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A Novel Transglutaminase Substrate from *Streptomyces mobaraensis* Inhibiting Papain-Like Cysteine Proteases

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Transglutaminase from *Streptomyces mobaraensis* is an enzyme of unknown function that cross-links proteins to high molecular weight aggregates. Previously, we characterized two intrinsic transglutaminase substrates with inactivating activities against subtilisin and dispase. This report now describes a novel substrate that inhibits papain, bromelain, and trypsin. Papain was the most sensitive protease; thus, the protein was designated *Streptomyces* papain inhibitor (SPI). To avoid transglutaminase-mediated glutamine deamidation during culture, SPI was produced by *Streptomyces mobaraensis* at various growth temperatures. The best results were achieved by culturing for 30–50 h at 42°C, which yielded high SPI concentrations and negligibly small amounts of mature transglutaminase. Transglutaminase-specific biotinylation displayed largely unmodified glutamine and lysine residues. In contrast, purified SPI from the 28°C culture lost the potential to be cross-linked, but exhibited higher inhibitory activity as indicated by a significantly lower K_i (60 nM *vs.* 140 nM). Despite similarities in molecular mass (12 kDa) and high thermostability, SPI exhibits clear differences in comparison with all members of the well-known family of *Streptomyces* subtilisin inhibitors. The neutral protein (pI of 7.3) shares sequence homology with a putative protein from *Streptomyces lavendulae*, whose conformation is most likely stabilized by two disulfide bridges. However, cysteine residues are not localized in the typical regions of subtilisin inhibitors. SPI and the formerly characterized dispase-inactivating substrate are unique proteins of distinct Streptomycetes such as *Streptomyces mobaraensis*. Along with the subtilisin inhibitory protein, they could play a crucial role in the defense of vulnerable protein layers that are solidified by transglutaminase.

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Transglutaminases (TGase, E.C. 2.3.2.13) are widely distributed enzymes that commonly catalyze protein cross-linking *via* γ-glutamyl ε-lysine isopeptide bonds [23]. The initial acyl-enzyme complex between a glutamine-containing substrate and the catalytic cysteine subsequently disintegrates by the nucleophilic attack of a lysine donor protein. Alternative incorporation of primary amines, ω-hydroxy fatty acids, and water results in substituted glutamines, glutamic esters, or glutamine hydrolysis, respectively.

Certain members of the multicellular Streptomycetes, formerly assigned to the genus *Streptoverticillium*, produce a Ca^{2+}-independent TGase that differs from its mammalian counterparts in many respects [1, 17]. Besides the absence of Ca^{2+}-binding sites, a low molecular mass (38 kDa) and lack of sequence homology are peculiar attributes of the bacterial enzyme. The sole common feature of eukaryotic TGases is the cysteine in the active core, which forms a catalytic triad with adjacent histidine and aspartyl residues. However, the crystal of microbial TGase likewise shows a modified pattern of the catalytic triad [18]. Arrangement of the aspartyl residue in proximity to the thiol group of cysteine has led to the assumption that the β-carboxylate is the decisive proton acceptor whereas histidine has only the function of directing the substrate into the active site.

Microbial TGase from *S. mobaraensis* is initially exported as a precursor protein containing an activation peptide of 45 amino acids [27]. The cleavage site in close vicinity to the mature domain exhibits the sequence motif SFRAP. *In vitro* activation of TGase by a series of *P1*- and *P1*'-endoproteases, such as dispase from *B. polymyxa* (Ser-Phe), bovine chymotrypsin (Phe-Arg), and bovine trypsin (Arg-Ala), suggests the incorporation of SFRAP in an

extended loop region containing at least three accessible peptide bonds [27, 41]. Accordingly, processing of the zymogen by the transglutaminase-activating metalloprotease (TAMEP) from *S. mobaraensis*, which shares with dispase the same *P1′* specificity, resulted in the formation of FRAP-TGase [40]. The truncated activation peptide may still function as a cross-linking inhibitor [28, 40]. Recently, an additional TGase-activating serine protease was purified from culture supernatants of *S. hygroscopicus* [39]. Unfortunately, specificity was not determined; it remains unclear whether chymotrypsin- or trypsin-like endoproteases release RAP-TGase or AP-TGase. Our studies have shown that only FRAP-TGase and RAP-TGase, but not AP-TGase, were substrates of a co-secreted tripeptidylaminopeptidase (TAP) *in vitro*, cleaving the remaining tetra- or tripeptide from the mature N-terminus [41].

TAMEP and the unspecified serine protease are inhibited by heat-resistant proteins, which have been assigned to the large family of *Streptomyces* subtilisin inhibitors (SSI) [39, 40]. Distinct SSI are well known to exhibit two binding sites, one for serine proteases and the other for metalloproteases [15]. The TAMEP inhibitory protein (SSTI) from *S. mobaraensis* is obviously this type of SSI, since it diminishes the activity of subtilisin from *B. licheniformis* and bovine trypsin equally. Recently, we have shown that SSTI is an excellent TGase substrate as well, having accessible glutamine and lysine residues [31]. An additional substrate was discovered that has not been found in frequently studied Streptomycetes such as *S. coelicolor* A3(2) [30]. The dispase autolysis inducing protein (DAIP) destroys neutral metalloproteases by triggering suicide, thus revealing more a defensive than a regulatory function.

