A new method for the extracellular production of recombinant thermolysin by co-expressing the mature sequence and pro-sequence in *Escherichia coli*

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Thermolysin, a representative zinc metalloproteinase from *Bacillus thermoproteolyticus*, is synthesized as inactive pre-proenzyme and receives autocatalytic cleavage of the peptide bond linking the pro- and mature sequences. The conventional expression method for recombinant thermolysin requires the autocatalytic cleavage, so that production of a mutant thermolysin is affected by its autocatalytic digestion activity. In this study, we have established a new expression method that does not require the autocatalytic cleavage. The mature sequence of thermolysin containing an NH2-terminal pelB leader sequence and the pro-prosequence of thermolysin were co-expressed constitutively in *Escherichia coli* as independent polypeptides under the original promoter sequences in the *npr* gene which encodes thermolysin. Unlike the conventional expression method, not only the wild-type thermolysin but also mutant thermolysins [E143A (Glu143 is replaced with Ala), N112A, N112D, N112E, N112H, N112K and N112R] were produced into the culture medium. The wild-type enzyme expressed in the present method was indistinguishable from that expressed in the conventional method based on autocatalytic cleavage, as assessed by hydrolysis of N-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide and N-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester. The present method should be useful especially for preparation of active-site mutants of thermolysin, which might have suppressed autocatalytic digestion activity. The results also demonstrate clearly that the covalent linking between the pro- and mature sequences is not necessary for the proper folding of the mature sequence by the propeptide in thermolysin.

**Keywords:** autocatalytic digestion activity/*Escherichia coli* metalloproteinase/pro-sequence/thermolysin

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### Introduction

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (Endo, 1962; Inouye, 2003). It requires one zinc ion for enzyme activity and four calcium ions for structural stability (Latt et al., 1969; Feder et al., 1971; Tajima et al., 1976), and catalyzes specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (Morihara and Tsuzuki, 1970; Inouye et al., 1996). Thermolysin is widely used for the peptide bond formation through reverse reaction of hydrolysis (Oyama et al., 1981; Inouye, 1992; Truek-Holownia, 2003).

We have reported that thermolysin activity in the hydrolysis and even in the synthesis of peptides is remarkably enhanced by high concentration (1–4 M) neutral salts, and the activity increases typically with increasing the NaCl concentration in an exponential fashion (Inouye, 1992; Inouye et al., 1997, 1998a, 1998b; Ondeda et al., 2004). It should be of note that the stability is also enhanced by neutral salts (Inouye et al., 1998a). These findings suggest that not only the dielectric constant of the reaction medium but also the interaction between ions and particular surface residues of thermolysin is involved in the salt-induced activation. If such residues are identified, genetic engineered thermolysins which have higher activity than the wild-type enzyme in the absence of salts might be generated. In addition, the inhibitory effect of alcohols on thermolysin activity is characterized and shown to be also related to the dielectric constant of the reaction medium and the interaction of alcohols with particular residues (Inouye et al., 1997; English et al., 1999; Muta and Inouye, 2002).

