J. Life Science. (2001), 11(1), 42~46

JOURNAL OF
Life Science

Secretory production of prosubtilisin YaB by a six extracellular protease-deficient mutant of *Bacillus subtilis*

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Received: March 7, 2001

Abstract Subtilisin YaB, produced by alkalophilic *Bacillus* strain YaB, is an extracellular alkaline serine protease having 55% homology to subtilisin BPN'. It is synthesized as a 378-amino acid preproenzyme and secreted into the culture medium as a 265-amino acid mature protease. To examine the role of pro-sequence for the secretion of subtilisin YaB, we have studied the expression, in *Bacillus subtilis*, of a mutant preprosubtilisin YaB in which active site Ser214 is substituted with Cys. The use of a six protease-deficient strain, WB600, was required for its efficient production. The prosubtilisin YaB, thus produced, was indeed secreted into the culture medium and was processed to its mature form upon treatment with exogenously added active subtilisin YaB. From these results, we have concluded that the processing of pro-sequence is not essential for the secretion of the enzyme.

Key words: subtilisin, preproenzyme, *Bacillus subtilis*, chaperon, secretion

Introduction

Subtilisin YaB, encoded by the *ale* gene, is an extracellular alkaline serine protease of alkalophilic *Bacillus* strain YaB [1,2]. The mature protease of 265 residues is processed from its 378-amino acid precursor containing a putative signal sequence of 27 residues and a pro-sequence of 83 residues. Though its primary structure shares 55% homology with subtilisin BPN' [3], it has three distinguishing characteristics; high elastin-hydrolyzing activity, elastin-binding activity, and elastase-like substrate specificity [2,4]. Thus we also call the enzyme alkaline elastase.

Subtilisins are produced from their preproproteins [5-7]. The pro-sequence functions as the signal peptide for protein secretion across the cytoplasmic membrane. There are many studies investigating the structure and function of pro-sequence [8,9]. Both *in vivo* and *in vitro* studies have established the feature of pro-sequence as an intra-molecular chaperone for the folding of the mature protease [10,11]; the pro-sequence is essential for the folding of the active enzyme, but, after folding, it is removed via an intra-molecular autoprocessing mechanism. Recently, the crystal structure of propeptide-subtilisin BPN' complex has been solved at 2.0 Å resolution [12]. Based on this, Gallangher *et al.* [13] have proposed a refined model where the two helices and their β-strand of subtilisin form a propeptide-stabilized folding nucleus to catalyze the folding of mature domain.

In spite of our increasing knowledge on the mechanism of folding of subtilisins, our understanding of its *in vivo* mechanism is very poor. Conflicting results have been obtained on the role of pro-sequence *in vivo* [14-17]. For instance, Egnell and Flock [18] have proposed, based on their results, a membrane anchorage model: the pro-sequence functions as a membrane anchorage to retain prosubtilisin in the membrane until the proteolytic maturation of subtilisin occurs. However, experiments performed with cloned subtilisin genes in *Escherichia coli* and *in vitro* [17,19], have shown that the pro-sequence can be cleaved off without any anchorage to a membrane.

To learn more about the secretion and folding *in vitro* of subtilisins, we are studying the expression of subtilisin YaB in *Bacillus subtilis*. In our previous paper, we have demonstrated that physical linkage between the pro and the mature sequence is not required, *in vitro*, for the active subtilisin production [20]. This strongly suggests that the membrane anchorage mechanism is not necessary for the production of the active subtilisin YaB *in vitro*.

Recent data show that the intramolecular chaperon domain can impart structural information onto its protease [21] and that this inprinting occurs after autoprocessing but before degradation of the IMC from the precursor [22]. Such results suggest that IMCs promote protein folding by direct stabi-

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lization of the rate-limiting folding transition state and support the notion that the IMC is essential only during late stages of the folding process [23,24].

In this paper, we have addressed whether the pro-sequence of subtilisin YaB functions as a membrane anchorage or not by expressing a mutant prosubtilisin YaB where the active site Ser of the wild type enzyme is replaced with Cys [25,26]. The prosubtilisin YaB, when produced by a six extracellular protease-deficient strain [27] of *B. subtilis*, was successfully secreted into the culture medium, indicating that the processing the pro-sequence is not essential for its secretion. In addition, we show that the prosubtilisin YaB, thus produced, has an aberrant electrophoretic mobility.

MATERIALS AND METHODS

Strains, media and Materials.

