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ORIGINAL PAPER

Overexpression and characterization of thermostable serine protease in *Escherichia coli* encoded by the ORF TTE0824 from *Thermoanaerobacter tengcongensis*

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Abstract A novel extracellular serine protease derived from *Thermoanaerobacter tengcongensis*, designated tengconlysin, was successfully overexpressed in *Escherichia coli* as a soluble protein by recombination of an N-terminal Pel B leader sequence instead of the original presequence and C-terminal 6× histidine tags. The purified protein was activated by 0.1% sodium dodecyl sulfate (SDS) treatment but not by thermal treatment. The molecular weight of tengconlysin estimated by SDS-polyacrylamide gel electrophoresis analysis and gel filtration chromatography was 37.9 and 36.2 kDa, respectively, suggesting that the enzyme is monomeric. The N-terminal sequence of mature tengconlysin was LDTAT, suggesting that it is a preproprotein containing a 29 amino acid presequence (predicted from the SigP program) and a 117 amino acid prosequence in the N-terminus. The C-terminal putative propeptide (position 469–540 in the preproprotein) did not inhibit the protease activity. The optimum temperature for tengconlysin activity was 90°C in the presence of 1 mM calcium ions and the optimum pH ranged from 6.5 to 7.0. Activity inhibition studies suggest that the protease is a serine protease. The protease was stable in 0.1% SDS and 1–4 M urea at 70°C in the presence of calcium ions and was activated by the denaturing agents.

Keywords Serine protease · *Thermoanaerobacter* · Thermophile · Recombinant protease · Overexpression

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Introduction

Thermophiles are widely distributed not only among the archaea but also among bacteria. The enzymes derived from such thermophiles have been recognized to be of considerable interest in clarifying the molecular mechanisms on the basis of their stability and activity at high temperature (Robb and Clark 1999); they demonstrate peculiar properties such as resistance to organic solvents, detergents, etc. that are of interest in potential biotechnological applications (Adams and Kelly 1998; Vieille and Zeikus 2001).

Protease is one of the widely used enzymes and thus is required to be stable in its chemical and physical properties. Five catalytic peptidases are now recognized in which serine, threonine, cysteine, aspartic acid, and metallo groups play primary roles in catalysis. Furthermore, the serine proteases can be divided into six clans based on a comparison of the tertiary structures and the order of the catalytic residues in the sequence (Barrett et al. 2000). The SB clan is also called S8 protease, or synonymously called subtilase. To date, over 200 members of the subtilase superfamily have been discovered, and ambitious sequence analysis based on the primary structures has demonstrated that the subtilases can be further divided into six families (Siezen and Leunissen 1997). The subtilases are widely used as detergents (Coolbear et al. 1992; Maurer 2004) and biological tools (Fung and Fung 1991; Borges and Bergquist 1992).

Some thermophilic subtilases, originating from thermoaerobic bacteria/archaea and thermoanaerobic archaea, have been isolated and characterized, e.g., aerolysin from *Pyrobaculum aerophilum* (Volkl et al. 1994), pyrolysin from *Pyrococcus furiosus* (Voorhorst et al. 1996), thermitase from *Thermoactinomyces vulgaris* (Kleine 1982),

aqualysin from *Thermus aquaticus* (Sakamoto et al. 1995), archaelysin from *Desulfurococcus* (Cowan et al. 1987), and pernisine from *Aeropyrum pernix* (Catara et al. 2003). However, little is known regarding the subtilases originating from the thermoanaerobic bacteria. In a recent study, an intriguing subtilase termed thermicin originating from thermoanaerobic bacterium *Thermoanaerobacter yonseiensis* was reported (Jang et al. 2002a). Thermicin is a calcium-independent thermophilic subtilase and thus is structurally unique (Jang et al. 2002a, b). Unlike *Thermoanaerobacter yonseiensis*, genomic information on *Thermoanaerobacter tengcongensis* (Xue et al. 2001) has previously been analyzed (Bao et al. 2002) and published online (http://www.genome.jp/kegg/catalog/org_list.html). Thus, we found the gene TTE0824 which encodes the putative subtilase with a different primary structure of thermicin (described in Sect. "Results") and aimed to express it in *Escherichia coli*.

Extracellular subtilases are synthesized as preproproteins, and it is difficult to express them as soluble proteins in heterologous organisms such as *Escherichia coli*. In the present study, we succeeded in expressing the gene encoded by TTE0824 as a soluble form in *Escherichia coli* as a recombinant protease. Furthermore, characterization of the protease revealed some unique properties. In this paper, we describe the overexpression and properties of the TTE0824 protease in *Escherichia coli*.

Materials and methods

Construction of expression plasmid and cells

The protease gene encoded by the ORF TTE0824 (accession no. = AAM24081) was amplified from the genomic DNA of *Thermoanaerobacter tengcongensis* MB4 by PCR with a primer pair of the forward (CCCAACCTAA CCAAATAAACCTGCC) and the reverse (CAACA CTCGAGATATTTTACAGGTTCACTACCTCC, where the underline indicates the *Xho*I site) and Phusion DNA polymerase (Finnzymes, Espoo, Finland). The genomic DNA was prepared using DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany) from *Thermoanaerobacter tengcongensis* MB4 obtained from the NITE Biological Resource Center (NBRC). The PCR condition for Phusion polymerases was as follow: 98°C (1 min) → [98°C (10 s) → 55°C (30 s) → 72°C (60 s)] × 25 cycle → 72°C (5 min). The PCR product was purified by GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, England) and then digested with *Xho*I to introduce pET22b(+) vector (Novagen, Darmstadt, Germany) that cleaved both *Msc*I and *Xho*I. The recombinant plasmid, pET22b(+)-TTE0824, was introduced into *Escherichia coli* DH5α [F^- Φ80 d*lac*Z Δ M15 Δ (*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(r_k^-, m_k^+) *phoA*, *supE*44 λ *thi*-1 *gyrA*96 *relA*1] (Takara, Shiga, Japan) and cloned. Finally, the host strain, *Escherichia coli* Rosetta 2(DE3) [(F^- *ompT* $hsdS_B$(r_B^-, m_B^-) *gal dcm*(DE3) pRARE2[7] (Cam^R)] (Novagen, Darmstadt, Germany), was transformed with the recombinant plasmid.