Exposed glutamines of SSTI and DAIP were modified during submerged culture of *S. mobaraensis*, most likely by TGase-mediated hydrolysis of the γ-carboxamide side chains [30, 31]. Rapid hydrolysis occurred after onset of proteolytic TGase activation, mostly 35–45 hours after bacterial growth [27]. In shaken cultures, the development of immobile Streptomycetes is well known to be mechanically impaired. The observed substrate modification may be a result of the disturbed bacterial growth.

We have now discovered an additional TGase substrate in *S. mobaraensis* that possesses an inhibitory function against the cysteine and serine proteases papain from *Papaya carica*, bromelain from *Ananas comosus*, and bovine trypsin. The novel protein was designated *Streptomyces* papain inhibitor (SPI). Rapid glutamine hydrolysis of SPI by TGase during culture at 28°C inspired us to study the export of both proteins at higher growth temperatures. Bacterial growth at 42°C considerably impaired export and activation of TGase and prevented SPI modification to a large extent as a result. SPI is, like SSTI, a small, thermoresistant protein, suggesting that is has a biological function in a heat-exposed bacterial organ such as outer protein skins of aerial hyphae and spores. It complements the inhibitory effects of SSTI and DAIP against proteases and supports the hypothesis that TGase participates in the construction of an antibiotic shield to protect vulnerable protein layers.

Materials and Methods

Materials

The hydrochlorides of N^{α}-carbobenzoxy-L-phenylalanyl-L-arginyl *p*-nitroanilide (Cbz-Phe-Arg-*p*NA), N^{α}-benzoyl-L-arginyl *p*-nitroanilide (Bz-Arg-*p*NA), and N^{α}-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide (Suc-Ala-Ala-Pro-Phe-*p*NA) were purchased from Bachem (Bubendorf, Switzerland), and N^{α}-carbobenzoxy-L-glutaminylglycin, and 1-*N*-biotinyl-6-*N′*-(carbobenzoxy-L-glutaminylglycyl)diamidohexane (ZQGB), and monobiotinylcadaverine (MBC) from Zedira (Darmstadt, Germany). Bovine trypsin, bovine chymotrypsin, collagenase (*C. histolyticum*), papain (*Papaya carica*), subtilisin A (*B. licheniformis*), and thermolysin (*B. thermoproteolyticus rokko*) were from Sigma-Aldrich (Munich, Germany), dispase from Worthington (Lakewood, NJ, USA), and proteinase K (*Tritirachium album*) from Merck (Darmstadt, Germany). Dabcyl-Ser-Phe-EDANS was synthesized as described [37]. All other chemicals were of the finest quality available.

Bacterial Strain and Exported Proteins

S. mobaraensis DSM 40847 [German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany] was allowed to grow on glucose-yeast-malt (GYM) agar up to 30 days. Overcrowded agar (1 cm^2) exhibiting a continuous cell layer was used to inoculate a starch-mineral salt medium as previously described [14]. TGase was purified from cell-free supernatants of 3–4-day-old submerged cultures, and activity was measured using the described hydroxamate assay [27]. Extracts of colonies, grown on GYM agar for 15–18 days, were used to purify TAMEP as previously described [40]. Protein content was determined according to the manufacturer's protocol (Interchim, Frampton, UK) using the bicinchoninic assay of Smith *et al*. [33].

Determination of Cell Mass

The entire contents of culture flasks (110 ml) was suctioned through pre-dried filter papers, and the repeatedly water-rinsed cell aggregates were dried for 24 h at 110°C. Total solid was determined by weighing after cooling the sample in a silica-dried atmosphere.

Measurement of Inhibitory Activity

Inhibitory activity was determined by measuring residual protease activity after 30 min of pre-incubation with the studied enzyme at ambient temperature or 37°C. For this purpose, the protease substrates alkaline casein, azocasein, Cbz-Phe-Arg-*p*NA (papain), Bz-Arg-*pNA* (trypsin), Suc-Ala-Ala-Pro-Phe-*p*NA (subtilisin), and Dabcyl-Ser-Phe-EDANS (TAMEP, dispase, thermolysin) in 0.1 M citrate, pH 6.5 (papain, bromelain), and 0.1 M Tris-HCl/2 mM $CaCl_2$, pH 7.5 (trypsin, chymotrypsin, subtilisin, proteinase K, TAMEP, dispase, thermolysin, collagenase), were used. With exception of the two methods described

below, protease assays were performed as previously described [30, 31, 37]. One inhibitory unit was defined as the reduction of one protease unit.

Azocasein Assay

A stock solution of azocasein (1%, 200 µl) in the appropriate buffer was incubated for 10 min with 200 µl of the respective protease inhibitor mixture at 37°C. After addition of 10% trichloroacetic acid and centrifugation, absorbance was monitored at 420 nm. One protease unit was defined as the difference in optical density of 0.1 between the sample and a control without protease.

Nitroanilide Assay

Cbz-Arg-Phe-*p*NA (4 mM in ethanol, 50 µl) in 0.1 M citrate, pH 6.5, and an additional 50 µl of ethanol was incubated for 10 min with 5.1 µM papain or an appropriate papain-inhibitor mixture at 37°C (final volume, 500 µl). The reaction was terminated by the addition of PMSF (5 mM in DMSO, 500 µl), and released *p*-nitroaniline was determined at 405 nm using the molar extinction coefficient of 14.25 ml μmol^{-1} cm^{-1}. One papain unit was defined as the release of 1 µmol *p*-nitroaniline per minute per milliliter.