B. subtilis DB104 [*nprE18 nprR2 aprE3 his-101*] [28], a gift from Roy H. Doi and Fujio Kawamura, and *B. subtilis WB600* [*nprE18 nprR2 aprE3 nprB nprE eprE bpf his-101*] [27], a gift from Sui-Lam Wang, were used as hosts for the expression of subtilisin YaB. For the preparation and construction of plasmids, *E. coli* JM109[*recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 relA1 supE44 / F' (traD36 proAB$^+$lac1^{q}lacZ ΔM15)*], DH5 [*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]and CJ236 [*dut ung thi relA;pCJ105(Cm^r)*] were used. All bacterial strains were propagated at 37℃ in Luria broth. The medium was supplemented with 100㎎ of ampicillin per liter, or 20㎎ of tetracycline per liter when necessary. All the restriction enzymes used were purchased from Boehringer Mannheim Biochemical (Mannhein, Germany). T4 DNA ligase, T4 polynucleotide kinase and DNA blunting kit were obtained from Takara Shuzo Co. (Kyoto, Japan). They were used according to the recommendations of the manufacturers.

Recombinant DNA techniques.

Preparation of plasmid and transformation of *E. coli* cells were done as previously described [29]. Transformation of *B. subtilis* was done by the method of Hass and Yoshikawa [30]. Site-directed mutagenesis was performed by the method of Kunkel [31] with Muta-Gene M13 In Vitro Mutagenesis Kit (Bio-Rad).

Construction of plasmides.

Plasmid pCH202 is a derivative of pUC119 carrying *ale* gene (coding for preprosubtilisin YaB). The 2.2-kb *EcoR* I -*Sal* I fragment of pCH202, containing the entire *ale* gene, was inserted into the *EcoR* I -*Sal* I sites of M13mp19 replicative-form DNA to obtain pME. After transfection of CJ236 with pME, single-stranded pME was isolated to serve as a template for mutagenesis. A synthetic oligonucleotide (5'-CAATGGTACATGCATGGCTACACCG-3') was used to introduce, into the subtilisin YaB gene, a mutation that converts the active site Ser214 to Cys to construct pME-S214C. Plasmid carrying the desired mutation was confirmed by DNA sequencing with dideoxy chain termination method [32]. Plasmid pKK601 is a derivative of pTUBE1500 [33] carrying *ale* gene under the control of *spac-1* promoter [34]. It was made by deleting an unnecessary 634 bp sequence on plasmid pEX1500 by exonuclease III digestion from the *Sal* I site using the Kilo-Sequence Deletion Kit (Takara Shuzo Co., Kyoto, Japan). The 260 bp *Dra*III-*Spe* I fragment of pKK601 was replaced with that of pME-S214C containing the active site mutation to construct pKK601-S214C. In pKK601-S214C, the entire prepro-Ser214Cys-subtilisin YaB gene is placed under the control of *spac-1* promoter. Plasmid pTUBE1500 is a gift from Kunio Yamane and Kouji Nakamura. The oligonucleotides for mutagenesis and sequencing were synthesized on an Applied Biosystems model 380A DNA synthesizer.

SDS-PAGE and Immunoblotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Leammli [35]. The gel used contained 10% acrylamide and 0.33% N, N-methylenebisacrylamide. Protein separated on SDS-polyacrylamide gel were transferred to a nitrocellulose membrane filter. The filter was treated with a rabbit anti-subtilisin YaB antibody and than with horse radish peroxidase(H계)-conjugated goat anti-rabbit lgG [36]. Membrane-bound antibody was raised against purified subtilisin YaB.

RESULTS

Construction of plasmid. To examine the role of pro-sequence for the secretion of subtilisin YaB, we aimed to express, in *B. subtilis*, a mutant prosubtilisin YaB that can not cleave off its pro-sequence. For this purpose a mutation that convert Ser214, a member of catalytic triad, to Cys was introduced into subtilisin YaB with site directed mutagenesis, since it has been well established that substitution of amino acid residue of the catalytic triad results in the loss of proteolytic activity and hence the processing of pro-sequence is inhibited in such a mutant [25,26]. The resulting mutation was then introduced into subtilisin YaB gene on plasmid pKK 601 to obtain pKK601-S214C. Plasmids, pKK601 and pKK 601-S214C contain the wild type and the mutant subtilisin YaB genes under the control of the *spac-1* promoter [34)] an IPTG-inducible promoter which functions in *B. subtilis*. Figure 1 show the structure of wild type and mutant subtilisin YaB gene products expressed from pKK601 and pKK601-S214C.