Gene expression and protein purification

Escherichia coli Rosetta 2(DE3) harboring the pET22b(+)-TTE0824 plasmid was cultivated in one liter of Luria–Bertani (LB) medium containing 1% glucose until optical density at 660 nm reached to 0.5. Sequentially, isopropyl-β-D-thiogalactopyranoside was added to the culture in a concentration at 1 mM, and the cells were continuously cultivated at 25°C for 20 h.

Cells were harvested by centrifugation at 4,000×*g* at 4°C for 15 min, washed with 50 mM sodium phosphate buffer (pH 7.6) containing 500 mM NaCl, and then suspended in 36 ml of the buffer. The suspended cells were disrupted by sonication and the supernatant was obtained after centrifugation at 10,000×*g* for 30 min at 4°C (first extract). The cell debris was re-suspended in the same buffer (pH 7.6) containing 0.1% sodium dodecyl sulfate (SDS) and then centrifuged at 10,000×*g* for 30 min at 4°C to obtain supernatant (second extract). The mixture of the first and second extracts (crude enzyme) was loaded onto HisTrap HP column (5 ml) (GE Healthcare, Buckinghamshire, England) equilibrated with 50 mM phosphate buffer (pH 7.6) containing 500 mM NaCl and 25 mM imidazole. The column was washed with 10 column volume of the equilibration buffer and then a desired protein was eluted with 3 column volume of 50 mM phosphate buffer (pH 7.6) containing 500 mM NaCl and 500 mM imidazole. Eluted protein solution was loaded onto Superdex 10/300 (GE Healthcare, Buckinghamshire, England) equilibrated with 20 mM phosphate buffer (pH 7.5) containing 300 mM NaCl. Elution was performed with the equilibration buffer at a flow rate of 1 ml/min and the desired protein fraction was corrected. The eluted protein was activated with 0.1% SDS treatment for 15 min at room temperature, concentrated by ultracentrifugation with Ultra-15 10,000 MW (Amicon), and then loaded onto PD-10 column (GE Healthcare, Buckinghamshire, England) twice to change the buffer to 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl.

The protein concentration was determined using the Coomassie Protein Assay Reagent (PIERCE, Rockford, IL, USA) and adjusted to 0.125 mg/ml. The purity of protein was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein solution obtained was divided into small volumes and kept at –80°C until being used for assays.

Molecular mass determination

The apparent molecular mass of the recombinant enzyme was determined by gel filtration chromatography on an HPLC column of Superdex 10/300 (GE Healthcare, Buckinghamshire, England) using a standard gel filtration calibration kit for low molecular weight protein (GE Healthcare, Buckinghamshire, England): bovine pancreas ribonuclease A 13.7 kDa; bovine pancreas chymotrypsinogen A 25.0 kDa; hen egg ovalbumin 43.0 kDa; bovine serum albumin 67.0 kDa; blue dextran (for void volume). The molecular mass was also calculated from the putative amino acid sequence of the protein encoded by TTE0824 and estimated by SDS-PAGE analysis by comparison with migration of standard protein marker, low molecular weight (GE Healthcare, Buckinghamshire, England). The proteins were stained with Coomassie brilliant blue R250 on the gel. Samples for SDS-PAGE analysis were prepared as in the following procedure: addition of 30% trichloroacetate (TCA) to the same volume of protein solution, chillness on ice for 15 min, centrifugation at 10,000 rpm for 10 min at 4°C, washing of precipitant with 1 ml acetone, and suspension of precipitant with appropriate amount of SDS-PAGE sample buffer.

Determination of N-terminus

The purified protease was separated by SDS-PAGE and electroblotted onto a PVDF membrane (Matsudaira 1987). The protease band was cut out and analyzed by Edman degradation using a peptide sequencer PPSQ-21A (Shimadzu, Kyoto, Japan).

Standard enzyme assay

Proteolytic activity was routinely measured using azocasein (Nacalai tesque, Kyoto, Japan) as substrate, according to Catara (Catara et al. 2003) and Kubo (Kubo et al. 1996), with some minor modification. The reaction mixture contained 0.2% (w/v) azocasein and enzyme in a total 500 µl of 50 mM Tris–HCl buffer (pH 7.7 at 80°C). The reaction was started by addition of 10 µl enzyme solution and performed at 80°C for 10 min. The reaction was then stopped by addition of 500 µl of 30% trichloroacetic acid and the solution was kept for 10 min on ice. After centrifugation at 10,000×*g* for 10 min at 4°C, the absorbance of the supernatant was measured at 335 nm, against a blank, using spectrophotometer UV-2200 (Shimadzu, Kyoto, Japan). The blank reaction was performed by a similar procedure to the complete reaction, but the enzyme was added to the mixture after addition of TCA. One unit was defied as the amount of enzyme which yielded an increase in the absorbance at 335 nm of 0.05 OD.