All extracellular bacterial proteases are synthesized as inactive pre-proenzymes. Sequence analysis of the *npr* gene in *B. thermoproteolyticus* encoding thermolysin reveals that thermolysin is synthesized as a pre-proenzyme consisting a signal peptide (28 amino acid residues), a pro-sequence (204 residues) and a mature sequence (316 residues) (O’Donohue et al., 1994). The signal peptide acts as a signal for translocation of pre-thermolysin to membrane. The pre-thermolysin is processed into the prothermolysin by a signal peptidase. The pro-sequence in the prothermolysin then acts as a molecular chaperone leading to an autocleavage of the peptide bond linking the pro- and mature sequences. Site-directed mutagenesis experiments of thermolysin have been extensively performed using either *B. subtilis* (Toma et al., 1989; O’Donohue et al., 1994; Beaumont et al., 1995; Kidokoro et al., 1995; Marie-Claire et al., 1997; Hanzawa and Kidokoro, 1999; Matsumiya et al., 2004, 2005) or *Escherichia coli* (Inouye et al., 2006; Kusano et al., 2006) as host organisms for extracellular production of recombinant molecule. Thermolysin-like protease (TLP-ste) [EC 3.4.24.4], a neutral metalloprotease from *B. steaetherophilus* that consists of 319 amino acid residues and differs at 44 out of 319 residues from thermolysin, has also been used for the same purpose. Glu143 and His231 of thermolysin were demonstrated to play significant catalytic roles (Toma et al., 1989; Beaumont et al., 1995) as predicted by the structural data (Holmes and Matthews, 1982; Hangauer et al., 1984; Mock and Aksamawati, 1994). The thermolysin mutants with higher activity (Kidokoro et al., 1995; Hanzawa and Kidokoro, 1999) and the TLP-ste mutants with higher thermal stability (Van den Burg et al., 1998; Eijink et al., 2004) have been generated by mutation of the residues at the active site and the N-terminal domain, respectively. Recently,
we generated a mutant thermolysin N112D with altered pH-activity profile by replacing Asn112 by Asp (Kusano et al., 2006). Therefore, there is no doubt that the conventional method of producing recombinant thermolysin, which is based on the expression of a single polypeptide pre-prothermolysin and autolysin cleavage of the peptide bond linking the pro- and mature sequences, is a powerful tool for protein engineering of thermolysin. However, active-site mutants that have suppressed or no autolysin digestion activity cannot be produced in the conventional method. We demonstrated that N112D and N112E were produced with small amounts, whereas N112A, N112H, N112K and N112R were not produced at all (Kusano et al., 2006). In addition, N112D and N112E revealed altered catalytic activities (Kusano et al., 2006). This led to the speculation that substantial numbers of mutant thermolysins have barely been produced in Bacillus or \textit{E. coli} probably due to suppression in autolysin digestion activity. Some of them might exert individual catalytic activities, if the peptide bond linking the pro- and mature sequences can be cleaved.

This report describes a novel method of producing recombinant thermolysin extracellularly. The method is based on co-expression of the mature sequence of thermolysin containing an NH$_2$-terminal pelB signal peptide and the pre-prosequence of thermolysin in \textit{E. coli}, and therefore does not require the autolysin digestion activity of thermolysin to be expressed.

**Materials and methods**

**Materials**

FAGLA (Lot 111K1764) was purchased from Sigma (St. Louis, MO, USA). The concentration of FAGLA was determined spectrophotometrically using the molar absorption coefficient, $\varepsilon_{345} = 766 \text{ M}^{-1} \text{ cm}^{-1}$ (Feder, 1968; Inouye, 1992). ZDFM was prepared as described earlier (Inouye, 1992). The concentration of ZDFM was determined using the molar absorption coefficient, $\varepsilon_{257} = 387 \text{ M}^{-1} \text{ cm}^{-1}$ (Inouye, 1992).

**Bacterial strains, plasmids and transformation**

\textit{Escherichia coli} K12 JM109 [recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, $\Delta$(lac-proAB), \textit{F}'(traD36, proAB$^+$ lac$^{+b}$, lacZ\textit{MD15})] was used. pTE1, an expression plasmid containing the complete \textit{npr} gene and the promoter region (Inouye et al., 2006), was used as a template for PCR. The 305-bp fragment containing the promoter region in the \textit{npr} gene was amplified using the following nucleotide primers:

\begin{align*}
5'\text{-TAGATCTCCTCATTGGTA GTGACCAAGAAC-3'} & \quad 5'\text{-TGAATTCCTACGACTCTCA CATCACCTGGTT-3'} \\
5'\text{-TTCTAGACCATGGC GATAACAGGAACATCAACTGTCGGAG-3'} & \quad 5'\text{-CCTCATGGAACAAACAATTGTA GGAG-3'} \\
5'\text{-CCGAAGCGCATTTGGA AGGAAAGAACC AAAATAT-3'} & \quad 5'\text{-CACTATGTTTGTACATCTCC CTTATTCTTGTATTTA-3'}
\end{align*}