Production and secretion of pro-Ser214Cys-subtilisin YaB by B. subtilis. For the expression of prosubtilisin YaB, *B. subtilis* strains DB104 [28] and WB600 [27], either of which lacks two or six extracellular proteases including the endogenous subtilisin, were used as hosts to avoid proteolytic degradation of the gene product. To study their expression, DB104 and WB600 cells harboring each plasmid,

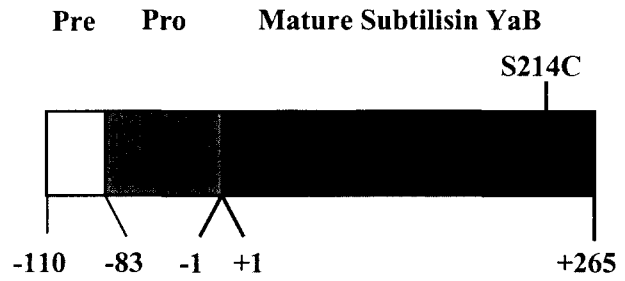

Fig. 1. Structure of wild type and mutant preprosubtilisin YaB gene products from pKK601 and pKK601-S214C. The black box represents mature subtilisin YaB of 265 residues. The hatched box indicates the pro-sequence of 83 residues. The white box represents the signal sequence of 27 residues. The residues were numbered from the amino-terminal residue of mature subtilisin (*+1*). In pKK601-S214C, Ser residue at position 214 of wild type subtilisin YaB in pKK601 is replaced with Cys.

were cultured in LB medium. When the optical density at 600 nm of the culture reached 0.5, 0.5 mM IPTG was added to induce the expression from the *spac-1* promoter. After 6 hr, the induced cells were collected by centrifugation, and proteins in the resulting supernatant, medium fraction, were analyzed by SDS-PAGE and subsequent Immunoblotting with a rabbit polyclonal antibody raised against subtilisin YaB. When proteins secreted into the culture medium of *B. subtilis* cells expressing mutant subtilisin YaB were analyzed, several protein bands that specifically cross-react with the anti-subtilisin YaB antibody were detected (Fig. 2). These mutant subtilisin gene products (leans 4 and 7) had higher apparent molecular masses than mature subtilisin YaB (26,7 kDa)(lanes 3, 6, and 8), indicating that the introduction of the active site mutation abolished, as expected, the ability of subtilisin to undergo autocatalytic cleavage of the pro-sequence. While the mutant subtilisin YaB gene product from WB600 cells showed a distinct band at 52 kDa (land 7), that from DB104 cells showed several bands with molecular masses ranging from 27 kDa to 36 kDa (land 4). Taken into consideration of the fact that WB600 lacks four additional extracellular proteases over DB104, the 52 kDa protein is the prosubtilisin YaB molecule secreted into the culture medium while the smaller ones are its proteolytic degradation products.

In vitro maturation of mutant prosubtilisin YaB with active subtilisin YaB. It is well known that pro-sequence can be processed from prosubtilisin *in trans* by exogenously added active subtilisin. To examine if it applies also for prosubtilisin YaB and to further confirm that the 52 kDa protein is the real prosubtilisin YaB molecule secreted into the culture medium, *in vitro* maturation assay was performed. For this purpose, the 52 kDa protein was incubated with a small amount of purified active subtilisin YaB (0.1 μg/mg). Then the maturation was examined by SDS-PAGE and subsequent immunoblotting with the anti-subtilisin YaB antibody (Fig. 3). Upon incubation with active subtilisin YaB,

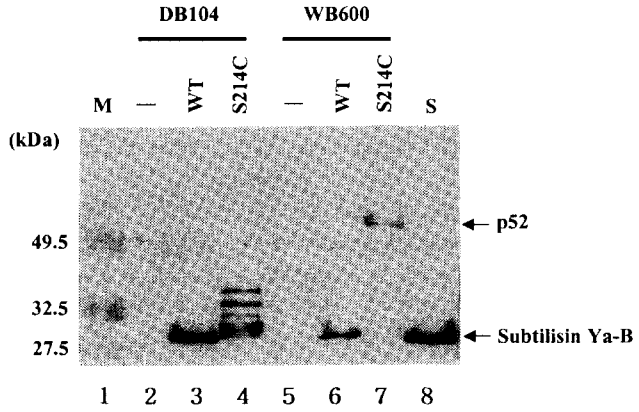

Fig. 2. Immunoblot analysis of the secreted proteins of *B. subtilis* producing wild type (*WT*) or mutant (*S214C*) subtilisin YaB. *B. subtilis* DB104 (lanes 2 to 4) and WB600 (lanes 5 to 7) harboring pTUBE1500 (lanes 2 and 5), pKK601 (lanes 3 and 6), or pKK601-S214C (lanes 4 and 7), were grown at 37℃ in LB medium. When the optical at 600 nm of the culture reached 0.5, 0.5 mM IPTG was added to the culture. After shaking at 37℃ for 6 hr, a portion of the culture was withdrawn and the medium proteins were separated on SDS-PAGE and subjected to an Immunoblotting using a rabbit anti-subtilisin YaB antibody. Lanes: 1, molecular mass standards (*M*); 8, purified subtilisin YaB (*S*). The positions of molecular mass standards are indicated on the left. The positions of the 52 kDa protein (*p52*) and mature subtilisin YaB are indicated by arrows on the right.

