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# Functional analysis of the propeptides of subtilisin E and aqualysin I as intramolecular chaperones

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**Abstract Several proteases require propeptides for the correct folding of their own protease domain. We have recently found that the propeptide from a thermostable subtilisin homolog aqualysin I can refold subtilisin BPN′ when added *in trans*. Here, we constructed chimeric genes with subtilisin E and aqualysin I to attempt the *in cis* folding of subtilisin E by means of the propeptide of aqualysin I. Our results indicate that the propeptide of aqualysin I can to some extent chaperone the intramolecular folding of the denatured subtilisin E. These results suggest that propeptides in the subtilisin family, despite their sequence diversity, have similar functions. Further, some enzymatic properties of some chimeras in which the subtilisin mature domain is partly swapped with that of aqualysin I were shown to be more similar to those of aqualysin I.** 

*Key words:* Propeptide; Intramolecular chaperone; Protein folding; Subtilisin E; Aqualysin I; Chimeric protease

## 1. Introduction

Recently, protein folding mechanisms mediated by propeptides have been found in a variety of proteases, including subtilisin [1–3], α-lytic protease [4,5], aqualysin I (AQI) [6], carboxypeptidase Y [7], cathepsin L [8], and thermolysin [9]. Such proteases require the assistance of the N-terminal propeptides of precursors to produce active, mature enzymes. Upon the completion of folding, the propeptides are autocatalytically degraded because they are not necessary for the activity or stability of folded, mature cognates of the original enzymes [10]. Therefore, the propeptide functions as an intramolecular chaperone (IMC) that guides correct folding of the mature domain [11].

IMC-mediated protein folding is best understood in subtilisin [10]. Subtilisin has been extensively investigated in terms of both basic and applied aspects as a promising target for protein engineering [12–15]. Subtilisin E (SBE), an alkaline serine protease of *Bacillus subtilis* I168, is first synthesized as a precursor, preprosubtilisin, which consists of a 29-mer signal peptide for protein secretion (presequence), a 77-residue propeptide as an IMC, and a 275-amino acid active protease domain [16]. Subtilisin also serves as an ideal model for the large superfamily of subtilisin-like serine protease 'subtilases' [17]. The in vitro folding pathway of prosubtilisin involves folding of the precursor, autoprocessing of the propeptide form of the N-terminus, and degradation of the cleaved propeptide (IMC domain), resulting in the protease domain [18–20]. It is important to note that the IMC domain is devoid of significant secondary and tertiary structures when released from subtilisin [21–23]. Hence, specific interactions between IMC and subtilisin appears to be crucial in stabilizing the complex.

To analyze in detail the mechanisms of protein-folding by IMC, we have previously focused on the propeptide from a thermostable subtilisin homolog, AQI, which is secreted by *Thermus aquaticus* YT-1 [24]. Similar to subtilisin, AQI is also produced as a precursor consisting of a signal peptide, an N-terminal propeptide functioning as an IMC, a mature domain, and an additional C-terminal propeptide [25–27]. Despite showing only 21% sequence identity with the propeptide of SBE (ProS), the propeptide of AQI (ProA) was found not only to bind to mature subtilisin BPN′ tighter than ProS, but to refold denatured-subtilisin BPN′ with approximately half the efficiency of ProS when added *in trans* [24]. In the present study, we constructed various chimeric genes with SBE and AQI and attempted to achieve the *in cis* refolding of denatured proteins using ProS and ProA in an intramolecular manner. Furthermore, we describe here the isolation and partial characterization of some of the chimeric proteases.