Hydrolysis of synthetic peptides and oxidized insulin B chain

The activities against a number of peptide *p*-nitroanilides (Sigma-Aldrich Inc., MO, USA) were determined at 70°C, basically according to conventional method (Peek et al. 1992). Stock peptide solutions were prepared in 50 mM Tris–HCl, pH 7.7, containing 1 mM $CaCl_2$, and diluted with the buffer as required. The assay was started by the addition of 10 µl protease solution into 1 ml pre-incubated (5 min) reaction mixture and the absorbance at 410 nm was continuously monitored for 3 min using the spectrophotometer UV-2200 (Shimadzu, Kyoto, Japan) equipped with a thermo-incubation cell folder. The rate of change in absorbance was calculated from the initial rate of reaction and activity was determined using an absorption coefficient for *p*-nitroaniline of 8,750/M/cm. The K_m and V_{max} for peptide substrates were determined by linear regression analysis of Lineweaver–Burk plots from at least five substrate dilutions.

Oxidized insulin B chain (500 µM) was incubated at 70°C for 24 h with protease (0.05 µM) in 10 mM Tris–HCl (pH 7.7) containing 1 mM $CaCl_2$. Aliquots were sampled at appropriate times and the reaction was terminated by the addition of HCl at 50 mM. The produced peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The peptides were separated and eluted by Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with the C18 column CHEMCOSORB 5-ODS-UH (10 µm, 4.6 mm × 250 mm) (Chemco Scientific Co. Ltd, Osaka, Japan), with a linear gradient from 0.1% formic acid to 39% acetonitrile/0.1% formic acid, for 90 min at a flow rate of 0.4 ml/min. The separated peptides were detected by API2000 tandem mass spectrometer equipped with a turbo ion spray interface (Applied Biosystems, CA, USA). The source temperature was set at 500°C and ionization voltage at 3 kV. Nitrogen was used as the collision gas for collision-induced dissociation with collision energy set at 25, 35, or 60 eV (peptide depended). The mass spectrometry was operated in Q1-total ion current positive-ion mode within 100–800 atomic mass units from 1 to 50 min or 800–1,000 atomic mass units for 50.1–90 min. The fragmentation pattern of each molecular ion was obtained by Q3-MS2 positive mode. The amino acid compositions were deduced by the molecular mass and a corresponding amino acid sequence was searched in oxidized insulin B chain.

Effects of pH and temperature on proteolytic activity, and thermostability

The effect of pH on proteolytic activity was determined by the standard enzyme assay using the following buffers:

50 mM sodium acetate (pH 4.5–5.6), 50 mM sodium phosphate (pH 6.0–8.0), 50 mM Tris–HCl (pH 6.0–8.2), 50 mM borate–NaOH (pH 8.0–9.5).

The optimum temperature for proteolytic activity was examined by the standard enzyme assay at temperatures ranging from 30 to 100°C in the presence and absence of 1 mM $CaCl_2$, $MgSO_4$, or $MnCl_2$.

For the thermostability test, 50 µl of the purified enzyme solution (20 µg/ml) in 50 mM Tris–HCl (pH 7.7) in the presence and absence of 1 mM $CaCl_2$ was incubated at 60–100°C in 0.2 ml PCR tubes in PCR Express II (Thermo Electron Corporation, MA, USA). The solutions were rapidly cooled on ice at intervals of 15 min (until 1 h) or 1 h (until 4 h). The remaining activity was measured by the standard enzyme assay.

Effect of protease inhibitors and denaturing agents

The purified enzyme solution (20 µg/ml) in 50 mM Tris–HCl (pH 7.5) was pre-incubated at room temperature for 15 min in the presence of specific protease inhibitors and then the residual proteolytic activity was determined by the standard enzyme assay. The following protease inhibitors were used: 1, 5 mM ethylenediaminetetraacetic acid (EDTA); 1, 10 mM *o*-phenanthroline; 1, 5 mM phenylmethylsulfonyl fluoride (PMSF); 1, 10 mM *N*-alpha-tosyl-L-lysine chloromethyl ketone (TLCK); 1 mM *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK); 1 mg/ml soybean trypsin inhibitor; 10 mM indoleacetic acid; 10 mM *N*-ethylmaleimide.

The proteolytic activity of azocasein at 80°C in 50 mM Tris–HCl (pH 7.7) was assayed in the presence of the following denaturing agents: 1, 5 mM dithiothreitol (DTT); 1, 5% 2-mercaptoethanol; 1, 2, 4 M Urea; 2, 4 M guanidine–HCl; 0.1, 1.0, 5.0% SDS.

Results

Phylogenetic analysis of TTE0824 protease

Using the genomic information of *Thermoanaerobacter tengcongensis*, we were able to identify three putative subtilase genes, TTE0576, TTE0824, and TTE2615. Among these, the amino acid sequence of TTE2615 almost completely agreed with that of thermicin (98.8% in the prepro-form) of *Thermoanaerobacter yonseiensis*. However, the functions of the proteins encoded by TTE0576 and TTE0824 have yet to be elucidated, and homologous genes whose functions have been analyzed could not be found in other *Thermoanaerobacter* strains. Thus, we attempted to elucidate the functions and properties of one of these proteins, that encoded by TTE0824.

The amino acid sequences of 28 subtilases belonging to four families, namely, subtilisin, pyrolysin, proteinase K, and thermitase families, were selected for alignment along with the amino acid sequences of TTE0824 protease using the ClustalX multiple alignment program (Jeanmougin et al. 1998). The sequences of the catalytic domains containing the catalytic triad (Asp, His, and Ser) were selected for the alignment, according to the method described by Siezen and Leunissen (1997), to identify the family to which the TTE0824 protease belongs. A phylogenetic tree presented in Fig. 1 indicates that the TTE0824 protease belongs to the pyrolysin family and that it is closely related to thermicin. Although the active domain sequence of the TTE0824 protease exhibited sufficient similarity to thermicin (43%), it possessed a putative calcium-binding motif (position 185–197 in the preproprotein), which does not exist in thermicin. Furthermore, the similarity of the mature sequence of the TTE0824 protease to thermicin was only 27% because of the bacterial C-terminal prodomain (position 469–540 in the preproprotein) of the TTE0824 protease. These suggest that the TTE0824 protease is a distinctly different type of subtilase from thermicin despite belonging to the same family. The most similar sequences obtained by the protein Basic Local Alignment Search Tool (blastp) search (http://www.ncbi.nlm.nih.gov/BLAST/) were the peptidases S8 and S53 (EAU57869) from *Thermoanaerobacter ethanolicus* X514 (84% amino acid mature sequence); however, the function of the protein is yet to be investigated.