The amplified DNA product was digested with restriction enzymes $\text{Bgl II}$ and NdeI and inserted in pET-22b (+) (Merck Bioscience, Japan) digested with $\text{Bgl II}$ and NdeI to produce pET-5'. The 1060-bp fragment containing the gene encoding the mature sequence was amplified using the following primers:

\begin{align*}
5'\text{-TTCTAGACCATGGG GATAACAGGAACATCAACTGTCGGAG-3'} & \quad 5'\text{-TGAATTCCTACGACTCTCA CATCACCTGGTT-3'} \\
5'\text{-TATGTCGAGAGATCTAACTA ACTATTCCTCCCGTG TACATCTG-3'} & \quad 5'\text{-CACTATGTTTGTACATCTCC CTTATTCTTGTATTTA-3'}
\end{align*}

The amplified DNA product was digested with XbaI and SacI and inserted in pUC19 to produce pUC-M. The 992-bp fragment containing the promoter region in the \textit{npr} gene and the gene encoding pre-prosequence was amplified using the following primers:

\begin{align*}
5'\text{-TAGATCTCCTCATTGGTA GTGACCAAGAAC-3'} & \quad 5'\text{-TGAATTCCTACGACTCTCA CATCACCTGGTT-3'} \\
5'\text{-TTCTAGACCATGGC GATAACAGGAACATCAACTGTCGGAG-3'} & \quad 5'\text{-CCTCATGGAACAAACAATTGTA GGAG-3'} \\
5'\text{-CCGAAGCGCATTTGGA AGGAAAGAACC AAAATAT-3'} & \quad 5'\text{-CACTATGTTTGTACATCTCC CTTATTCTTGTATTTA-3'}
\end{align*}

**Fermentation**

For seed culture, 5 ml of L broth was inoculated with the glycerol stock of the transformed JM109 and grown with shaking at 37°C to reach an optical density at 600 nm (OD$_{600}$) of 0.6. The culture (2 ml) was then diluted 100 times with 200 ml of L broth in a 500-ml flask and incubated at 37°C. At an appropriate time, the culture (1 ml) was pipetted out to determine OD$_{600}$ and then centrifugated. The supernatant was assessed for casein hydrolysis activity.

**Hydrolysis of casein**

The activity of supernatants toward casein hydrolysis was determined according to the methods described earlier (Hagihara et al., 1958). Samples (0.5 ml) were added to 1.5 ml of a solution containing 1.33% (w/v) casein and 40 mM Tris–HCl (pH 7.5), and incubated at 25°C for 30 min. The reaction was stopped by the addition of 2 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The reaction mixture was filtered through Whatman No. 2 filter paper (70 mm in diameter), and the $A_{275}$ was measured. One unit of activity is defined as the amount of enzyme activity needed to liberate a quantity of acid-soluble peptide corresponding to an increase in $A_{275}$ of 0.0074 (A$_{275}$ of 1 µg of tyrosine)/min.

**SDS–PAGE**

SDS–PAGE was performed in a 12.5% polyacrylamide gel under reducing conditions according to the method of Laemmli (1970). A constant current of 40 mA was applied for 1 h. Supernatants were reduced by treatment with 2.5% 2-mercaptoethanol at 100°C for 10 min. Proteins were stained with Coomassie Brilliant Blue R-250. The molecular-mass marker kit consisting of rabbit muscle phosphorylase $b$ (97.4 kDa), bovine serum albumin (66.3 kDa), rabbit muscle aldolase (42.4 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and hen egg
Fig. 1. Structure of the thermolysin expression plasmids. (A) pTE1: pTE1 is designed to express pre-prothermolysin. (B) pTMP1: pTMP1 is designed to co-express the mature sequence of thermolysin containing a pelB leader sequence at its N-terminus and the pre-prosequence of thermolysin. (C) pTMP2: pTMP2 is designed to co-express the mature sequence of thermolysin containing the original pre-sequence of thermolysin at its N-terminus and the pre-prosequence of thermolysin. (D) pTM1: pTM1 is designed to express the mature sequence of thermolysin containing a pelB leader sequence at its N-terminus. The underline shows the promoter sequences, and that labeled with SD shows the Shine–Dalgarno sequence. The arrow indicates the putative cleavage site between the pre-sequence and the pro-sequence (A) or the pre-sequence and the mature sequence (B–D).
white lysozyme (14.4 kDa) was a product of Daiichi Pure Chemicals (Tokyo, Japan).