## 2. Materials and Methods

### 2.1. Materials

An *Escherichia coli* strain JM109 [*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44*/(*F′ traD36 proAB*$^+$ *lacI*$^q$ *ZΔM15*)] and two plasmids pHI212 [28] and pNK006 [29] containing the wild-type SBE and AQI genes, respectively, were used for construction of the chimeric genes by polymerase chain reaction (PCR). An *E. coli* strain BL21 (DE3) and the plasmid vector pET-3d (Novagen, Madison, WI, USA) were used for overexpression of the chimeric genes induced by isopropyl-β-D-thiogalactopyranoside (IPTG). AQI was purified from the culture medium of *T. aquaticus* YT-1 according to the method described previously [25]. All enzymes for DNA ma-

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*Abbreviations:* IMC, intramolecular chaperone; SBE, subtilisin E; AQI, aqualysin I; ProS, propeptide of subtilisin E; ProA, propeptide of aqualysin I; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction


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nipulations were obtained from Takara Shuzo (Kyoto, Japan) and used under conditions recommended by the supplier. Synthetic peptide substrates, *N*-succinyl-L-Ala-L-Ala-L-X-L-Y-*p*-nitroanilide (AAXY; X = Pro (P) and Val (V), Y = Ala (A), Phe (F), Lys (K), Leu (L), and Met (M)) were purchased from Bachem AG (Bubendorf, Switzerland) and Sigma (St. Louis, MO, USA).

*2.2. Construction of the chimeric genes and the expression plasmids*

In this study, two genes in which ProS or ProA was fused to the N-terminus of SBE mature domain were constructed by PCR using a Gene Amp PCR system 2400 (PE Biosystems, Foster City, CA, USA) and subcloned into the expression plasmid pET-3d (Fig. 1). We also created four chimeric proteases (Sα–Sδ) based on the mature domain of SBE by PCR (Fig. 2).

Next, to create the chimeric protease genes, PCR was carried out with pNK006 as a template and primers 5′-CATGGCCGGCTAGCTACACCTACACCGCT-3′ and 5′-CCCAAGCTTGCCCGGCCGCCGAACT-3′ for Sα, 5′-CCCAAGCTTCGTAGGCTATGACGCC-3′and 5′-CTGCAGAACCATATTGTTGGACGTGACCCAGTCCA-3′ for Sβ, 5′-CTGCAGAACCAACAATATGGATCCGGCCGTTGCCAACA-3′ and 5′-CATGCCATGGAGGTGCCGTTAAGGGTCTG-3′ for Sγ, and 5′-CATGCCATGGCGACCCCCCATGTGGC-3′ and 5′-CATGCAGCTGCGAGACGGTTGGGGGA-3′ for Sδ, respectively. After the PCR, each unique amplified band was digested with *Nae*I and *Hin*dIII, *Hin*dIII and *Bst*XI, *Bst*XI and *Nco*I, and *Nco*I and *Pvu*II to recover the 90-, 186-, 297-, and 141-bp fragment, respectively. Each fragment was replaced with the corresponding SBE gene of pET-proSSBE or pET-proASBE by partial digestion and ligation to construct plasmid pET-proSSα, pET-proSSβ, pET-proSSγ, and pET-proSSδ, respectively, or pET-proASα, pET-proASβ, pET-proASγ, and pET-proASδ, respectively. All the nucleotide sequences were confirmed with a Model 377 DNA sequencer (PE Biosystems) using dideoxy chain termination sequencing directly on the plasmids.

*2.3. Expression of the chimeric genes in E. coli*

The chimeric genes were expressed in the *E. coli* strain BL21 (DE3). Each recombinant strain was grown at 37°C in M9 medium supplemented with 2% casamino acids, 0.4% glucose, 0.02% $MgSO_4$, and 50 μg/ml ampicillin (M9CA) [28]. When absorbance at 600 nm reached 0.5, IPTG was added to the culture medium to a final concentration of 1 mM to induce the gene expression. After cultivation for 4 h at 37°C, the cells were harvested by centrifugation.

*2.4. In vitro refolding of the denatured proteins*

The cells were disrupted by sonic oscillation (200 W; Kubota, Tokyo, Japan) and the sonicated cells were centrifuged at 10 000 × *g* for 20 min. Precursors produced as inclusion bodies were recovered in the pellet and solubilized in 6 M guanidine–HCl, pH 4.8. The insoluble materials were removed by centrifugation at 100 000 × *g* for 30 min. The renaturation was initiated through rapid dilution of 200 μl of the denatured proteins (1 mg/ml) into 20 ml of refolding buffer containing 50 mM Tris–HCl, pH 7.0, 500 mM $(NH_4)2SO_4$ and 1 mM $CaCl_2$ [30]. The diluted protein was kept with stirring for overnight at 4°C.

