

Novel Processing and Localization of *catA*, *ccdA* Associated Thiol-Disulfide Oxidoreductase, in Protein Hyper-Producing Bacterium *Brevibacillus choshinensis*

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Abstract: Previously, we have cloned *ccdA* and its associated thiol-disulfide oxidoreductase gene, *catA*, in *Brevibacillus choshinensis*. CcdA is known to be an integral membrane protein and its associated oxidoreductase homologues are believed to be membrane anchoring proteins, both providing reducing equivalents across the membrane to control correct disulfide bond formation. Here, we found that CatA is first localized as a membrane bound form and then slowly released into the cellular periphery and culture medium with cleavage at a novel processing site.

Keywords: *Brevibacillus choshinensis* (*Bacillus brevis*), *ccdA*, *catA*, thiol-disulfide oxidoreductase, localization, processing.

INTRODUCTION

Brevibacillus choshinensis (formerly *Bacillus brevis*) was isolated as a protein-hyperproducing bacterium [1]. Several mammalian proteins such as human epidermal growth factor (hEGF, 2 g/liter) as well as several bacterial proteins (1.0-3.5 g/liter) have been successfully produced extracellularly in this bacterium [2-4]. We have previously cloned the genes *ccdA* and *catA* (*ccdA* associated thiol-disulfide oxidoreductase) [5]. We succeeded in the over-expression of CatA as a secretory form using the signal sequence of a *B. choshinensis* major cell wall protein [5]. Co-expression of CatA protein with recombinant hEGF in the *B. choshinensis* production system increased the yield of correct disulfide-bonded (native) hEGF [5]. Thus, CatA, most likely together with CcdA, provides reducing equivalents across the membrane to form correct disulfide bonds in extracellular proteins [5]. CcdA is a widely distributed thiol-disulfide oxidoreductase in archaea, bacteria, and eukaryotes functioning as an integral membrane protein [6]. CcdA-related CatA-like oxidoreductases, such as ResA, YkvV, YneN and HelX have been identified in several bacteria, and are believed to be amino-terminal-anchored membrane proteins (type I membrane proteins) [7, 8, 9]. CatA contains the amino-terminal sequence MNKLVLALLV₁₀TVGVGYAVWQ₂₀QPQESAA₂₇, which can be assigned as either a co-translationally processed signal peptide or a non-cleavable amino-terminal membrane anchor sequence. To resolve this problem, we (i) raised rabbit anti-CatA antiserum to localize CatA in *B. choshinensis* cellular fractions, (ii) prepared an *in vitro* synthesized precursor form of CatA, and (iii) overproduced intact CatA in *B. choshinensis* to determine its amino terminal amino acid sequence. Here we found that

~70% and ~15% of CatA was localized to the culture medium and cellular periphery, respectively, as a processed form, and the rest (~15%) was anchored in the membrane fraction as a precursor form. The processing site of the anchor sequence was found to be different from that predicted to be cleaved by the signal peptidase (SPase). Thus, it is likely that CatA was first anchored to the membrane as a type I membrane protein, and then processed post-translationally at a novel cleavage site and released into the cellular periphery and culture medium.

MATERIALS AND METHODS

Strains and Medium

B. choshinensis HPD31-S5 was cultured in TM medium [10]. For transformants, TM medium containing 50 µg/ml of Neomycin was used.

Fractionation of *B. choshinensis* Cells and Culture Medium

B. choshinensis HPD31-S5 and *B. choshinensis* HPD31-S5(pNYcatA) were grown in 5 ml of medium at 30 °C for 2 days, and the culture supernatant (cs) and cells were separated by centrifugation at 5,400 x g for 10 min. To remove any cell debris (csP), the culture supernatant was ultra-centrifuged at 150,000 x g for 1 hr (csS). Cells were suspended in 1 ml of ice-cold 50 mM Na phosphate buffer, pH 7.5, and disrupted by sonic oscillation for 3 min with a 30% pulse to prepare a crude cell homogenate (ch). Soluble cellular (chS) and membrane (chP) fractions were prepared from the crude homogenate by ultra-centrifugation at 150,000 x g for 1 hr.