Purification of SPI

S. mobaraensis was allowed to grow in shaking flasks for 30–50 h at 28–42°C. Cell mass was removed by suction through a Büchner funnel, and the filtrate was immediately heated to 70°C for 30 min. After centrifugation (10,000 ×*g*, 10 min), the supernatant was separated by Fractogel EMD TMAE at pH 9 using 50 mM Tris, pH 9. Non-binding proteins were pumped onto Fractogel EMD SO_3^- (bed volume, 35 ml) at pH 4.0. The retained TGase substrates SSTI and SPI were subsequently eluted by linearly increasing NaCl concentrations from 0–1 M in 50 mM acetate. If large amounts of SSTI were present, the procedure was repeated under the same conditions by reloading the non-binding fraction on the regenerated column. Active fractions were combined, and, if necessary, dialyzed and lyophilized. For protein sequence analysis, minute contaminants were removed by borate gel electrophoresis as previously described [27]. Edman degradation was performed by Dr. Stoll (NMI, Reutlingen, Germany).

Determination of SPI Glutamines and Lysines

TGase-mediated labeling of SPI was performed to indicate the presence of glutamines and lysines as previously described [30, 31]. After enzymatic incorporation of MBC or ZQGB, protein blotting revealed exposed glutamine or lysine residues, respectively.

Other Procedures

Electrophoresis on 12.5% SDS polyacrylamide gels, silver or Coomassie staining, isoelectric focusing, and size-exclusion chromatography (SEC) were performed as described elsewhere. The determination of oligoglutamic acids by MALDI–TOF–TOF analysis was kindly performed by Patrick Keller (Merck, Darmstadt).

RESULTS

Protein Export by *Streptomyces mobaraensis* at Elevated Growth Temperatures

S. mobaraensis simultaneously produces TGase and the TGase substrates SSTI and DAIP during submerged culture at 28°C. Activated TGase, emerging after 35–45 h by proteolytic processing of the precursor protein [27], impairs the intrinsic substrates by hydrolysis of accessible glutamine side chains [30, 31]. Unmodified SSTI or DAIP can be purified as yet only from young culture broth when mature TGase is absent to a large extent.

Our screening for additional TGase substrates from *S. mobaraensis* resulted in the discovery of a novel protein, possessing an inhibitory function against papain, that was designated *Streptomyces* papain inhibitor (SPI). Preliminary experiments suggested that SPI is, like SSTI or other members of the *Streptomyces* subtilisin inhibitor family, a heat-resistant protein. This result, along with our observations of enzymatic substrate modification, prompted us to study bacterial growth, the processing of TGase, and the export of TGase substrates at various temperatures (Fig. 1). The activity of functional proteins was determined by monitoring TGase-induced formation of ferric glutaminyl peptide hydroxamate complexes and the degree to which the culture supernatant reduced papain-, subtilisin- or dispase-mediated azocasein hydrolysis. Activation of TGase during culturing was visualized in addition by Western blotting using anti-proTGase antibodies. Because *S. mobaraensis* produces continuously increasing amounts of cell deposits at the flask wall above the culture broth surface, bacterial growth was estimated by harvesting and drying the total cell mass of many culture flasks at various times and temperatures.

As can be seen from Fig. 1A, the most favorable temperatures for bacterial growth were 28 and 32°C, as the increase in cell mass indicates. Neither culture reached the stationary plateau after 96 h. In contrast, a temperature of 42°C obviously caused hyperthermal stress as indicated by the short exponential growth phase, and early onset of the stationary and death phases. Under these conditions, TGase activity in 42°C cultures was absent or near the detection limit (Fig. 1B). This measurement was confirmed by immunoblotting, which showed, apart from proTGase, no or merely tiny bands of the mature enzyme (not shown). Cross-linking activity of up to 2.5 U/ml was only found when *S. mobaraensis* was cultured at moderate temperatures.

Compared with TGase, secretion of SPI was completely different. Considerable amounts of the protein were detected after 24 h at 32–42°C, and maximum papain inhibitory activity was reached a few hours later (Fig. 1C). Only growth at 28°C caused significant delay of SPI export. Decline of SPI beyond 80 h at 42°C, most likely caused by the release of internal endoproteases, coincided with the observed death phase. Similar results were obtained for SSTI, suggesting that both inhibitors (*i.e.*, SPI and SSTI) are stress proteins (Fig. 1D). Even export of DAIP was favored by moderately elevated growth temperatures (Fig. 1E). That dispase inactivating activity was comparably low in supernatants of 42°C cultures must be ascribed to a more heat-labile DAIP structure [30]. The most intriguing finding,