*2.5. Purification of the refolded proteins*

The refolded proteins were concentrated by cold acetone precipitation followed by dialysis against 10 mM sodium phosphate buffer (pH 6.0) containing 1 mM $CaCl_2$. The dialysate was applied onto a cation-ion exchange Mono S HR5/5 column (Amersham Pharmacia Biotech, Buckinghamshire, England) that was connected to AKTA™ Design (Amersham Pharmacia Biotech) and preequilibrated with the buffer. The enzyme was eluted with a linear 0 to 200 mM KCl gradient in 30 ml of the buffer at a flow rate of 1 ml/min. The activity-containing fractions were collected and used as the purified enzyme for characterization.

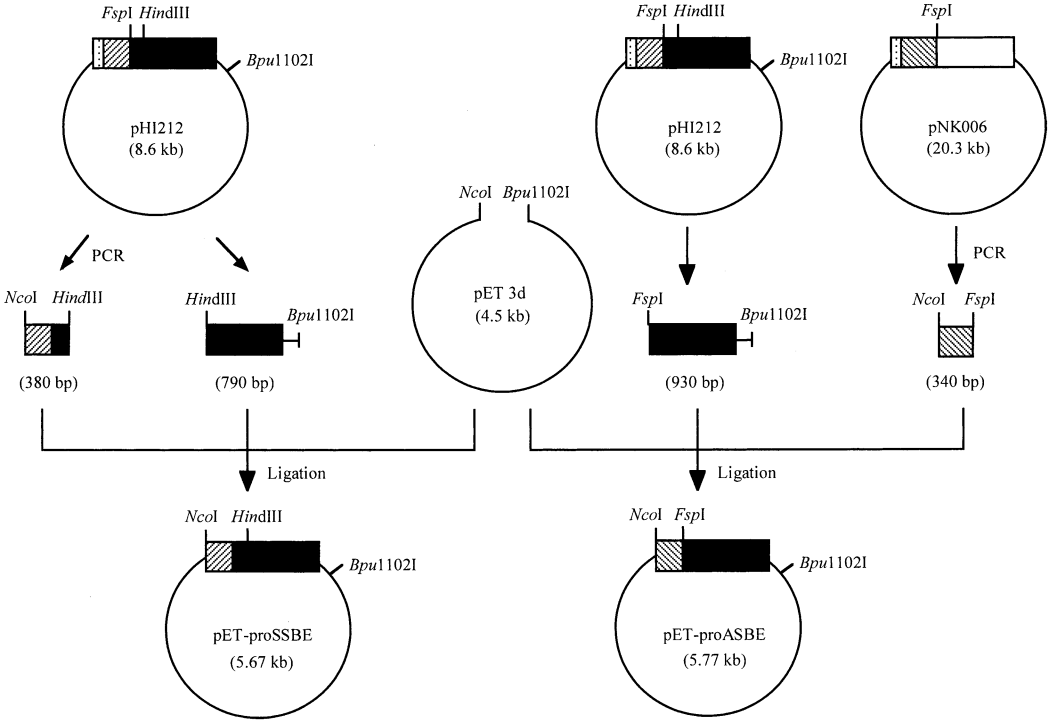


Fig. 1. Construction of expression plasmids for chimeric genes in *E. coli*. PCR was first carried out with pHI212 [28] containing the wild-type SBE gene as a template and primers 5′-CATGCCATGGCCGGAAAAAGCAGTAC-3′ and 5′-CCCAAGCTTGCTCCGCCTCTGACGTT-3′. After the PCR, the unique amplified band was digested with *Nco*I and *Hin*dIII to recover the 380-bp *Nco*I–*Hin*dIII fragment and then ligated with the 790-bp *Hin*dIII–*Bpu*1102I fragment of pHI212 and the 4.5-kb *Nco*I–*Bpu*1102I fragment of pET-3d to construct plasmid pET-proSSBE. To isolate the ProA domain only, PCR was performed with pNK006 [29] harboring the wild-type AQI gene as a template and primers 5′-CATGCCATGGTTTTGGGTGGTTGTCAG-3′ and 5′-AGATTGCGCAGCCCAGGCCCGTACCACCTT-3′. The expected band of PCR products was digested with *Nco*I and *Fsp*I to recover the 340-bp *Nco*I–*Fsp*I fragment and then ligated with the 930-bp *Fsp*I–*Bpu*1102I fragment of pHI212 and the 4.5-kb *Nco*I–*Bpu*1102I fragment of pET-3d to construct plasmid pET-proASBE. The solid and open boxes represent the SBE and AQI mature protease domains, respectively. The shaded boxes represent the IMC domains (ProS or ProA). The dotted boxes represent the signal peptides of SBE or AQI.

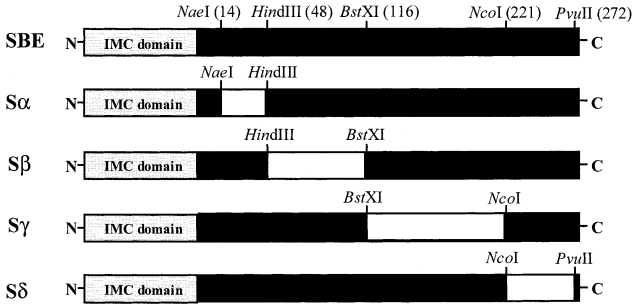


Fig. 2. Structure of the wild-type SBE (SBE) and chimeric proteases (Sα–Sδ). The solid and open boxes represent the SBE and AQI mature protease domains, respectively. The residues of mature proteases are numbered in parentheses.

### 2.6. Assay of protease activity

For synthetic peptide substrates, assays were performed as described previously [28,31]. The activity was calculated as unit/mg protein. One unit is defined as the activity releasing 1 μmol of *p*-nitroaniline/min. Protein concentrations were determined by Bio-Rad Protein Assay kit (Hercules, CA, USA) using subtilisin BPN′ (Sigma) as a standard.