Construction of intact CatA expression vector pNYcatA

The *B. choshinensis* expression vector pNY301 [11] was used to express intact CatA. A *catA* gene was amplified by

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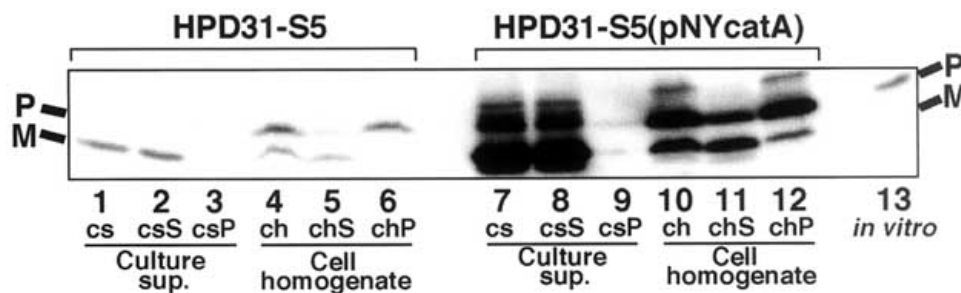


Figure 1. Cellular localization of precursor and mature forms of CatA. HPD31-S5 (lanes 1-6) and HPD31-S5(pNYcatA) (lanes 7-12) cells were fractionated, and CatA was detected by immuno-blotting using an ECL detection kit. The culture supernatant (cs) was ultracentrifuged (150,000 x g) to obtain supernatant (csS) and pellet (cell debris, if any. csP) fractions. The cell homogenate (ch) was ultracentrifuged (150,000 x g) to obtain cellular soluble (chS) and membrane (chP) fractions. Lane 13, unprocessed CatA synthesized *in vitro*. See details in Materials and Methods.

PCR with a forward primer, 5'-GGAAGGTCATGAACAAACTGGTGTGGCG-3', which contains a *Bsp*HI site (underlined) followed by the coding sequence starting at the start codon, and a reverse primer, 5'-CGACGGATCCTATGGTTTTTCATTCAAAAGCTTGC-3', which contains the coding sequence up to the termination codon followed by a *Bam*HI site (underlined). The amplified fragment was digested with *Bsp*HI/*Bam*HI, and ligated with *Bsp*HI/*Bam*HI-digested pNY301 to construct pNYcatA.

Preparation of Anti-CatA Antiserum

His-tag-CatA protein was purified from an *E. coli* BL21(DE3) transformant harboring pETcatA as described previously [5], and used to immune rabbits. Antiserum was obtained as described previously [12].

Other Methods

The thiol-disulfide oxidoreductase activity was assayed using insulin as a substrate according to Holmgren [13]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [14] and western blotting with an ECL detection kit (Amersham) were performed using methods described elsewhere [15]. The amount of protein was measured with the Lowry method [16].

RESULTS AND DISCUSSION

Cellular Fractionation and Subcellular Localization of CatA

We have previously succeeded in the cloning of *catA* from *B. choshinensis* [5]. To clarify the processing and subcellular localization of native CatA, here, we carried out the fractionation of *B. choshinensis* cells (culture medium, soluble cellular, and membrane fractions) and detection of CatA with immuno-blotting (see Materials and Methods). As shown in Figure 1, two CatA bands (P and M) were detected. About 90% of the fast migrating band (M) was detected in the culture medium (lanes 1 and 2), while almost all of the slow migrating band (P) was detected in the pellet of crude cell homogenate obtained by ultra-centrifugation (chP, membrane fraction, lane 6). In Figure 1, the cellular fractions were applied in 5-fold larger amounts than the culture supernatant fractions for SDS-PAGE. From these results, we

postulated that band P represents the precursor form of CatA and band M represents the processed mature form. About 10% of band M was detected in the soluble fraction of crude homogenate (lane 5); it is most likely localized at the periphery of the cells.