Preparation of purified thermolysin
Preparation of purified thermolysin was performed as described earlier (Inouye et al., 2006; Yasukawa et al., 2006). Briefly, for seed culture, 5 ml of L broth was inoculated with the glycerol stock of the transformed JM109 and grown with shaking at 37°C for 12 h. The culture (5 ml) was then diluted 100 times with 500 ml of L broth in a 1 L flask and incubated under the conditions at 37°C, with 0.1% (w/v) anti-foam A (Sigma) and vigorous aeration by air-pump. Active and mature wild-type thermolysin was purified to homogeneity by sequential column chromatography procedures of the supernatant of E. coli cells with hydrophobic interaction chromatography followed by affinity chromatography. Prior to kinetic measurements, the preparations were desalted using pre-packed PD-10 gel filtration columns (Amersham Biosciences, Uppsala, Sweden).

Hydrolysis of FAGLA
Hydrolysis of FAGLA by thermolysin was measured following the decrease in absorbance at 345 nm (Feder, 1968; Inouye, 1992) with a Shimadzu spectrometer UV mini-1240 (Kyoto, Japan). The amount of FA-dipeptide amides hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310 \text{ M}^{-1} \text{ cm}^{-1}$, at 25°C (Feder, 1968; Inouye, 1992). The reaction was carried out in 40 mM acetate–NaOH buffer at pH 4.0–5.5, 40 mM MES–NaOH buffer at pH 5.5–7.0, 40 mM HEPES–NaOH buffer at pH 7.0–8.5 and TAPS–NaOH buffer at pH 8.0–9.0, each of which contained 10 mM CaCl$_2$. The reaction with FAGLA was carried out under pseudo-first order conditions, where the substrate concentration is lower than the Michaelis constant $K_m (>30 \text{ mM})$ (Inouye, 1992), because of the sparing solubility (<6 mM) of FAGLA (Feder, 1968; Inouye et al., 1998a; Inouye, 1992). Under the conditions, the kinetic parameters, $K_m$ and the molecular activity, $k_{cat}$, cannot be determined separately. Instead, the enzyme reaction was performed under pseudo-first order conditions at pH 4.0–9.0, and the enzyme activity was evaluated by the specificity constant, $k_{cat}/K_m$.

Hydrolysis of ZDFM
Hydrolysis of ZDFM catalyzed by thermolysin was measured by following the decrease in absorbance at 224 nm (Inouye, 1992) with a Shimadzu spectrometer UV mini-1240. The amount of ZDFM hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{224} = -493 \text{ M}^{-1} \text{ cm}^{-1}$, at 25°C (Inouye, 1992). The reaction was carried out with thermolysin at 0.1 $\mu$M in 40 mM Tris–HCl (pH 7.5) buffer containing 10 mM CaCl$_2$ at 25°C. The kinetic parameters, $k_{cat}$ and $K_m$, were determined based on the Michaelis–Menten equation using the non-linear least-squares methods (Sakoda and Hiromi, 1976).