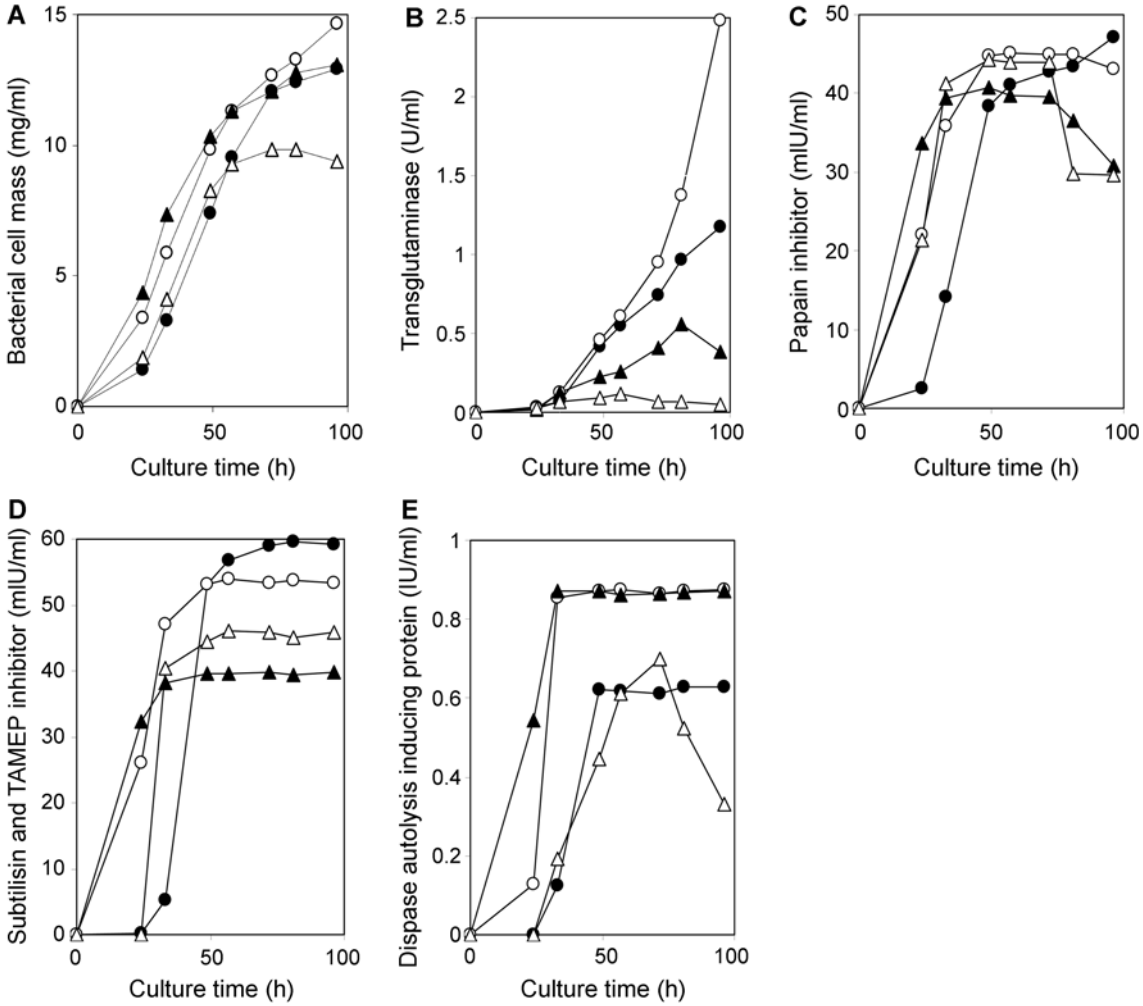

Fig. 1. Effect of temperature on (**A**) bacterial growth and export of (**B**) transglutaminase, (**C**) SPI, (**D**) SSTI, and (**E**) DAIP during submerged growth of *S. mobaraensis*.
Culture was performed in mineral salt starch medium at 28°C (●), 32°C (○), 37°C (▲), and 42°C (△). Cell mass and the activity of the exported proteins were determined as described in the Materials and Methods.

however, was the discovery that *S. mobaraensis* produces the papain inhibitor SPI under hyperthermal stress conditions, almost without substrate-modifying TGase.

Production and Purification of SPI

Numerous attempts were made to purify the papain inhibitory protein SPI from culture broths of *S. mobaraensis*. Largely unmodified SPI, still equipped with TGase-accessible glutamine and lysine residues, was obtained from 42°C cultures interrupted after 30 h. Immediate heating of cell-free supernatants to 70°C for 30 min was necessary to destroy tiny amounts of activated TGase. On the other hand, SPI from 28°C cultures proved to be a better papain inhibitor whereas the potential to be cross-linked was rapidly lost with increasing culture time.

SPI was purified by Fractogel EMD SO_3^- chromatography at pH 4 (Table 1). When supernatants of 42°C cultures were used, considerable amounts of silver-stainable compounds, migrating at the front of 12.5% polyacrylamide gels, prevented binding of SPI and SSTI to the ion-exchange material. Removal by Fractogel EMD TMAE at pH 9 and MALDI–TOF/TOF analysis revealed the presence of small oligoglutamates exhibiting molecular masses of 0.5–1.2 kDa (not shown). The preceding separation of the oligoglutamates by TMAE chromatography was maintained, at least in the purification of SPI from stress culture supernatants. Furthermore, binding of SPI to Fractogel EMD SO_3^- sulfonates was weak in the presence of large amounts of SSTI, so that most of the protein was in the non-binding fraction. Re-chromatography of the forerun yielded highly purified SPI samples whether supernatants from 28°C or 42°C cultures were used (Table 1 and Fig. 2). Two SPI isoforms were displayed by SDS–PAGE, differing by approximately 0.5 kDa in molecular mass. Distinct

Table 1. Purification protocols for the papain inhibitor from *Streptomyces mobaraensis*[a].