Expression and purification of recombinant TTE0824 protease

In order to obtain the protein encoded by the gene TTE0824, the expression plasmid pET22b(+)-TTE0824 was constructed. For the construction of the plasmid, the original presequence, MKKHQLAKILLSLALIISLISLNEILVQA, predicted from the SignalP3.0 program (Bendtsen et al. 2004) was modified to the leader peptide (MKYLLP TAAAGLLLLAAQPAMA) of *Erwinia carotovora* pectate lyase B (Pel B, AAA24848) (Lei et al. 1987). The cells harboring the recombinant plasmid did not grow well in LB medium. However, the cells grew well and expressed the protease gene as a soluble and active form when 1% glucose was added to the medium in order to maintain the low basal expression level of the target gene (Grossman et al. 1998). When the crude extract treated with TCA was analyzed on 12.5% SDS-PAGE, the expressed protein was distinctively observed on the Coomassie-stained PAGE gel (Fig. 2), whereas without TCA treatment the corresponding band was not observed (data not shown).

The enzyme in the crude extract exhibited low specific activity (3 U/mg). However, treatment with 0.1% SDS

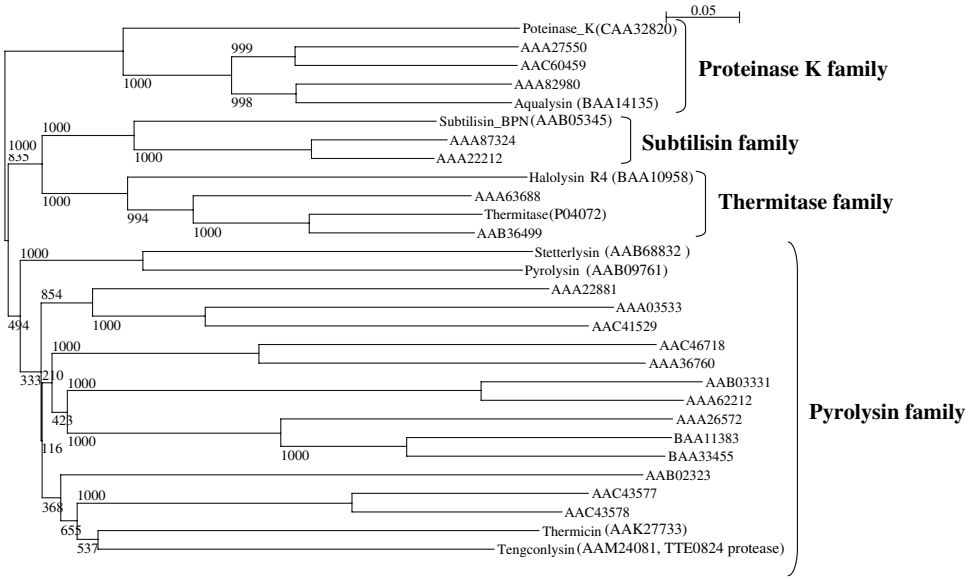

Fig. 1 Phylogenetic analysis of subtilases inferred from the amino acid sequence alignment of catalytic domains. The catalytic domains of the respective enzymes used in the present study were referred to by Siezen and Leunissen (1997). The sequences of positions 103–412 and 147–441 of the preproprotein were used for thermicin and tengconlysin, respectively. Sequence alignment was performed using ClustalX version 1.81 and the tree was constructed by the neighbor-joining method. *Numbers* on selected nodes indicate bootstrap values. Source sequences for the alignment were as follows: CAA32820, proteinase K from *Tritirahium album* Limber; AAA27550, serine exoprotease A from *Vibrio alginolyticus*; AAC60459, alkaline serine protease II from *Alteromonas* sp.; AAA82980, serine proteinase from *Thermus* sp. Rt41A; BAA14135, aqualysin from *Thermus aquaticus*; AAB05345, subtilisin BPN' from *Bacillus amyloliquefaciens*; AAA87324, subtilisin from *Bacillus subtilis* YaB; AAA22212, subtilisin from *Bacillus alcalophilus* PB92; BAA10958, halolysin R4 from *Haloferax mediterranei*; AAA63688, serine proteinase from *Bacillus* sp. Ak1; P04072, thermitase from *Thermoactinomyces vulgaris*; AAB36499, thermostable alkaline protease from *Thermoactinomyces* sp. E79; AAC68832, stetterlysin from *Thermococcus stetteri*; AAB09761, pyrolysin from *Pyrococcus furiosus*; AAA22881, serine protease from *Bacillus subtilis* GP264; AAA03533, proteinase from *Lactococcus lactis* (cremoris) SK11; AAC41529, proteinase from *Lactobacillus delbrueckii*; AAC46718, tripeptidase from *Caenorhabditis elegans* Bristol N2; AAA36760, tripeptidyl peptidase II from Homo sapiens; AAA62212 and AAB03331, serine protease/transporter from *Dictyostelium discoideum* AX4; AAA26572, BAA11383, and BAA33455, serine protease, SSP-h2 protease, and SSP-h1 from *Serratia marcescens* IFO3046; AAB02323, stable protease from *Staphylothermus marinus*; AAC43577 and AAC43578, serine protease A and B from *Bacillus* sp LG12; AAK27733, thermicin from *Thermianaerobacter yonseiensis*; AAM24081; tengconlysin from *Thermoanaerobacter tengcongensis*