## 3. Results and discussion

### 3.1. Construction and expression of chimeric genes with SBE and AQI in E. coli

To examine whether ProA can chaperone *in cis* subtilisin folding, we constructed the chimeric gene ProASBE, which consisted of ProA and the SBE protease domain, with ProA being covalently attached to the N-terminus of the mature domain of SBE (Fig. 1). In addition, to analyze the specific interactions between the IMC and mature domains of SBE and AQI during protein folding, we designed four types of chimeric proteases Sα, Sβ, Sγ, and Sδ, in which the SBE mature domain was partly swapped with the sequence of the AQI protease domain (Fig. 2). Although the crystal structure of SBE is known [32], that of AQI has not yet been determined. Therefore, four chimeras were chosen by considering their influence on the secondary structure of SBE and the locations of restriction-enzyme recognition sites. The swapped regions displayed approximately 40% sequence identity (ranging from 37 to 46%).

After gene expression by IPTG, a large amount of product was observed at the position corresponding to the molecular size of each protein and was accumulated at a level of approximately 30–50% of total cellular proteins (Fig. 3). The products were isolated in pellets after disruption by a sonicator, indicating that they aggregated to form insoluble inclusion bodies (data not shown).

### 3.2. The in cis folding of denatured SBE by ProA

After the pellets were solubilized in a guanidine–HCl solution, denatured precursors were attempted to refold through a rapid dilution procedure. Enzymes in the refolding buffer were assayed without further purification (Table 1). The total subtilisin activity of ProASBE was recovered to approximately 60% that of ProSSBE, suggesting that ProA is intrinsically capable of refolding SBE in an intramolecular event, despite the lower efficiency of ProS under identical conditions. Although we need the refolding and inhibition kinetics analyses of SBE using ProS and ProA, the findings obtained from the *in trans* folding of subtilisin BPN′ suggest that ProA can initially fold denatured SBE within range of efficiency comparable to that of ProS, though ProS can later chaperone folding more efficiently than ProA [24,33]. These results also suggest that, despite their sequence diversity, propeptides functioning as IMC domains in the subtilisin family carry out similar functions.