Results
Construction of plasmids for co-expression of the mature and pro-sequences
Figure 1A shows the expression plasmid of Glu143 of thermolysin is catalytically important residue polarizing the water molecule which attacks a scissile bond in catalysis (Holmes and Matthews, 1982; Hangauer et al., 1984; Toma et al., 1989). JM109 cells were transformed with each plasmid containing a DNA sequence encoding the wild-type thermolysin or E143A, in which Ala is substituted for Glu143. Each transformant was cultured in a test tube for 24 h and harvested, and the supernatant was analyzed by SDS–PAGE (Fig. 2A). In the conventional expression method based on an autocatalytic cleavage, the 34 kDa protein band corresponding to the mature thermolysin was detected for the wild-type thermolysin plasmid pTE1 (lane 4) but not for the mutant thermolysin E143A expression plasmid pTE1–E143A (lane 5), which was the same result as expression of thermolysin in B. subtilis (Toma et al., 1989). In the new method, however, the 34 kDa protein band was detected both for the wild-type thermolysin expression plasmid pTMP1 (lane 6) and the E143A expression plasmid pTMP1–E143A (lane 7). This suggests that mutant thermolysins lacking autocatalytic digestion activity can be potentially produced by independent expression of mature and pro-sequences. Unexpectedly, another wild-type thermolysin expression plasmid in the present co-expression-based method, pTMP2, did not express the 34 kDa protein band (lane 8), which might be explained by that the signal peptidase recognized the peptide bond linking pelB leader sequence and the mature sequence but not the peptide bond linking the original pre-sequence of thermolysin and the mature sequence. pTM1 did not express...
the 34 kDa protein band (lane 9), demonstrating that the propeptide was required for the mature sequence to be properly folded.

The 26 kDa protein band was visible only in pTMP1–E143A (lane 7). Although the calculated molecular mass of the propeptide of thermolysin is 22,852 Da, we speculate that this band corresponds to the propeptide because it was previously reported that the 26 kDa band on SDS–PAGE corresponded to the propeptide expressed in E. coli by Coomassie blue staining (O’Donohue and Beaumont, 1996) and by the western blots (Marie-Claire et al., 1999). Figure 3 shows the putative maturation pathway of thermolysin based on the results shown in Fig. 2A. E143A might exist as a complex with the propeptide in the supernatant (Fig. 3D) because E143A was reported to lose the catalytic activities but retain the substrate-binding ability (Marie-Claire et al., 1999) whereas the propeptide is thought to be finally degraded by the active thermolysin and/or bacterial proteases in pTE1 (Fig. 3A) and pTMP1 (Fig. 3C).

Figure 2B shows the SDS–PAGE of the culture supernatant of E. coli cells transformed with pUC19 (lane 3), pTE1 (lane 4), pTE1–E143A (lane 5), pTMP1 (lane 6), pTMP1–E143A (lane 7), pTMP2 (lane 8) and pTM1 (lane 9). The marker proteins (lanes 1 and 12), native thermolysin from B. thermoproteolyticus (lanes 2 and 11), supernatants of E. coli cells transformed with pUC19 (lane 3), pTMP1 (lane 4), pTMP1–N112A (lane 5), pTMP1–N112D (lane 6), pTMP1–N112E (lane 7), pTMP1–N112H (lane 8), pTMP1–N112K (lane 9) and pTMP1–N112R (lane 10).

Figure 4 shows a time-course for a flask-shake culture of the transformants. In the transformants with the wild-type expression plasmids pTE1 and pTMP1 in the conventional method and the new method proposed in this study, respectively, casein hydrolysis activity appeared in the supernatants and increased progressively even after OD600 reached the maximum level. In the transformants with the E143A expression plasmids pTE1–E143A and pTMP1–E143A in the conventional and new methods, respectively, OD600 increased with time, but casein hydrolysis activity did not appear in the supernatants.

**Purification of recombinant thermolysin**

Two wild-type thermolysins that were expressed by pTE1 and by pTMP1, respectively, were purified to homogeneity by sequential column chromatography procedures of the supernatant of the E. coli transformants with hydrophobic interaction chromatography followed by the Gly-D-Phe affinity chromatography. Each fraction was assessed for casein hydrolysis activity on the casein plates, and the active fractions were pooled and analyzed by SDS–PAGE (Fig. 5). Thermolysin was eluted as an apparently single protein (lanes 4 and 7) from the culture supernatant (lanes 3 and 6), and the thermolysin preparation thus obtained yielded a single band with a molecular mass of 34 kDa (lanes 5 and 8). Table I shows the representative purification data of the wild-type thermolysin from the culture supernatant of the transformants with pTMP1. Starting with 350 ml of supernatant containing 8.0 mg thermolysin, 2.4 mg was recovered. This is comparable with the purification data of the wild-type thermolysin from the culture supernatant of the transformants with pTE1 (1.5 mg from 350 ml of supernatant) (data not shown).

