is observable if minor fractions of the *endo*-protease are used that are not able to activate pro-TGase completely under the conditions applied (Fig. 3A, lines 1, 2, 7, 8). An obvious interpretation might be the successive removal of the activation peptides by the protease, forming mixed aggregates of the zymogen and processed TGase. Probably, dimer formation occurs during heating in SDS, as autocatalytic labelling with fluorescent amine or glutaminyl peptide substrates could not be observed (R. Pasternack & H.-L. Fuchsbauer, unpublished results).

The TGase-activating protease purified on DEAE Sepharose was occasionally contaminated with traces of the mentioned Arg-C *endo*-protease (< 10% of the original activity) but was free of Phe-C and Leu aminopeptidase activity. Arg-C was easily inhibited by the addition of 1 mM phenylmethanesulfonyl fluoride or 10 μM leupeptin. Alternatively, Arg Sepharose chromatography could be utilized to separate the proteases. Neither enzyme bound to the arginine residues. Arg-C was eluted first, followed by the TGase-activating protease (results not shown). The results of TAMEP purification are illustrated in Fig. 4.

Identification of the cleavage site and the protease family

The TGase precursor protein was digested in the presence of inhibitors (5 mM phenylmethanesulfonyl fluoride, 20 μM E-64, 25 μM pepstatin and 20 μM bestatin) and purified TAMEP. The activated enzyme was blotted onto a poly(vinylidene difluoride) membrane and analysed by Edman degradation. The N-terminal sequence was clearly identified as Phe-Arg-Ala, reflecting a cleavage site between Ser(−5) and Phe(−4) (Figure 2). A 41 amino acid peptide of about 4.1 kDa must have been removed from pro-TGase by TAMEP, as indicated by a shift to the corresponding lower molecular mass for the activated enzyme (Fig. 3). Furthermore, the results show that TGase must be processed in a two-step procedure if no other activating protease is secreted during the life-cycle of *S. mobaraensis*. TGase was cleaved by the endogenous protease at the same position as by dispase from *B. polymyxa* [27], and, accordingly, we expected to observe P1′ specificity, i.e. hydrolysis of the peptide bond at the N-side of phenylalanine. In order to substantiate the assumption, the P1′ substrates of Table 1, furylacryloyl peptides, were used, only allowing proteolysis at the sole peptide bond [35]. A decrease in absorption at 340 nm demonstrated that all peptides are digested by TAMEP. The results also suggest that, beside phenylalanine, a short side-chain of the P(−1) amino acid, i.e. alanine (FA-Ala-Phe-NH_2) or serine (TGase, Fig. 2), may favour substrate binding to the endoprotease.

Numerous proteases from *Streptomycetes* have been characterized, potentially allowing identification of the TGase-activating enzyme by sequence alignment. Purified TAMEP was separated again by SDS gel electrophoresis and blotted, and the protein at 39 kDa was excised and analysed by N-terminal sequencing. A sequence of 18 amino acids was obtained which shared 61% sequence homology with the known zinc-dependent metalloprotease of *S. griseus* (SGMPII) (Fig. 5) [40]. Additionally, sequence alignment using the protein blast programmes of NCBI and EMBL also revealed two putative neutral zinc metalloproteases of *S. coelicolor* A3(2), coding for proteins homologous to TAMEP and SGMPII. SGMPII is related to

Table 1. Hydrolysis rates of furylacryloyl peptides (μmol·min^{-1}·ml^{-1}) in the presence of 0.13 μg·mL^{-1} transglutaminase (TGase)-activating metalloprotease (TAMEP) from *Streptomyces mobaraensis* and 1.38 μg·mL^{-1} thermolysin from *Bacillus thermoproteolyticus rokko*. Incubations were performed in 50 mM Tris/Mes, pH 7.2, containing 0.01% Triton-X-100 and 2 mM $CaCl_2$, at 37 °C. Values represent the mean ± SEM of three experiments.