resulted in an increase in the specific activity to approximately 258 U/mg (approximately 85-fold higher than the untreated enzyme) (Table 1). The nonSDS treated enzyme exhibited no loss in activity following PMSF treatment, suggesting that the active site was protected, possibly by the propeptide conformation of the enzyme (Ohta et al. 1991). The demand of the activation treatment by SDS was convenient because the possibility of autoproteolysis could be avoided in the purification procedure. Thus, the activation treatment was placed on the final step of the purification procedure, and a portion of the enzyme solution on the each purification step was activated by 0.1% SDS to correctly evaluate the proteolytic activity. After purification by nickel affinity and size-exclusion chromatography, the specific activity increased to approximately 2,680 U/mg (10.4-fold). Finally, the activation of the enzyme by SDS and the desalting column manipulation resulted in a highly active form (3,810 U/mg, 14.8-fold). The purified enzyme exhibited no loss in activity following a single freeze-thaw and maintained its stability at –80°C. The TTE0824 protease was, thus, termed tengconlysin in the present study.

The enzyme sample obtained after size-exclusion chromatography resolved as two bands on SDS-PAGE analysis (Fig. 2). The smaller band of approximately 14.0 kDa disappeared within 5 min during the treatment with 0.1% SDS and the desalting column manipulation and, finally, a homogeneous purified enzyme was obtained in the SDS-PAGE analysis.

The calculated molecular mass (56.7 kDa) of tengconlysin without the putative presequence did not correspond to the molecular mass estimated by the SDS-PAGE analysis (37.9 kDa), suggesting that tengconlysin is a preproprotease. This suggestion was supported by the fact that the N-terminal sequence of the active tengconlysin was LDTAT, which is located at the position 147–151 in the original tengconlysin. The calculated molecular mass (44.2 kDa) of the expected mature tengconlysin almost

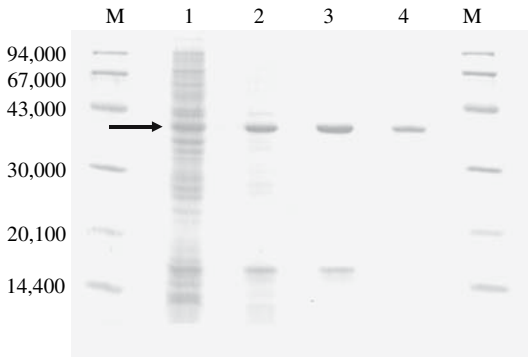

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified tengconlysin overexpressed in *Escherichia coli* Rosetta 2(DE3): protein molecular markers (*M*), crude enzyme (*lane 1*), tengconlysin after nickel column purification (*lane 2*), tengconlysin after size-exclusion chromatography (*lane 3*), and tengconlysin after activation by 0.1% SDS and desalting column manipulation (*lane 4*). The proteins were denatured by 15% trichloroacetic acid, washed twice with acetone, re-suspended in an appropriate amount of sample buffer, and then applied to 12.5% polyacrylamide gel containing SDS. Proteins were stained with Coomassie brilliant blue R250

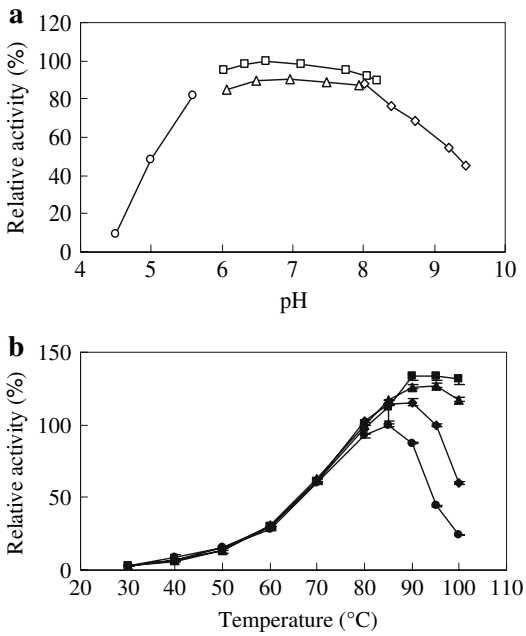

Fig. 3 Effects of pH (**a**) and temperature (**b**) on the proteolytic activity of tengconlysin. **a** Proteolytic activity was assayed at 80°C using azocasein as a substrate in 50 mM sodium acetate (*open circle*), sodium phosphate (*open triangle*), Tris–HCl (*open square*), and borate-NaOH (*open diamond*) buffers under standard conditions. **b** Proteolytic activity was assayed in 50 mM Tris–HCl (pH 7.7) using azocasein as a substrate at the indicated temperature in the absence of cations (*dark filled circle*) and in the presence of 1 mM $CaCl_2$ (*dark filled triangle*), 1 mM $MnCl_2$ (*dark filled square*), and 1 mM $MgSO_4$ (*dark filled diamond*)

coincided with that estimated by the SDS-PAGE analysis. Furthermore, the molecular mass of the native form of the protease was estimated to be 36.2 kDa by gel filtration chromatography, suggesting that the protease is monomeric. The purified tengconlysin specifically bound with the nickel column so that C-terminal putative prosequence was not accepted by the processing, dissimilar to aqualysin (Lee et al. 1994).

Effects of pH and temperature on proteolytic activity

Figure 3a shows the azocasein proteolysis as a function of pH at 80°C. The optimum pH for the enzyme ranged from 6.5 to 7.0. The enzyme exhibited 95% of the maximum activity at pHs 6.0 and 8.0. The relative activity of approximately 50% was observed at pHs 5.0 and 9.5.