**Characterization of recombinant thermolysin**

We compared the recombinant thermolysin from the expression plasmid pTMP1 in the new method with the one from the expression plasmid pTE1 in the conventional method. The pH dependence of their activity at 25°C is shown in Fig. 6A. The pH dependence was similar for both enzyme preparations, with the optimal pH around 6.5–7.0, the acidic pKa around 5.3–5.4 and the alkaline pKa around 8.2–8.3. Figure 6B shows the hydrolysis at various ZDFM concentrations (0–1.5 mM) with 0.1 μM thermolysin. The Km and kcat values of the thermolysin from pTE1 (0.54 ± 0.08 mM and 4.9 ± 0.3 s⁻¹) and those of the thermolysin from pTMP1 (0.40 ± 0.03 mM and 4.8 ± 0.1 s⁻¹) were respectively the same. These results suggested that the two preparations of the wild-type thermolysin were substantially identical in catalytic properties, although they underwent the different maturation processes.

**Discussion**

**Production of mutant thermolysins without autocatalytic digestion activity**

In protein engineering of extracellular bacterial proteases, one of the obstacles is that some mutations may cause significant failure or complete loss of autocatalytic digestion activity. In the case of thermolysin, some active-site mutants have not been processed to the mature enzyme (Beaumont...
et al., 1995; Inouye et al., 2006). The commonly used approach for this issue is expression of pro-sequence and mature sequence as separate polypeptides in inclusion bodies in *E. coli* and the subsequent refolding process, which was reported first in subtilisin (Zhu et al., 1989) and α-lytic protease (Silen and Agard, 1989) as a pioneering work on the effect of propeptide as chaperones and later in thermolysin (Marie-Claire et al., 1999), aqualysin I (Kim et al., 2002) and streptopain (Anderson et al., 2005). However, this method is not suitable for manipulating a number of mutants, because they require empirical adjustment of refolding conditions. Another approach is optimization of amino acid sequences at the autocatalytic cleavage site for the mutated enzymes with altered substrate specificity, which was reported in *Streptomyces griseus* protease B (Sidhu and Borgford, 1996), although it does not seem universal.

In this study, we co-expressed mature sequence of thermolysin containing an NH₂-terminal pelB leader sequence and the pre-prosequence of thermolysin as independent polypeptides constitutively in *E. coli*. The mutant thermolysins at the active site E143A, N112A, N112H, N112K and N112R have not been produced in the conventional expression method based on autocatalytic cleavage (Toma et al., 1989; Kusano et al., 2006). In the new method proposed in the present study, however, such mutant thermolysins were produced in the culture medium with almost the same expression levels as the wild-type enzyme. One of the important points in this system is the selection of a signal peptide. Both mature- and pro-peptides should be translocated to periplasm across the cytoplasmic membrane and receive the processing by the signal peptidase. We used the original pre-sequence of thermolysin as the signal peptide for the pro-sequence, because the active mature wild-type thermolysin was produced extracellularly when pre-proenzyme was expressed in *E. coli* (Inouye et al., 2006). As to the signal peptide for the mature sequence, a pelB leader sequence, one of the most commonly used signal peptides in expression of recombinant proteins in *E. coli* functioned well (Fig. 2A lane 6 and Fig. 3C), but the original pre-sequence of thermolysin did not (Fig. 2A lane 8 and Fig. 3E). Another important point is avoidance of