Purification step	Volume (ml)	Activity (IU)	Protein (mg)	Specific activity (IU/mg)	Yield (%)
Heated supernatant (28°C culture)	150	1,857	899	2.1	100
1. Fractogel EMD SO_3^- 2. Fractogel EMD SO_3^-	12	17	2.5	6.6	1
Pool A	6	177	4.1	43	10
Pool B	6	63	1.8	35	3
Pool C	6	95	4.8	20	5
Heated supernatant (42°C culture)	89	781	551	1.4	100
Fractogel EMD TMAE (forerun)	180	717	544	1.3	99
1. Fractogel EMD SO_3^- 2. Fractogel EMD SO_3^-	6	39	0.96	41	5
Pool A	10	77	3.6	21	10
Pool B	10	45	2.7	17	6
Pool C	4	23	1.1	21	3

[a]*S. mobaraensis* was allowed to grow in liquid starch mineral salt medium for 30 h at 28 and 42°C. Cell-free supernatants were heated to 70°C for 30 min, centrifuged at 10,000 ×*g*, and separated twice by Fractogel EMD SO_3^- (35 ml column) at pH 4.0 using a linear NaCl gradient of 0–1 M in 50 mM sodium acetate. In the case of 42°C cultures, oligoglutamates were removed from heated supernatants by TMAE chromatography using 50 mM Tris, pH 9. Inhibitory activity was determined by preincubation with papain for 30 min at 37°C and measuring residual azocasein degradation activity as described in the Materials and Methods. One inhibitory activity unit is defined as the A_{420} reduction of 0.1 resulting from azocasein degradation by papain.

fractions, either from 28°C or 42°C cultures, contained only one of these proteins (Fig. 2). Since papain activity was equally reduced by such homogeneous samples, truncation of SPI at the N- or C-terminus during culture, resulting in the observed isoforms, was obvious. That SSTI is significantly larger than unprocessed SPI is shown in lane 7 of Fig. 2.

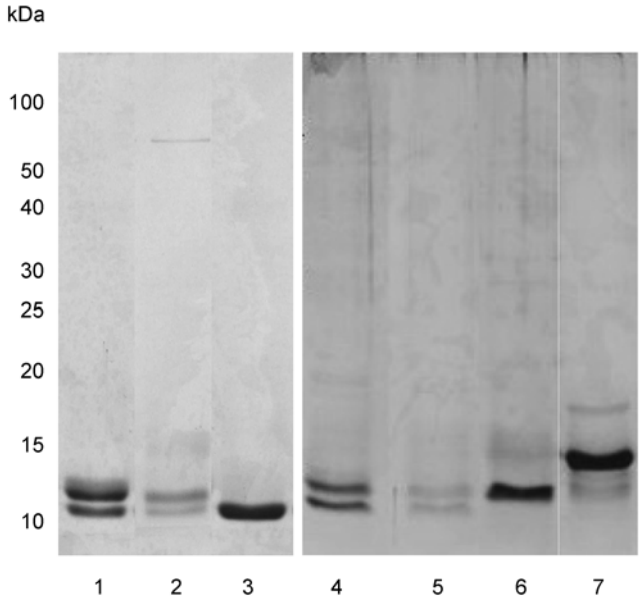

Fig. 2. Purified papain inhibitor from *Streptomyces mobaraensis*. Lanes 1–3, pools A, B, and C from 30 h culture at 28°C (cf. Table 1); lanes 4–6, pools A, B, and C from 30 h culture at 42°C; lane 7, subtilisin and TAMEP inhibitor from 30 h culture at 42°C.

Structural Attributes of SPI

SPI from *S. mobaraensis* is a novel papain inhibitor and is related only to the putative protein Q9X5U4 from *S. lavendulae*, sharing 71% identity with the N-terminal peptide (Fig. 3). The apparent molecular mass of 12 kDa, equally obtained by SDS–PAGE and SEC, was well in accordance with the calculated molecular mass of the mature *S. lavendulae* protein (11,943 Da). The SSTI forms, like other members of the SSI family, homodimers of 28 kDa. Only SPI consists of a single protein chain, as SEC experiments suggested (results not shown). The isoelectric

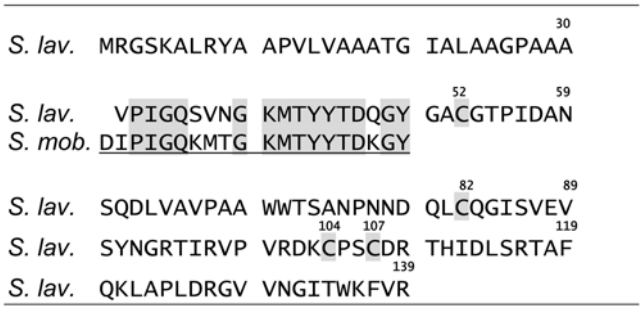

Fig. 3. N-Terminal peptide of SPI from *S. mobaraensis* (underlined). Sequence identity of 71% reveals relationship to a putative protein (Swiss-Prot, Q9X5U4) from *S. lavendulae* [21]. Identical amino acids and cysteine residues, most likely involved in disulfide bridges, are highlighted in gray.