The enzyme was active at temperatures ranging from 30 to 100°C. As shown in Fig. 3b, maximal activity was observed at 85°C in the absence of cations. The maximal activity increased up to 90°C in the presence of 1 mM $CaCl_2$, $MgSO_4$, or $MnCl_2$. In particular, the enzyme exhibited more than 90% of the maximal activity at 100°C in the presence of 1 mM $CaCl_2$ or $MnCl_2$, whereas it exhibited only 20% activity in the absence of cations. Other cations, such as $FeSO_4$, $ZnSO_4$, $CuSO_4$, and $CoCl_2$,

Table 1 Summary of the purification of tengconlysin from *Escherichia coli* overexpressing the gene encoded by TTE0824

Preparation	Volume (ml)	Total protein (mg)	Total activity (U)[b]	Yield (%)	Specific activity (U/mg)	Purification fold[a]
Crude extract	71	149.8	38618.4	100	257.8	1.0
Affinity (HisTrapHP)	12	17.4	36675.7	95.0	2107.8	8.2
Size exclusion (Superdex 10/300)	33	9.5	25460.0	65.9	2680.6	10.4
SDS treatment and desalting column manipulation[a]	12	5.1	19420.8	50.3	3808.0	14.8

[a] The SDS-activated enzyme solution was concentrated by ultracentrifugation and most of SDS was removed by twice manipulations of a desalting column

[b] The activity was measured by the standard assay using azocasein after activation by 0.1% SDS, except to the finally purified enzyme

did not affect the enzyme activity at a concentration of 1 mM. The addition of 5 mM $CaCl_2$ resulted in a similar temperature–activity curve (data not shown) as compared to that in 1 mM $CaCl_2$. In the presence of 5 mM $MnCl_2$, a precipitate was formed and most of the proteolytic activity was lost.

Thermostability

The thermal stability of the enzyme was examined in 50 mM Tris–HCl (pH 7.7) in the presence and absence of 1 mM $CaCl_2$ (Fig. 4). The enzyme was stable below 60°C in the absence of $CaCl_2$, with a calculated half-life of 1,520 min. However, the enzyme was unstable above 70°C in the absence of $CaCl_2$, with a half-life of 281 min at 70°C, 24 min at 80°C, and less than 15 min at 90°C. The addition of 1 mM $CaCl_2$ resulted in an increase in the stability of the enzyme for up to 4 h at 80°C, and the half-life increased to 2,470 min at 80°C and 143 min at 90°C. A higher concentration (5 mM) of calcium affected the thermostability of the enzyme, which was the same as that in 1 mM calcium. Although the effect of $MnCl_2$ on thermostability was examined by the same procedure, manganese was rapidly oxidized and precipitated under the experimental conditions.

Effect of inhibitors

In order to determine the type of protease, tengconlysin was tested using different protease inhibitors (Table 2). The serine protease inhibitor PMSF almost completely inhibited the enzymatic activity at a concentration of

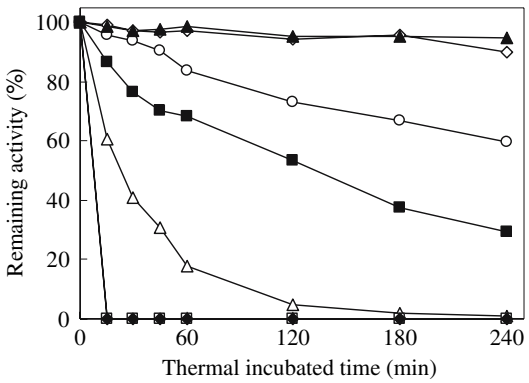

Fig. 4 Thermal stability of tengconlysin at temperatures between 60 and 100°C. Purified enzyme (12.5 μg/ml) was incubated at 60°C (*open diamond*), 70°C (*open circle*), 80°C (*open triangle*), and 90°C (*open square*) in the absence of 1 mM $CaCl_2$ and at 80°C (*dark filled triangle*), 90°C (*dark filled square*), and 100°C (*dark filled diamond*) in the presence of 1 mM $CaCl_2$. After incubation, the enzyme solution was immediately cooled on ice and residual activity was measured by the standard azocasein assay. Enzyme incubated nonthermally was used as a control sample that exhibited 100% activity

Table 2 Effect of inhibitors on enzymatic activity

Inhibitor	Class	Concentration	Residual activity (%)
None			100
EDTA	Metalloprotease	1 mM	14
		5 mM	5
o-Phenanthroline	Metalloprotease	1 mM	94
		10 mM	90
PMSF	Serine protease	1 mM	1
		5 mM	0
TLCK	Serine protease	1 mM	94
		10 mM	100
TPCK	Serine protease	1 mM	94
Soybean trypsin inhibitor	Serine protease	1 mg/ml	103
Indoleacetic acid	Cysteine protease	10 mM	99
N-ethylmaleimide	Cysteine protease	10 mM	97

1 mM. In contrast, TPCK and TLCK—chymotrypsin and trypsin-like protease inhibitors—did not distinctly affect the activity, similar to the effect of soybean trypsin inhibitor. The protease activity was strongly inhibited by EDTA—a typical metalloprotease inhibitor—at a concentration of more than 1 mM. However, the zinc chelator, *o*-phenanthroline, usually used as a classic indicator for metalloprotease inhibition, did not inhibit the proteolytic activity at a high concentration of 10 mM. Therefore, the apparent inhibition by EDTA was considered to be due to a loss of stabilizing Ca^{2+} ions rather than the removal of catalytic Zn^{2+} ions (Peek et al. 1992). The cysteine protease inhibitors—indoleacetic acid and *N*-ethylmaleimide—also did not act on the enzyme. These data suggest that tengconlysin is a serine protease.

Effect of denaturing agents

The activity of the enzyme in the presence of different concentrations of denaturing agents was examined (Table 3). DTT (5 mM) and 2-mercaptoethanol (5%) reduced the activity by 36 and 61%, respectively. The enzyme in the presence of urea exhibited an increase in the activity of approximately 1.5- (concentration, 1 M), 2.4- (2 M), and fourfold (4 M). Similarly, the proteolytic activity of the enzyme increased by approximately 3.5- and 5.7-fold in the presence of 0.1 and 5.0% SDS, respectively.