Peptide	TAMEP	Thermolysin
FA-Ala-Phe-NH_2	3.24 ± 0.12	3.78 ± 0.19
FA-Gly-Phe-NH_2	0.76 ± 0.07	1.46 ± 0.10
FA-Gly-Leu-NH_2	0.42 ± 0.03	1.89 ± 0.11

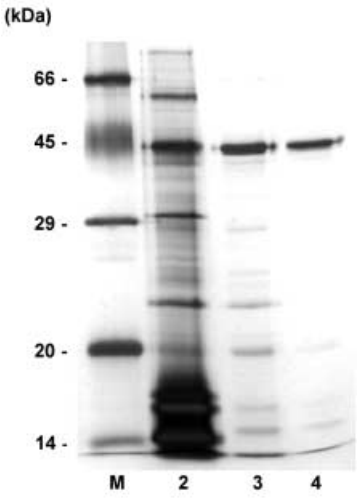

Fig. 4. Silver-stained SDS gel of purified transglutaminase-activating metalloprotease (TAMEP). Lane 2, filtered extract; lane 3, combined fractions of DEAE Sepharose chromatography; lane 4, combined and dialyzed fractions of Arg Sepharose chromatography.

TAMEP (*S. mobaraensis*)	GTGNS QHSGQ VQIGT TKS
SGMPII (*S. griseus*)	GTGNT QYNGQ VTLGT APS
SC3D11.04 (*S. coelicolor*)	GTGNT LYSGT VTLGT AQS
SC7A8.13 (*S. coelicolor*)	GDGKS LYGGT VPLET TPS

Fig. 5. Relationship of transglutaminase-activating metalloprotease (TAMEP) to the M4 family. Alignment of the N-terminal sequence with primary structures of a zinc metalloproteases from *Streptomyces griseus* (SGMPII) and putative proteases from *S. coelicolor* A3(2) (EMBL loci SC3D11.04 and SC7A8.13).

the M4 family, which also includes thermolysin from *B. thermoproteolyticus* [41].

Properties of TAMEP

The metalloprotease from *S. griseus* SGMPII has a calculated molecular mass of 37 kDa [40,42]. TAMEP from *S. mobaraensis* appears to be slightly larger (with a molecular mass of ≈ 39 kDa, as estimated by SDS/PAGE), where the protein is found between the TGase molecules (calculated masses of 38 and 42.5 kDa, Fig 1). IEF and proteinchemical investigations provided further evidence that TAMEP is a member of the M4 family. A pI of 5.0 (Fig. 6) is consistent with that of other metalloproteases, substantiating the close relationship to the enzymes [43]. Furthermore, as outlined above (Table 1), TAMEP-mediated hydrolysis of the furylacryloylpeptides FA-Ala-Phe-NH_2 or FA-Gly-Phe-NH_2 proceeds at a higher rate than that of FA-Gly-Leu-NH_2. The result is in line with the previously determined preference of SGMPII hydrolyzing Phe-Xaa peptide bonds of Cbz-Phe-Xaa-Ala in the order Phe > Tyr > Leu.

In addition to being inhibited by the metalloprotease inhibitor, EDTA, the TGase-activating protease was susceptible to a *S. subtilisin* inhibitor (SSI)-like protein of about 14 kDa (P_{14}), purified from liquid cultures of *S. mobaraensis* (see below). The unusual binding of the serine protease inhibitor SSI by a metalloprotease has been demonstrated for the SGMPII from *S. griseus* and for a protease from *S. caespitosus* [44]. Comparative inhibitory experiments revealed a low sensitivity of thermolysin against P_{14} (not shown). In contrast, phosphoramidon, a powerful antagonist of thermolysin, was a weak inhibitor for TAMEP (Fig. 7). From all these findings we conclude that transformation of the precursor protein of TGase to an active enzyme may be performed by the action of an endogenous metalloprotease that is enzymatically more related to SGMPII than to thermolysin.

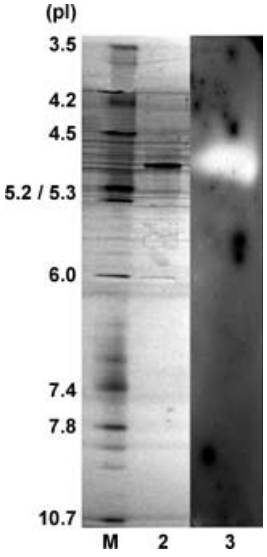

Fig. 6. Isoelectric focusing of transglutaminase-activating metalloprotease (TAMEP) purified by DEAE Sepharose chromatography. Lane 2, TAMEP; lane 3, casein agarose overlay stained by Coomassie Brilliant Blue.