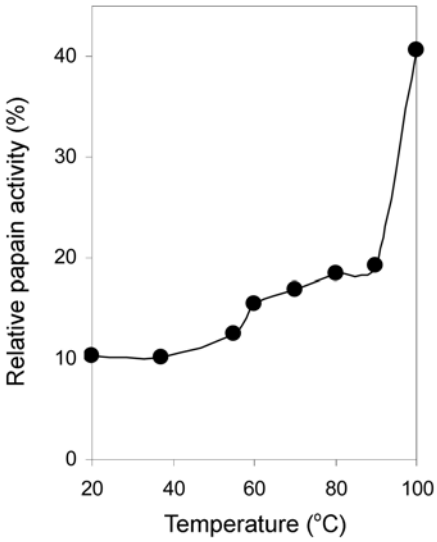

Fig. 4. Thermostability of SPI from *Streptomyces mobaraensis*. The purified protein was heated for 60 min in 0.1 M citrate, pH 6.5, at the indicated temperatures. After further incubation for 30 min with papain at ambient temperatures, residual papain activity was monitored by the degradation of casein. The data represent the means of two independent measurements.

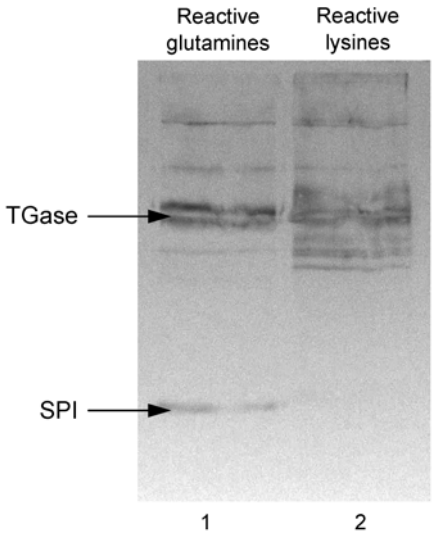

Fig. 5. TGase-mediated biotinylation of SPI from *S. mobaraensis*. The purified inhibitor (2 μg), monobiotinylcadaverine (2 mM, lane 1) or 1-*N*-biotinyl-6-*N'*-(carbobenzoxy-L-glutaminylglycyl)diamidohexane (0.13 mM, lane 2), and TGase (0.7 μg) in 0.1 M HEPES, pH 7.5, were incubated for 2 h at 37°C. Biotinylated proteins were separated by SDS–PAGE (12.5 %), blotted on nitrocellulose, and visualized using streptavidin alkaline phosphatase conjugates, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium as previously described [30, 31].

point of SPI was 7.3, further highlighting the clear differences from the basic SSTI (pI of 9.0).

Furthermore, the deduced sequence of the putative protein Q9X5U4 exhibits four cysteine residues, C52, C82, C104, and C107, which suggests the formation of two disulfide bridges that stabilize the functional SPI conformation (Fig. 3). The cysteine positions differ markedly from the highly conserved SSI regions, thus providing additional evidence for the novel structure of SPI. That SPI has, like SSTI, a heat-resistant structure, was shown by measurements of papain inhibitory activity at elevated temperatures (Fig. 4). Loss of activity was only moderate when SPI was incubated for 1 h at 20–90°C. Even boiling could not reduce the inhibitory activity by more than 30%.

Reactive Sites for Transglutaminase-Mediated Biotinylation

SPI was examined for TGase-accessible glutamine and lysine residues. When SPI was purified from culture supernatants containing activated TGase, the enzymatic incorporation of the biotinylated equivalents MBC, a glutamine probe, and ZQGB, a lysine probe, failed. This result confirmed our former observations with the TGase substrates SSTI and DAIP that mature TGase hydrolyzes the γ-carboxamide side chains of exposed glutamines during submerged culture of *S. mobaraensis* [30, 31]. As was the case with DAIP, electrostatic attraction of exposed ε-amino groups by the newly formed glutamyl residues most likely prevented labeling of exposed lysines in parallel (all results not shown). Only SPI produced by *S. mobaraensis* under hyperthermal growth conditions at 42°C remained unmodified, at least to a large extent, due to the absence for the most part of activated TGase. The purified protein proved to be a glutamine and lysine donor substrate of TGase (Fig. 5). Besides amounts of cross-linked aggregates, labeled SPI monomers were displayed when the biotinylated amine MBC was incorporated into accessible glutamines. In contrast, TGase-mediated lysine probing using the biotinylated glutamine dipeptide ZQGB could not prevent SPI polymerization, thus also indicating the presence of several available lysine residues. These results are in agreement with SSTI or DAIP labeling and clearly show that the papain inhibitor SPI is an excellent substrate of the intrinsic TGase.

Specificity of the Papain Inhibitor

Initially, inhibition of various endoproteases, among them serine, cysteine, and metalloproteases, were studied by pre-incubation with SPI and measurement of residual activity. As can be seen in Table 2, the targets of SPI were the cysteine and serine endoproteases papain, bromelain, and trypsin. An inhibitory effect on the other proteases used was absent or negligibly small. The most sensitive protease, papain, and the papain-specific substrate Cbz-Phe-Arg-*p*NA were further used to characterize SPI. As judged from Dixon plots, the inhibitory constants K_i were 60 nM and 140 nM for SPI from 28°C and 42°C cultures, respectively (Fig. 6). The uncompetitive type of inhibition was indicated by parallel lines at various inhibitor concentrations in a

Table 2. Specificity of the papain inhibitor from *S. mobaraensis*.