![Fig. 3. Schematic illustration of the maturation pathway of thermolysin. Putative maturation pathways of thermolysins expressed in *E. coli* cells transformed with pTE1 (A), pTE1–E143 (B), pTMP1 (C), pTMP1–E143A (D), pTMP2 (E) and pTM1 (F) are described. The open and dotted boxes represent the signal sequences of thermolysin and pelB, respectively. The filled and shaded diagrams represent the mature sequence and the pro-sequence of thermolysin, respectively. The asterisk means the mutated site (Ala143). (A) The pre-prothermolysin is processed into the prothermolysin. The pro-sequence acts as an intramolecular chaperone leading to the autocatalytic cleavage. The pro-sequence then acts as an intermolecular chaperone and is finally degraded. The correctly folded mature thermolysin is secreted. (B) The preprothermolysin is processed into the prothermolysin. The pro-sequence acts as an intramolecular chaperone, but the autocatalytic cleavage does not occur. (C) The mature sequence of thermolysin containing an NH₂-terminal pelB leader sequence and the pre-prosequence of thermolysin are processed into the mature sequence and the pro-sequence, respectively. The pro-sequence acts as an intermolecular chaperone and is finally degraded. The correctly folded mature thermolysin is secreted. (D) The mature sequence of thermolysin containing an NH₂-terminal pelB leader sequence and the pre-prosequence of thermolysin are processed into the mature sequence and the pro-sequence, respectively. The pro-sequence acts as a molecular chaperone but is not finally degraded. The correctly folded mature thermolysin is secreted as a complex with the pro-sequence. (E) The mature sequence of thermolysin containing an original pre-sequence at its NH₂-terminal is not processed into the mature sequence whereas the pre-prosequence is processed into the pro-sequence. (F) The mature sequence of thermolysin containing an NH₂-terminal pelB leader sequence is processed into the mature sequence, which is not subsequently folded.
formation of inclusion bodies, which was attained by expressing both pro- and mature sequences under the original promoter sequences in the npr gene from B. thermoproteolyticus. As described in the previous article (Inouye et al., 2006) and shown in Fig. 1, the npr gene contains the putative promoter and Shine–Dalgarno sequences in E. coli, and both peptides were constitutively expressed at levels possibly low enough to prevent formation of inclusion bodies. To compare the new expression method using the original promoter in the npr gene with the conventional method using the T7 promoter, we constructed another expression plasmid for the mature wild-type thermolysin containing an NH₂-terminal peIB leader sequence and the pre-prosequence of thermolysin both under the T7 promoter. When E. coli BL21(DE3) cells transformed with the plasmid were cultured and induced with 1mM IPTG, both peptides were expressed in the forms of inclusion bodies, and no active thermolysin was produced in the culture medium (data not shown). Unexpectedly, when the cells were cultured for 24 h without IPTG induction even after reaching the maximum OD₆₀₀, active thermolysin was produced (data not shown), which might be due to the leaking expression of T7 promoter.

**Insights into the maturation pathway**

On the basis of the study using the wild-type subtilisin E or its mutant S221C folded by either the wild-type propeptide or its mutant Ile(-48)T [the numbering of amino acid residues of pro-sequence of subtilisin E begins at −77 for the N-terminal Al and ends at −1 for the C-terminal Tyr], its folding process can be divided into two stages, i.e. the intramolecular folding before the autocatalytic cleavage and the intermolecular folding after the autocatalytic cleavage (Shinde et al., 1997, 1999). This suggests that thermolysin produced in the new method might be differently folded from the one produced in the conventional method. However, we demonstrated that the wild-type enzyme expressed in the new method was indistinguishable from that expressed in the conventional method, as assessed by the pH profile in the hydrolysis of FAGLA and kinetic parameters in the hydrolysis of ZDFM (Fig. 6), suggesting that the intramolecular folding (Fig. 3A) can be substituted with the intermolecular folding (Fig. 3C). This coincides with the previous finding that structural imprinting by the propeptide, which they termed ‘protein memory’, occurred after the autocatalytic cleavage (Shinde et al., 1999).