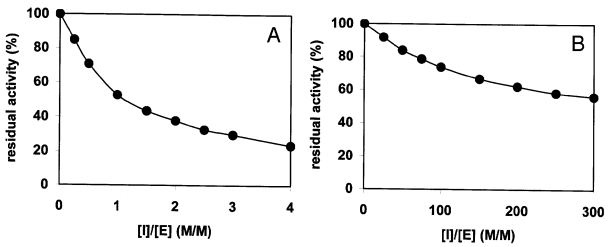

Fig. 7. Inhibition of transglutaminase-activating metalloprotease (TAMEP) and thermolysin by phosphoramidon. Thermolysin (10 nM) (A) or TAMEP (1 nM) (B) were incubated in 0.1 M Tris-Mes buffer, pH 7.2, at 37 °C for 15 min with phosphoramidon, and activities were measured using 0.5 mM FA-Ala-Phe-NH_2, as described in the Materials and methods.

Purification of the TAMEP inhibitor, P_{14}

P_{14} is one of the dominant proteins secreted by submerged cultures and surface colonies of *S. mobaraensis* and an effective inhibitor of TAMEP, as shown below. Considerable amounts of P_{14} were obtained if *S. mobaraensis* was allowed to grow in a liquid complex medium for 48 h at 28 °C. The protein could then be isolated by sequential ion-exchange chromatographies at pH 5.0 and pH 6.0 from supernatants of the centrifuged and filtered culture broths, as described in the Materials and methods (results not shown). The highly purified protein was used to prepare polyclonal antibodies. N-terminal sequencing assigned P_{14} to the SSI family (Fig. 8).

Regulation of TAMEP and TGase by P_{14}

Inhibition of TAMEP also results in repression of the bacterial cross-linking activities by preventing the activation of TGase. Investigations were therefore carried out to examine the influence of the SSI-like inhibitor P_{14} on the activity of both enzymes. First, activation of TGase by a 1000-fold lower concentration of TAMEP was studied (Fig. 9a). A steep increase in TGase activity slowed down after a few minutes, followed by a more gentle rise. If 0.5 μg of pro-TGase was incubated with various amounts of the activating protease, complete processing was achieved with ≈ 2 ng of TAMEP. However, TGase activity could be further enhanced by higher concentrations of TAMEP (Fig. 9b). It appears obvious that the

P_{14} (*S. mobaraensis*)	QLYAP SALVL TVGQQ D
SIL-V2 (*Sv. orinoci*) [45]	SLYAP SALVL TIGQG D
SIL-3 (*S. coelicolor*) [46]	YAP SALVL TVGHG E

Fig. 8. Alignment of the N-terminal sequence of P_{14} isolated from culture broths of *Streptomyces mobaraensis* with the *S. subtilisin*-type inhibitor sequences of *Sv. orinoci* and *S. coelicolor*.