In Vitro Synthesis of Precursor Form of CatA

To confirm that band P represents the precursor form of CatA, the protein was produced *in vitro* using an *E. coli* protein synthesis kit (PURESYSTEM classic mini kit, Post Genome Institute Co., Ltd.) according to the manufacturer's instructions [17]. The product, synthesized with the whole *catA* gene as a template, migrated to the same position as band P (Figure 1, lane 13), indicating band P corresponds to the unprocessed precursor form of CatA.

Expression of Intact CatA in *B. choshinensis* and Its Oxidoreductase Activity

To examine the processing site of the CatA precursor, we attempted to determine the amino-terminal amino acid sequence of extra-cellular CatA. Since the amount of CatA secreted by HPD31-S5 cells was too small to determine the sequence, we constructed the expression vector pNYcatA (Figure 2) encoding an intact *catA* by preceded the P5

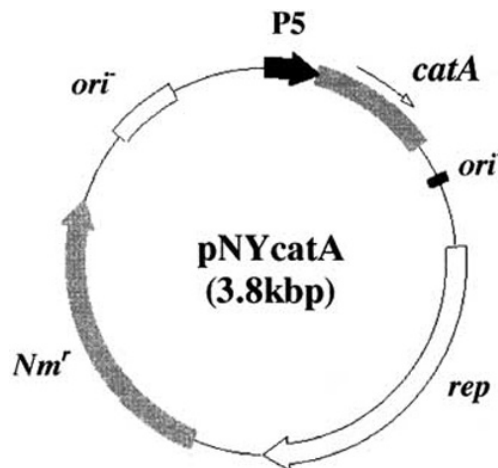


Figure 2. Schematic illustration of plasmid pNYcatA. P5, the fifth promoter of major cell wall protein; Nm^r, neomycin resistant marker.

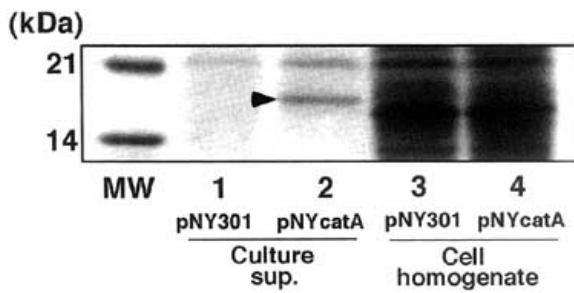


Figure 3. SDS-gel electrophoretogram of *B. choshinensis* HPD31-S5(pNY301) and HPD31-S5(pNYcatA). The protein band is stained with Coomassie blue and the CatA band is shown by an arrowhead. MW, molecular weight marker; lane 1, culture medium of HPD31-S5(pNY301); lane 2, culture medium of HPD31-S5(pNYcatA); lane 3, cell homogenate of HPD31-S5(pNY301); lane 4, cell homogenate of HPD31-S5(pNYcatA).

promoter of a major cell wall protein. As shown in Figure 3, lane 2, a new band (shown by an arrowhead), migrating at the same position as band M, was detected in the culture supernatant of HPD31-S5(pNYcatA) cells at about 30 µg/ml. This band was confirmed to be CatA by immuno-blotting (data not shown). This culture supernatant showed high thiol-disulfide oxidoreductase activity as shown in Figure 4. CatA amount in culture medium of HPD31-S5(pNY301) was estimated to be less than 1/10 of that of HPD31-S5(pNYcatA) by immuno-blotting (data not shown), and, therefore, enzymatic activity in control culture medium was too low to be detected within the sensitivity of this assay conditions (Figure 4). The localization of CatA overproduced in HPD31-S5(pNYcatA) (Figure 1, lanes 7-12) was essentially the same as that of HPD31-S5 cells (Figure 1, lanes 1-6). Interestingly, a small amount of the precursor form (band P) was detected in the culture supernatant (Figure 1, lanes 7 and 8), demonstrating that even with a membrane anchor sequence, the CatA precursor was released into the culture medium with leakiness most likely due to the overproduction of CatA.