Protease	Origin	SPI/Protease (mol/mol)	Residual protease activity[a] (%)
Papain	*Papaya carica*	0.125	62
		0.25	43
		0.5	17
		1	4
Bromelain	*Ananas comosus*	0.25	44
		0.5	33
		1	24
Trypsin	*Bos taurus*	1	25
Chymotrypsin	*Bos taurus*	1	76
Subtilisin	*Bacillus licheniformis*	1	82
Proteinase K	*Tritirachium album*	1	95
TAMEP	*Streptomyces mobaraensis*	1	93
Dispase	*Bacillus polymyxa*	1	99
Thermolysin	*B. thermoproteolyticus* Rokko	1	100
Collagenase	*Clostridium histolyticum*	1	100

[a]Residual protease activity was measured after preincubation for 30 min at 37°C using Cbz-Phe-Arg-*p*NA for papain, Dabcyl-Ser-Phe-EDANS for TAMEP, dispase, or thermolysin, and azocasein for the other proteases as described in the Materials and Methods.

double reciprocal plot of papain activity versus substrate concentration (not shown).

DISCUSSION

Streptomycetes are filamentous soil bacteria growing into available substrates to exploit essential nutrients [7]. The life cycle begins with the formation of germinating tubes and vegetative hyphae by spores, resulting in a branched, cotton-like mycelium [7, 22]. Stored, energy-rich compounds and parts of the immobile soil mycelium are then used to develop aerial hyphae and spores in the reproductive phase. Escape from the moist environment is supported by Sap B, a small, surface-active lanthionin morphogen composed of 21 amino acids [38]. Additional amphiphilic proteins, called chaplins [12] and rodlins [8], are thought to protect the cell wall of aerial hyphae and spores against desiccation and loss of essential compounds by forming an outer protein coat [11].

Key processes of *Streptomyces* differentiation have been intensively studied in *S. coelicolor* A3(2), among them

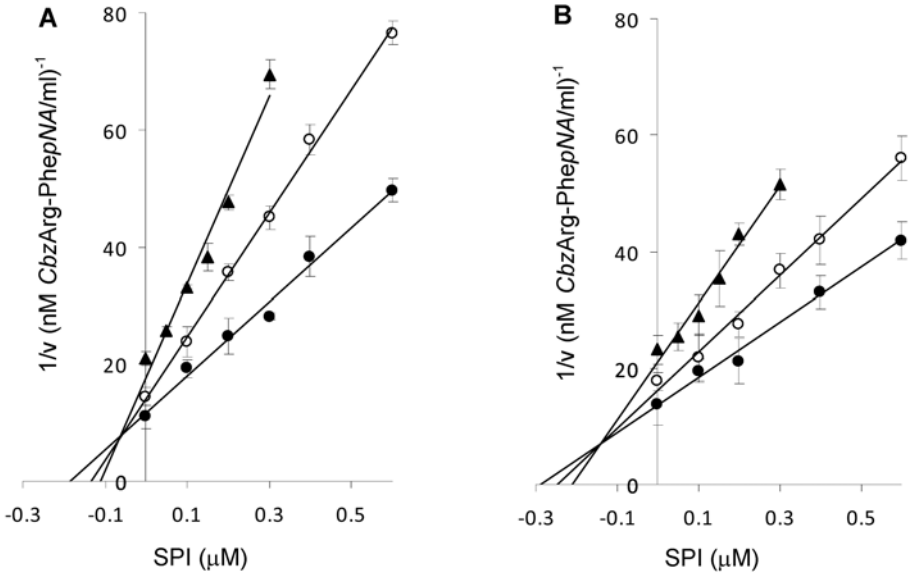

Fig. 6. Dixon plots of papain inhibition by SPI from (**A**) 28°C and (**B**) 42°C cultures of *S. mobaraensis*.
The concentrations of Cbz-Phe-Arg-*p*NA were 0.2 mM (▲), 0.3 mM (○), and 0.4 mM (●), and release of *p*-nitroaniline at pH 6.5 and 37°C was initiated by the addition of 5.1 μM papain. The apparent K_i value was determined by the intersection in the second quadrant. The data represent the means of three independent measurements.

self-assembly of chaplins and rodlins [7, 9]. The absence of coding genes for TGase and DAIP in the annotated genomes of the model bacterium and other strains suggests important differences in *S. mobaraensis* [3, 16, 25]. Conversely, the occurrence of chaplins and rodlins in *S. mobaraensis* has not been investigated as yet. Moreover, the strategy of Streptomycetes to protect the outer cell wall proteins against proteases from bacterial and fungal nutrient competitors or symbionts remains in question.