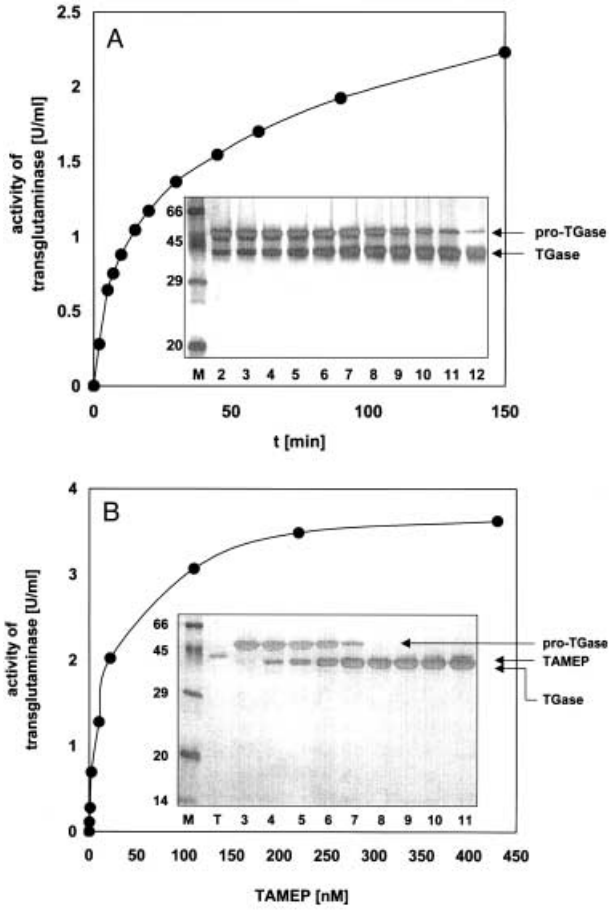

Fig. 9. Activation of transglutaminase (TGase) by transglutaminase-activating metalloprotease (TAMEP). pro-TGase (0.1 mg·mL^{-1}) was incubated with TAMEP, as described in the Materials and methods. (A) Incubation for 1, 3, 7, 10, 15, 20, 30, 45, 60, 90 and 150 min (lanes 2–12) using 0.1 μg·mL^{-1} TAMEP; and (B) incubation for 30 min using 0.5 μg of pro-TGase and 0, 0.043, 0.21, 0.43, 2.1, 4.3, 21, 43, 85 ng of TAMEP (lanes 3–11, respectively). Lanes M and T show the molecular weight marker mixture and TAMEP, respectively.

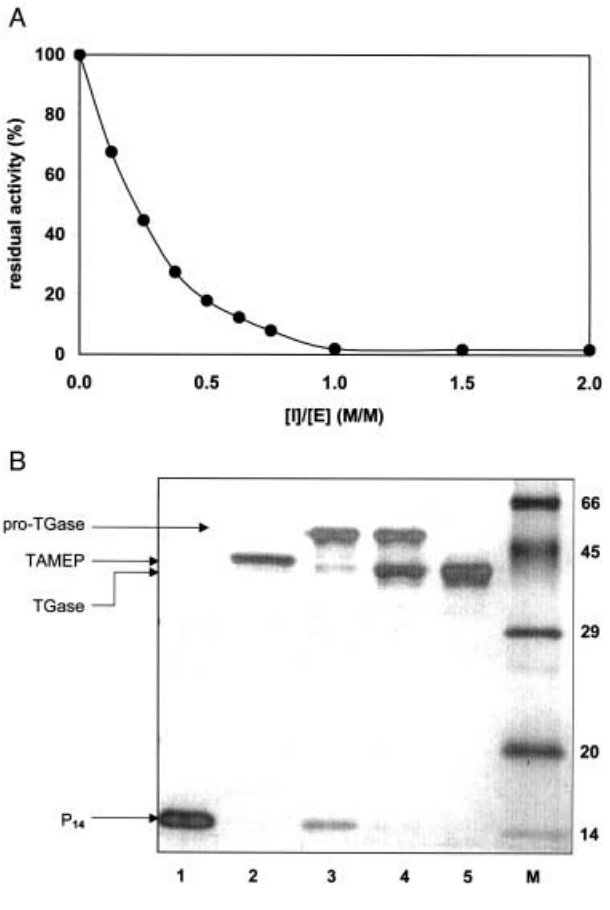

Fig. 10. Regulation of transglutaminase (TGase) by transglutaminase-activating metalloprotease (TAMEP) inhibitor of 14 kDa (P_{14}) inhibition of TAMEP. TAMEP was inhibited by P_{14}, prior to incubation with pro-TGase, as described in the Materials and methods. (A) 2.5 μM pro-TGase, 0.1 μM TAMEP and 0.01–0.20 μM P_{14}. (B) Lane 1, P_{14} (0.6 μg); lane 2, TAMEP (≈ 0.2 μg); lanes 3–5, 0.5 μg of pro-TGase, 21 ng of TAMEP and 8, 2.6 and 0.8 ng, respectively, of P_{14}; lane 6, molecular weight marker mixture.

released activation peptide can still inhibit TGase until it is digested by TAMEP. Interestingly, TGase processed by the highest concentration of TAMEP had the same specific activity (36 U·mg^{-1}) as highly purified mature enzyme from culture broth. Truncation of the remaining tetrapeptide is therefore probably an artefact of a peptidyl aminopeptidase coexpressed by *S. mobaraensis*. The experiments also revealed that the low amounts of TAMEP necessary to activate TGase are not detectable on silver-stained gels. The long periods of *S. mobaraensis* culture still suggest much lower concentrations of active TAMEP.

Complete suppression of TGase activation by TAMEP was found if the protease and the inhibitory peptide were preincubated for 15 min in equimolar concentrations (Fig. 10). Mature TGase alone could not be inhibited by the 14-kDa protein (not shown). The result clearly demonstrates the important function of P_{14} for *S. mobaraensis* to control TGase activity by inhibition of TAMEP.