Tengconlysin was highly resistant to SDS and urea in the presence of 1 mM $CaCl_2$. The half-life of tengconlysin in 0.1 and 1.0% SDS at 80°C was 1,140 and 170 min, respectively. Tengconlysin exhibited no loss in activity in 1 M urea at 70°C. The half-life of tengconlysin in 4 M urea at 70°C was 474 min and that in 1 and 4 M urea at 80°C was 407 and 174 min, respectively.

Table 3 Effect of denaturing agents on proteolytic activity

Reagent	Concentration	Relative activity (%)[a]
None		100
DTT	1 mM	70
	5 mM	64
2-Mercaptoethanol	1.0%	65
	5.0%	39
Guanidine–HCl	1 M	39
	4 M	6
Urea	1 M	152
	2 M	242
	4 M	395
SDS	0.1%	350 (289)
	1.0%	365 (518)
	5.0%	570 (844)

[a] Values in the parentheses indicate the relative activity in the presence of 1 mM $CaCl_2$ in the reaction mixture

Substrate specificity

The kinetic constants for the hydrolysis of several synthetic peptides by tengconlysin are shown in Table 4. Of the substrates tested, Suc-Ala-Ala-Pro-Phe-*p*NA, originally synthesized as a substrate for chymotrypsin, was the most susceptible to hydrolysis having the highest catalytic constant (k_{cat}) and the lowest K_m. The substitution of phenylalanine with leucine at the P1 position resulted in a lower k_{cat} and a higher K_m. This was different from the results with Rt41A proteinase (Peek et al. 1992) from *Thermus* sp., which exhibited a lower k_{cat} with minor change in K_m by the same substitution at the P1 position. Tengconlysin also hydrolyzed an elastase substrate Suc-Ala-Ala-Ala-*p*NA with a lower k_{cat}/K_m value.

The hydrolysis of the oxidized insulin B chain by tengconlysin is shown in Fig. 5. The insulin B chain was rapidly cleaved by the enzyme within 1 min, producing two major peptides cleaved between L15 and Y16. This site was the most cleavable one using other subtilases (Peek et al. 1992; Matsuzawa et al. 1988). The detection of the peptides VNQ after 40-min digestion and FV after 400-min digestion indicated that tengconlysin cleaved the insulin B chain at positions F1-V2 and V2-N3, which were unique cleavage positions. Most cleavage sites susceptible to tengconlysin such as Q4-H5, S9-H10, and F24-F25 were similar to those cleaved by other subtilases. The other major cleavage positions—E13-A14 and K30-A31—suggest that, compared to other subtilases, tengconlysin prefers alanine at the P1′ position.

Discussion

In this study, we succeeded in overexpressing tengconlysin (the protein encoded by TTE0824) originating from *Thermoanaerobacter tengcongensis* as a soluble recombinant protein in *Escherichia coli*. Tengconlysin was highly thermostable, and an inhibition test indicated that the enzyme should be classified as a serine proteinase. Several unique properties were found during the expression and characterization of the enzyme, e.g., the activation by SDS, no inhibitory effect on the enzyme activity by the putative C-terminal prosequence, the substrate specificity for the insulin B chain, and the enhancement of activity by urea.

Some extracellular thermophilic serine proteases have been studied by overexpression in heterologous cells such as *Escherichia coli*. Aqualysin I was successfully expressed as a soluble C-terminal proprotein in the membrane (Terada et al. 1990) or cytosolic fraction (Sakamoto et al. 1995) of *Escherichia coli*. Furthermore, extracellular alkaline serine protease Rt41A from *Thermus* sp. was expressed in *Escherichia coli* as a fusion protein with glutathione-*S*-transferase (Munro et al. 1995), which was similar to pernisine from *A. pernix* K1 (Catara et al. 2003). In all cases, the expressed proproteases required thermal activation for maturation. The recombinant thermicin from *Thermoanaerobacter yonseiensis* overexpressed in *Escherichia coli* also required heat treatment to form a mature structure (Jang et al. 2002a); however, thermal activation was not effective in tengconlysin. Instead of thermal activation, SDS treatment was required for the conversion of tengconlysin to a highly active form. Tengconlysin was not inhibited by PMSF before SDS treatment; this suggests that the active site of the enzyme was blocked. In addition, a small peptide (14 kDa) consisting of AQPAMAQPNQINLPIDS, which includes the

Table 4 Kinetic parameters for synthetic peptides

Synthetic peptide[a]	K_m (per mM)	k_{cat} (per second)	k_{cat}/K_m (10^3/s/M)	Range (mM)
Suc-Ala-Ala-Pro-Phe-*p*NA	1.4 ± 0.1	1433 ± 82	1003.7 ± 35.7	0.25–2.0
Suc-Ala-Ala-Pro-Leu-*p*NA	3.5 ± 0.1	361 ± 5	102.6 ± 1.3	1.0–7.5
Suc-Ala-Ala-Ala-*p*NA	11.9 ± 0.2	70 ± 1	5.9 ± 0.0	3.2–12.8

Values were obtained from three independent experiments, and ± means standard deviation