Table 1
Refolding of the denatured mature proteases using ProS and ProA

| IMC | Specific activity (units/mg) | | | | |
|---|---|---|---|---|---|
| | SBE | Sα | Sβ | Sγ | Sδ |
| ProS | 330 | 33 | < 1.0 | < 1.0 | 160 |
| ProA | 200 | < 1.0 | < 1.0 | < 1.0 | < 1.0 |

The denatured precursors were refolded through a rapid dilution procedure. Enzymes in the refolding buffer were assayed without further purification. Assays were performed in 50 mM Tris–HCl (pH 8.5) and 1 mM $CaCl_2$ at 45°C using *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Met-*p*-nitroanilide. Previous reports showed that no enzyme activity was detected in the absence of any IMC domain [11,21,31].

### 3.3. Specific interactions between the IMC and mature domains of SBE and AQI

To further examine the influence of the sequence and structure of the protease domain on the IMC-assisted folding, we

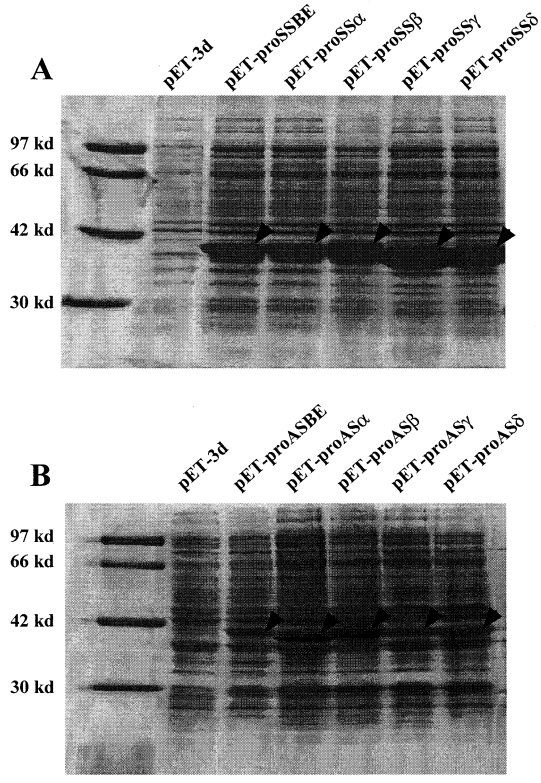


Fig. 3. SDS–PAGE patterns of the total cellular proteins from *E. coli* BL21 (DE3) cells expressing the chimeric genes with ProS (A) and ProA (B). The cells carrying pET-3d only are loaded as a control. In all case, samples applied to the gel are equivalent to 20-μl cultures. The filled arrows show the positions of precursors. SDS–PAGE was performed in a 15% polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Molecular mass standards are shown at the left.

Table 2
Properties of SBE, AQI and chimeric proteases

| Property | SBE | Sα[a] | Sδ | AQI |
|---|---|---|---|---|
| Optimum temperature for AAPM (°C) | 45 | 45 | 45 | 70 |
| Optimum pH for AAPM | 9.0 | 10.5 | 9.0 | 10.0 |
| $k_{cat}/K_m$ (s/mM) for AAPF | 21.0 | NT | 14.0 | NT |
| Relative activity (%) for | | | | |
| AAPM | 100 | 100 | 100 | 100 |
| AAPF | 96 | 90 | 89 | 145 |
| AAPL | 76 | 46 | 39 | 35 |
| AAPK | 60 | 29 | 19 | 22 |
| AAPA | 48 | 24 | 22 | 36 |
| AAVA | 78 | 45 | 39 | 104 |

Assays were performed under the conditions with individual optimum temperature and pH. NT, not tested.
[a]Sα protease was assayed without further purification after refolding.

next constructed four chimeric proteases with SBE and AQI, as described in Section 2.