The present report describes a novel papain inhibitor, SPI, that is, like DAIP and SSTI, a substrate of TGase from *S. mobaraensis*. SPI is related only to a putative protein of *S. lavendulae* NRRL 2564, further highlighting the exceptional position of the TGase producer among studied Streptomycetes. Two putative disulfide bonds, likewise available in all *Streptomyces* subtilisin inhibitors [19], provide for heat resistance up to boiling temperatures. However, divergently placed cysteines and the absence of dimer formation clearly confirm the affiliation of SPI with a novel type of cysteine protease inhibitor. Since it appears to be further obvious that SPI and SSTI could be factors responding to thermal stress, the growth of and protein export by *S. mobaraensis* was studied at elevated temperatures. The most intriguing result was the discovery of severely compromised TGase export at 42°C, revealing an experimental approach to the production of TGase substrates without modified glutamine residues. Under stress conditions, SPI and SSTI emerge in culture broth at the earliest possible time, indicated by the absence of the majority of other secreted proteins (data not shown). The greatest amounts were achieved after 30–40 h of bacterial growth. Furthermore, hyperthermal stress caused *S. mobaraensis* to secrete oligoglutamic acids that exhibit molecular masses ranging from approximately 500 Da to 1,200 Da. Failure of SPI and SSTI to attach to Fractogel EMD SO_3^- revealed a high affinity of the anionic peptides to the inhibitory proteins. Whether oligoglutamates contributed to the ability of the microbe to withstand high temperatures remains unclear. Several Gram-positive bacteria are well known to produce L- and D-enantiomers of linear γ-polyglutamic acids (γPGA) [6]. The released compounds are most likely involved in the virulence of pathogenic bacteria, the assembly of exopolymeric matrices in microbial communities, protection from the environment, and storage of carbon and nitrogen precursors or energy [26, 32, 34, 36]. However, γPGA from *Bacillus* spp. has a molecular mass of 10^4–10^6 Da, which is considerably larger than the small peptides from *S. mobaraensis*. Oligoglutamates of 3–6 residues linked to folic acid have been reported to enhance the inhibition of thymidylate synthetase and dihydrofolate reductase from *E. coli* [13]. The participation of both enzymes in the intracellular, folate-dependent biosynthesis rules out a similar function of *S. mobaraensis* oligoglutamates. Most recently, γPGAs from *S. roseoverticillatus* were shown to be composed of 10–13 glutamic acid residues and to have, in contrast to the *Bacillus* polymers, the L-configuration exclusively and a lariat-shaped structure [24]. Unfortunately, the relevance of the cyclic oligoglutamates to bacterial growth has not been studied as yet. We have begun to investigate the biological function of oligoglutamates from *S. mobaraensis*. For this purpose, the influence of oligoglutamates on the inhibitory or cross-linking activity of SPI, SSTI, DAIP, or TGase, respectively, will be examined.

The specificity of SPI raises the question, which is the putative target of the inhibitory protein? SSTI and DAIP counteract endoproteases from *Bacillus* spp. (*i.e.*, subtilisin and neutral metalloproteases such as dispase and thermolysin), suggesting a community of *S. mobaraensis* in close vicinity to the mobile, unicellular bacteria [30, 31]. To the best of our knowledge, cysteine proteases from Bacilli or Streptomycetes have not been characterized up to now. In comparison, papain-like enzymes are the most numerous family of cysteine endoproteases and are ubiquitously distributed in the kingdoms of bacteria, animals, and plants [35]. The existence of a wide range of pathogenic viruses, bacteria, and parasites causing disorders such as severe acute respiratory syndrome (SARS), periodontitis, malaria, Chagas' disease, and schistosomiasis demonstrates the invasive character of this type of protease [2, 4, 5, 10, 20, 29]. Accordingly, SPI might be more a defensive than a regulatory tool of *S. mobaraensis*. We assume that SPI, SSTI, and DAIP participate in the self-assembly of outer protein layers, thus protecting aerial hyphae and spores (Fig. 7). TGase has the role of cross-linking glutamine and lysine residues directed to the cell wall, resulting in the internal formation of rigid isopeptide bonds. In the case of disorders, rapid hydrolysis of available glutamines of faultily assembled building blocks occurs in all likelihood.

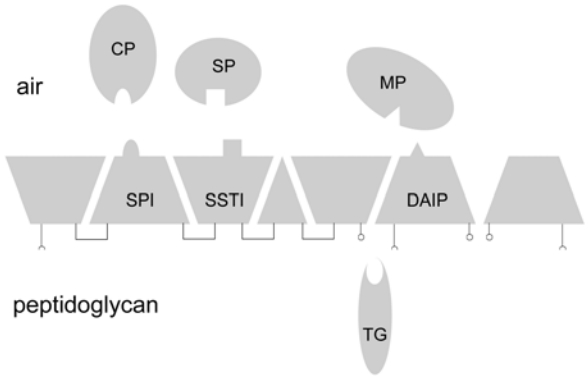

Fig. 7. Putative model for self-assembly of the inhibitory proteins SPI, SSTI, and DAIP, and the transglutaminase (TG)-mediated cross-linking during development of aerial hyphae and spores of *Streptomyces mobaraensis*.
The protein film exposed to the environment at the peptidoglycan-averted side counteracts papain-like cysteine proteases (CP), subtilisin-like serine proteases (SP), and neutral metalloproteases (MP).

To prove our hypothesis, we will study self-assembly, protein patterns, and the extent of cross-linked and hydrolyzed inhibitors in artificially prepared protein layers or aerial hyphae and spores.

Additional processing of SPI and SSTI takes place during growth of *S. mobaraensis*, most likely by the action of the co-secreted tripeptidylaminopeptidase (TAP), a Ca^{2+}-stimulated serine protease [41]. TAP removes the tetrapeptide FRAP from the N-terminus of TAMEP-activated TGase [41], and SSTI truncation has been verified as well by incubation with the purified exoprotease (unpublished result). Since the inhibitory constants of the SPI isoforms differ significantly, the involvement of TAP in the architecture of the bacterial protein coat by tailoring the building blocks appears to be possible. However, this hypothesis must be investigated further by *in vitro* and *in vivo* experiments.

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