Discussion

Previous studies have shown that bacterial TGase of *S. mobaraensis* is secreted as a zymogen in liquid cultures and is activated during the culture [27]. However, an endogenous protease involved in TGase activation could not be determined. This report now presents evidence that a TGase activating metalloprotease (TAMEP) is inhibited by a 14-kDa polypeptide (P_{14}), one of the major proteins in culture broths and homologous to the *Streptomyces* protease inhibitor SSI. TAMEP, the first important factor of regulating extracellular TGase activities in *Streptomycetes*, was identified by sporulating colonies grown on nutrient-deficient agar, and in many experiments the enzyme was active despite the occurrence of P_{14} in the extracts. In previous studies, it has been shown that the activity of SSI may be reduced by a carboxypeptidase truncating a tetrapeptide from the C-terminal region [47]. A similar degradation of P_{14} could occur correspondingly and would explain our different results with submerged and

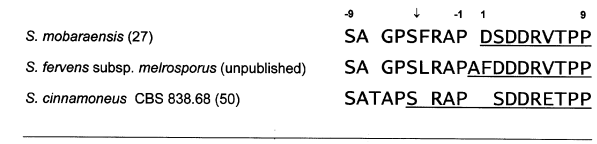

Fig. 11. Primary structure of transglutaminase (TGase) from *Streptomyces* strains at the cleavage site. The N-terminal peptides of the mature enzymes are underlined. The arrow indicates the cleavage site of the TGase-activating metalloprotease (TAMEP) from *Streptomyces mobaraensis*.

surface colonies. Expression of proteases and digestion of protease inhibitors seems to be strongly correlated with the onset of aerial mycelium growth and the development of spores [5,6]. It is therefore highly probable that TGase is involved in the early stages of *S. mobaraensis* differentiation when TAMEP is active as a consequence of P_{14} degradation. Investigations are in progress to study, in greater detail, the inactivation of the TAMEP inhibitor.

According to the sequence data established for the N-terminal peptide, the TGase-activating protease may be placed into a subfamily of Cys-containing metalloproteases that belong to the M4 family of metalloproteases [42]. Sequence homology was found for the Zn^{2+}-dependent *endo*-protease SGMPII from *S. griseus*, which may be inactivated by EDTA, *o*-phenanthroline and phosphoramidon, as well as by active-site-directed inhibitors such as $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 and $ClCH_2CO$-DL-(N-OH)Leu-Ala-Gly-NH_2 [40,48]. In contrast to related proteases such as thermolysin, SGMPII has a binding site for the SSI, although the gene for an SSI-like polypeptide is absent in *S. griseus* [49]. The TAMEP may likewise be inhibited by EDTA and the endogenous 14 kDa SSI-like polypeptide from *S. mobaraensis*, indicating the close relationship to SGMPII. It is remarkable that TGase of *S. griseus* has not been detected by now, substantiating the assumption that the SSI-like inhibitor is a second important tool for regulating TGase activity.

TGase from *S. mobaraensis* is activated by hydrolysis of the peptide bond between Phe(−4) and Ser(−5) (Figs 2 and 11). Sequence homology of TGase from *S. fervens* ssp. *melrosporus* at the cleavage site suggests the same processing procedure as for SM-TGase. However, if the phenylalanine (or leucine) residue is deleted, as is the case for TGase from *S. cinnamoneus*, a tetrapeptide with the RAP motif remains at the N-terminus of the mature enzyme [50]. It has been shown that peptide bonds with alanine in the P1′ position may also be cleaved by SGMPII, and, assuming the same specificity for TAMEP, the cleavage site of SC-TGase may be predicted to be between Ala(−6) and the inserted Thr(−7) or between Ala(−8) and Ser(−9). The latter possibility is more plausible as the peptide bond is located in the same position within the structural motif S↓YXAP, as that found at the other cleavage sites.

Processing of TAMEP-activated TGase has to be completed by the action of aminopeptidases to obtain the mature N-terminus. We have recently detected a peptidyl aminopeptidase in liquid cultures of *S. mobaraensis* enabled to remove the tetrapeptide SRAP. As TGase activity is not enhanced by this action, the last step in TGase processing is most probably an artefact of the peptidase secreted with the cross-linking enzyme.

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