When ProS was used as an IMC domain, the chimeric enzymes Sα and Sδ had approximately 10 and 50% of the wild-type SBE activity, respectively (Table 1). These results indicate that ProS can to some extent fold these chimeric proteases and that the C-terminal region of the SBE mature domain is not as important to the interaction with ProS, as the rest (α–γ) of the domain. In contrast, when ProS was replaced with ProA, no measurable enzyme activity was detected in any of the chimeras (less than 1.0 units/mg) (Table 1). In the case of Sα, the lack of activity was likely due to the folding efficiency of ProA being significantly lower than that of ProS, as described above. However, it is worth noting that ProA could not facilitate refolding of the denatured chimera Sδ under the conditions used, indicating that ProA can refold mature SBE, but not a SBE-based chimera substituting for a 48-residue AQI mature sequence at the C-terminus. This result seems incomprehensible, however, in that ProA failed to assist the folding of its own mature region. To further elucidate the mechanism involved, an attempt to add ProA or ProS exogenously (*in trans*) to the denatured chimeric mature proteases is currently in progress.

On the other hand, the chimeric Sβ and Sγ precursors failed to be autoprocessed and folded to become active, mature enzymes under the conditions tested, even though ProS or ProA was covalently attached as an IMC domain. Similar results were obtained by the folding method through a stepwise dialysis (data not shown) [30]. These results suggest that the β and γ regions in the SBE mature domain play a crucial role in the catalytic activity, including autoprocessing. It is unlikely that the substrate specificity is altered or the stability decreased in the chimeric proteases, as no mature protease bands were observed, while the precursor still remained visible on SDS–polyacrylamide gel electrophoresis (PAGE) after the refolding event (data not shown).

It is difficult to separate any effect of the replacements on the interaction of the propeptide with the rest of the molecule from other possible effects on some folding or activity determinant within the mature domain itself. Much more research on the refolding kinetics or the structural analysis is needed to clarify this point.

### 3.4. Partial characteristics of chimeric proteases

The wild-type SBE and the chimera Sδ were purified from the acetone precipitates after refolding to give a single band upon SDS–PAGE (data not shown), and their enzymatic properties were characterized (Table 2). The wild-type AQI was also purified to homogeneity from the liquid culture of *T. aquaticus* YT-1 and was examined as a control. However, the Sα protease was assayed without further purification after refolding because the amount of mature enzyme was extremely low.

The optimum temperature of SBE and chimeric proteases was approximately 45°C, while AQI had maximal activity at 70°C. AQI contains two disulfide bonds, which seem to be responsible for the thermostability of AQI [34]. In previous works [35,36], we introduced disulfide bonds engineered based on a structural similarity to AQI in SBE and found that the N-terminal disulfide bond enhances the thermostability without any change in the catalytic efficiency of SBE [35]. Sα and Sδ themselves, however, have no disulfide bonds. One noteworthy finding of the present study is that Sα shows an optimum pH of 10.5, which is nearly equal to that of AQI and is 1.5 higher than that observed for SBE. Although much more research on the pH profile using purified enzyme is needed, one possibility is that four Arg residues in the C-terminal region of AQI may be involved in the catalysis or stability of AQI at alkaline pH. Kinetic data for the hydrolysis of AAPF indicates that chimeric Sα has a slight decrease in the catalytic efficiency relative to SBE (Table 2) due to the decrease in *k*cat (data not shown). Table 2 also shows the relative activities of various proteases toward six peptide substrates. The relative activities of two chimeras for AAPL, AAPK, and AAPA were significantly diminished compared to that of SBE, which is similar to that of AQI (except for AAVA). Recently, the substrate specificity of AQI was found to be similar to that of subtilisin BPN′ and proteinase K, but different from that of subtilisin Carlsberg [37]. Our results also show that SBE has a broad specificity relative to that of AQI. Considering that a β-sheet including catalytic Asp32 or an α-helix containing catalytic Ser221 in SBE was swapped to that of AQI, it is possible that these regions in AQI may influence the specificity of AQI.

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