[a] Suc and *p*NA means succinate and *p*-nitroanilide, respectively

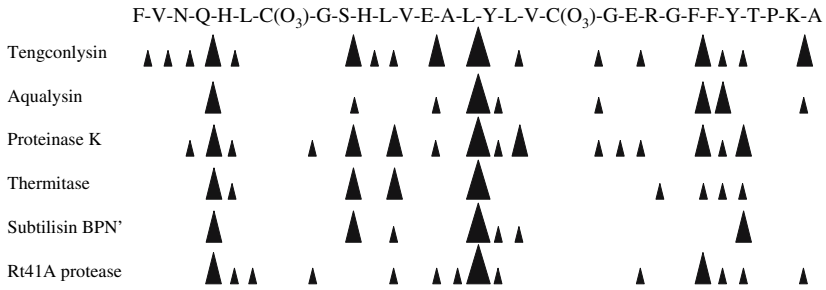

Fig. 5 Cleavage site of the oxidized insulin B chain hydrolyzed by tengconlysin and authentic subtilases. The oxidized insulin B chain was incubated with tengconlysin in the molar ratio of $1{:}10^4$ for 1, 5, 10, 20, 40, 80, 160, 400, and 1,320 min. The resulting peptides were analyzed and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The most cleavable sites are indicated by *large arrows*. The cleaved sites detected within 1 min are indicated by *medium-sized arrows*, and other detectable cleaved sites are indicated by *small arrows*. The cleavage sites of the authentic subtilases were referred to by Matsuzawa et al. (1988) and Peek et al. (1992)

partial sequence of Pel B leader peptide (single-underlined sequence) and prosequence of tengconlysin (double-underlined sequence), in the N-terminus was observed in the SDS-PAGE analysis (Fig. 2) before SDS-activation. Although the SDS-dependent mechanism of tengconlysin activation remains obscure, these results suggest that the cleaved N-terminal propeptide blocked the active site causing inhibition; thus, the SDS treatment promoted the release and/or degradation of the propeptide. This manner of activation is interesting in the recombinant thermophilic proteases because the prosequence of tengconlysin was cleaved automatically in *Escherichia coli* without applying high temperature.

The comparison of the primary structure of tengconlysin with those of other authentic extracellular serine proteases demonstrated that tengconlysin possessed an extra sequence in the C-terminus similar to that in aqualysin I, which possesses a C-terminal propeptide. The C-terminal propeptide was essential for aqualysin I secretion in *Thermus aquaticus* but not in *Escherichia coli* (Lee et al. 1994). Aqualysin I expressed in *Escherichia coli* accumulated in the membrane fraction as a proprotein containing the C-terminal propeptide, which prevented its activity (Terada et al. 1990). A similar C-terminal propeptide was observed in *Vibrio* serine protease (Kristjansson et al. 1999). In contrast, the C-terminal peptide of tengconlysin did not inhibit the activity. Thus, the role of the C-terminal peptide of tengconlysin appears to be considerably different from that of aqualysin I when expressed in *Escherichia coli*.

The substrate specificity of tengconlysin was different from that of thermicin despite the similar primary structure of the active domains in both the enzymes. Thermicin cleaved the oxidized insulin B chain between positions P30 and K31 (Jang et al. 2002a), which was not observed in tengconlysin. On the other hand, thermicin did not cleave the synthetic peptide, Suc-Ala-Ala-Pro-Leu-*p*NA, which was a susceptible substrate of tengconlysin. The substrate specificity of tengconlysin for the synthetic peptides was similar to that of the Rt41A protease from *Thermus* sp. (Peek et al. 1992) but different from that of thermicin. Although the k_{cat}/K_m values of tengconlysin for the synthetic peptides were fivefold higher than those of the Rt41A protease (Peek et al. 1992), the ratio of the k_{cat}/K_m values for the peptides Suc-Ala-Ala-Pro-Phe-*p*NA, Suc-Ala-Ala-Pro-Leu-*p*NA, and Suc-Ala-Ala-Ala-*p*NA for tengconlysin was the same as that for the Rt41A proteinase (200:20:1). The data suggests that the role of tengconlysin was similar to that of Rt41A protease rather than thermicin, although the Rt41A protease and tengconlysin are classified into different families of subtilase, proteinase K and pyrolysin, respectively (Fig. 1).

The activity of tengconlysin against the substrate azocasein was enhanced not only by SDS but also by urea. In several subtilases, SDS is known to enhance the proteolytic activity of proteases (Sako et al. 1997); however, to date, a significant enhancement in proteolytic activity by urea has not been observed. Compared to mesophilic enzymes, thermophilic enzymes are relatively resistant to urea denaturation (Beadle et al. 1999). Tengconlysin was stable in 1 M urea at 80°C and in 4 M urea at 70°C. Under such conditions, urea may cause partial denaturation of the protease and/or substrate and the substrate may be able to gain access to the active site of the protease more rapidly and easily. The effect of urea on the activity of tengconlysin is an interesting subject. In order to elucidate the relationship between tengconlysin and urea, we need to acquire more information, such as that relating to the change in the enzyme conformation, substrate specificity, and the kinetic constants for synthetic peptides in the presence of urea.

In the present study, we showed the phylogenetic and enzymatic properties of tengconlysin. The primary structure of tengconlysin was similar with that of aqualysin I of

Thermus thermophilus; that is, both proteases consist of the N-terminal signal sequence (presequence), the N-terminal prodomain, the active domain, and the C-terminal prodomain. However, the role of the C-terminal prodomain of tengconlysin in *Escherichia coli* differed to that of aqualysin I. Furthermore, in *Escherichia coli* expression system, the N-terminal prodomain of tengconlysin was removed without heat treatment, dissimilar to aqualysin I and other thermophilic subtilases. Although the primary structure of the active domain of tengconlysin was similar to that of thermicin, tengconlysin possessed the calcium-binding motif and showed calcium dependent thermostability. The substrate specificity of tengconlysin for the synthetic peptide was similar to that of Rt41A protease from *Thermus* sp., but the hydrolysis of oxidized insulin B chain by tengconlysin was unique. Tengconlysin was highly stable in urea, and the activity of tengconlysin was enhanced by urea. These results indicated that tengconlysin was a novel type of thermophilic subtilase (pyrolysin family) having unique properties.

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