Amino-Terminal Sequence of Secreted CatA

The CatA band in Figure 3, lane 2 was transferred onto a ProBlott membrane (Applied Biosystems), and the amino-

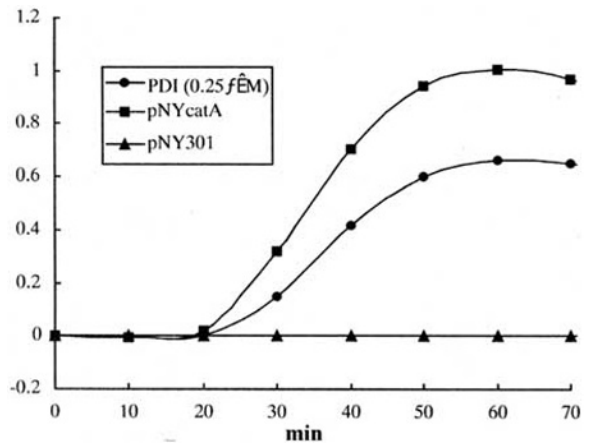


Figure 4. Thiol-disulfide oxidoreductase activity of secreted CatA. Culture supernatants (100 µl) of HPD31-S5(pNY301) (Figure 3, lane 1) and HPD31-S5(pNYcatA) (Figure 3, lane 2) were used for the activity assay. Bovine protein disulfide isomerase (PDI, Sigma P3818) was used as a positive control.

terminal sequence was determined. As shown in Figure 5, the anchor sequence was cleaved between Ala₂₇ and Val₂₈. This processing site was different from the one predicted by cleavage with SPase: SPase in Gram-positive bacteria was predicted to cleave CatA between Ala₂₆ and Ala₂₇ ([18], <http://www.cbs.dtu.dk/>). This general prediction for the cleavage site of SPase was supported by the secretome analysis of *Bacillus subtilis* [19]. The finding suggested that the anchor sequence was cleaved post-translationally by a putative protease which differed from SPase.

In conclusion, a thiol-disulfide oxidoreductase, CatA, probably functioning with CcdA integral membrane protein showed a novel processing and cellular localization. It is likely that CatA was first anchored to the membrane, and then processed post-translationally at a novel cleavage site and released into the cellular periphery and culture medium.

ACKNOWLEDGEMENTS

We are grateful to Matsujiro Ishibashi and Hiroko Tokunaga for helpful discussions.

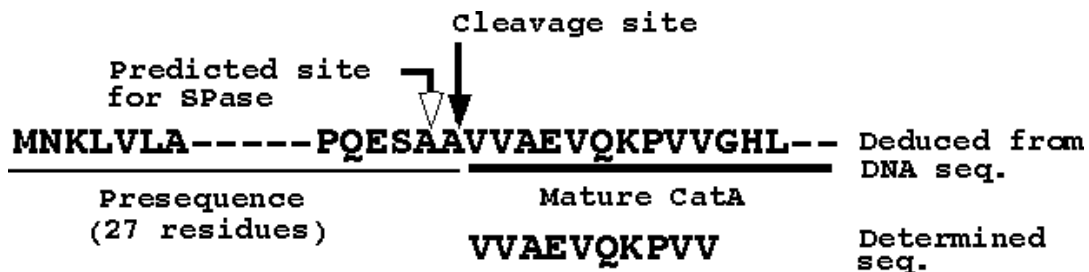


Figure 5. Sequencing of the amino-terminus of secreted CatA. The CatA band (Figure 3, lane 2) was transferred onto a ProBlott membrane, and after being cut out, its amino-terminal sequence was determined. Thin line, pre-sequence; thick line, mature CatA region. The white arrow shows the predicted cleavage site for signal peptidase (SPase), and the black arrow shows the cleavage site determined.

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Received on December 3, 2003, accepted on April 15, 2004.