There have been several reports on the maturation mechanism of thermolysin. First, the propeptide alone was expressed as the cellular soluble proteins, and the purified propeptide inhibited thermolysin activity in a non-competitive manner but facilitated the refolding of denatured thermolysin (O’Donohue and Beaumont, 1996). Secondly, when expressed as N-terminal histidine-tagged prothermolysin in the forms of inclusion bodies and then solubilized and immobilized on a cobalt-containing resin, the wild-type thermolysin was transformed into mature enzyme spontaneously by dialysis against a refolding buffer whereas E143A was not (Marie-Claire et al., 1998). Because E143A was not transformed to its mature form even in the presence of active wild-type thermolysin exogenously added, it is concluded that thermolysin maturation proceeded through intramolecular, but not intermolecular, cleavage (Marie-Claire et al., 1998). Thirdly, when the mature wild-type thermolysin or E143A was co-expressed with the propeptide in E. coli as independent polypeptides, the hydrolyzing activity or the substrate-binding ability was detected in the cells, and therefore it is concluded that the pro-sequence acted as an intermolecular chaperone (Marie-Claire et al., 1999). However, the physicochemical analysis, such as stoichiometry and dissociation constants of the propeptide and the mature sequence, and crystallographic analysis of the complex have not been performed because the mutant thermolysin that only autocatalytically cleaves but does not degrade the propeptide, like subtilisin E mutant S221C (Shinde et al., 1999, Jain et al., 1998), has not been available. The new method potentially provides enough amounts of the mutant thermolysin, such as E143A, that binds but does not degrade the propeptide and might be useful for further study on maturation of...
thermolysin. Purification and characterization of the complex of E143A and the propeptide is an important subject to demonstrate the maturation pathway of thermolysin (Fig. 3).

Recently, active mature TLP-ste was produced as the cellular soluble proteins in E. coli in the absence of its propeptide (Mansfeld et al., 2005), in which the expression was successful only when using lacZ/ψ-deficient E. coli strain Tuner cells, but not the conventional E. coli strains BL21 and JM109, and optimizing culture conditions such as the concentration of IPTG and the incubation temperature. In that study, active mature TLP-ste was also renatured from the inclusion bodies in the absence of its propeptide. This is not in agreement with the previous report on thermolysin (Marie-Claire et al., 1999). The reason of the difference between thermolysin and TLP-ste is not clear.

Conclusion
The new method of producing recombinant thermolysin in E. coli comprises constitutive co-expression of mature and prosequences both containing an appropriate signal peptide at their N-terminus. We speculate this strategy is applicable to extracellular production of not only mature thermolysin but also other bacterial proteases by selecting suitable signal peptide and promoter.

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Table 1. Representative purification of thermolysin from the supernatant of the E. coli transformed with pTMP1

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<thead>
<tr>
<th>Purification method</th>
<th>Volume (ml)</th>
<th>Activity (PU)</th>
<th>Recovery (%)</th>
<th>Protein (mg)</th>
<th>Specific activity (PU/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>350</td>
<td>85 100</td>
<td>100</td>
<td>55.6</td>
<td>1500</td>
<td>1</td>
</tr>
<tr>
<td>Phenyl chromatography</td>
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<td>62 800</td>
<td>74</td>
<td>9.3</td>
<td>6700</td>
<td>4</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>6</td>
<td>25 300</td>
<td>30</td>
<td>2.4</td>
<td>10 500</td>
<td>7</td>
</tr>
</tbody>
</table>

Fig. 6. Enzymatic activities of the recombinant wild-type thermolysins. The reactions were carried out with wild-type thermolysin from pTE1 (open circle) or pTMP1 (filled circle) at 0.1 μM, 25°C. (A) Effect of pH on the thermolysin-catalyzed hydrolysis of FAGLA. The acidic and alkaline pH values of the thermolysin from pTE1 were 0.54 ± 0.08 mM and 4.9 ± 0.3 s⁻¹, and those of the thermolysin from pTMP1 were 0.40 ± 0.03 mM and 4.8 ± 0.1 s⁻¹, respectively.

References
Co-expression of mature and pro-sequences of thermolysin


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