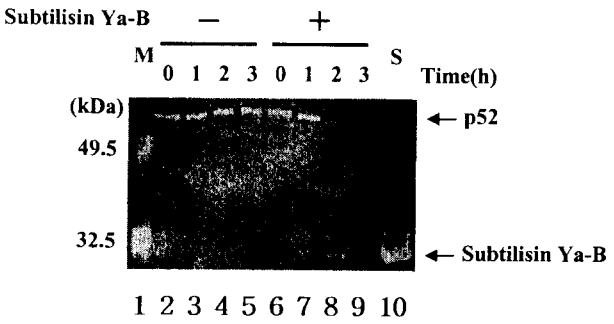

Fig. 3. *In vitro* maturation of pro-Ser213Cys-subtilisin YaB by active subtilisin YaB. Secreted prosubtilisin YaB (0.1 μg), obtained from the culture medium of the induced *B. subtilis* WB600 harboring pKK601-S214C, was incubated at 37℃, in the presence (lanes 6 to 9) or absence (lanes 2 to 5) of small amount of active subtilisin YaB (0.01 μg/ml), for the indicated times: 0 h (lanes 2 and 6), 1 h (lanes 3and 7), 2 h (lanes 4 and 8), and 3 h (lanes 5 and 9). Then the proteins in the each reaction mixture were analyzed by SDS-PAGE and subsequent immunoblotting with anti-subtilisin YaB antibody. Lanes: 1, molecular mass standards (*M*); 10, purified subtilisin YaB (*S*). The positions of molecular mass standards are indicated on the left. The positions of the 52 kDa protein (*p52*) and mature subtilisin YaB are indicated by arrows on the right.

the 52 kDa protein gradually disappeared to give a molecule of 26.7 kDa (leans 6 to 9), which completely coincides with

the mobility of mature subtilisin YaB (lane 10). On the other hand, without active subtilisin YaB, we could not detect any processing (lanes 2 to 5). From these results we concluded that the 52 kDa protein is the real secreted prosubtilisin YaB molecule that could be processed to mature size with the help of active subtilisin YaB.

DISCUSSION

Subtilisins are usually synthesized as preproenzyme which are later post-translationally activated to the active enzyme by the cleavage of propeptides [5-7]. Several lines of experiments, either *in vivo* or *in vitro,* have established the feature of propeptide as an intra-molecular chaperone which guide the folding of the mature portion into its active conformation prior to its autocatalytic cleavage [10,11]. In thais study we have addressed whether the pro-region of subtilisin YaB functions as a membrane anchorage [18] or not by expressing a mutant prosubtilisin YaB where the active site Ser of the wild type enzyme is replaced with Cys. Use of six extracellular protease-deficient strain [27] of *B. subtilis* was necessary for its efficient production. The prosubtilisin YaB thus produced was indeed secreted into the culture medium and was processed to its mature from upon treatment with active subtilisin YaB. The present result that the prosubtilisin YaB was really secreted into the culture medium inevitably shows that the processing of the pro-sequence is not required for its secretion, indicating clearly that the pro-sequence does not function as a membrane anchorage at least in the case of subtilisin YaB.

In addition to our *in vivo* results, many *in vitro* studies have shown that subtilisins can be folded to active enzymes without membrane [15-17,19]. From these results, we are thinking that membrane anchorage is not required even for the activation of the other subtilisins *in vivo.* Contrary to this notion, when to fusion proteins, comprising of subtilisin Carsberg prepro-region and either protein G or fibronectin binding protein, were expressed in *B. subtilis* [18], proteins with pro-sequence were recovered in membrane fraction, while proteins without pro-sequence were secreted into the culture medium. This observation led Egnell and Flock [18] to propose the membrane anchrage model already mentioned above. At present we do not know the reason for the discrepancy. Several explanation can be considered for it : the fusion proteins with pro-sequence in themselves tend to make insoluble aggregate and thus likely to be recovered in membrane fraction : or pro-sequence of subtilisin Carsberg functions indeed as a membrane anchrage while that of subtilisin YaB dose not. Further experiments are necessary to confirm this point.

The prosubtilisin YaB produced in this study showed an aberrant electrophoretic mobility. It migrated at an apparent molecular mass of 52 kDa which is 42 % greater than that predicted from its amino acid sequence (36.6 kDa). Such aberration has not been reported for any prosubtilisin. whether the polypeptide itself takes conformation that results in retardation of mobility or some modification, such as those described recently in *E.coli* [33], occurred on prosubtilisin YaB through the expression in *B. subtilis*, remained to be determined.

ACKNOWLEDGMENT

We thank Drs. Roy H. Doi and Fujio Kawamura for providing *B. subtilis* DB104, Dr. Sui-Lam Wang for *B. subtilis* WB600, and Drs. Kunio Yamane and Kouji Nakamura for plasmid pTUBE1500.

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