Involvement of NH₂-Terminal Pro-sequence in the Production of Active Aqualysin I (a Thermophilic Serine Protease) in Escherichia coli

Young-Choon Lee, Yuko Miyata, Ichiro Terada,*
Takahisa Ohta and Hiroshi Matsuzawa

Department of Agricultural Chemistry, The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan
Received June 13, 1991

Aqualysin I is a heat-stable subtilisin-type protease produced by Thermus aquaticus YT-1. The precursor of aqualysin I consists of four domains: an NH₂-terminal signal peptide, an NH₂-terminal pro-sequence, a protease domain, and a COOH-terminal pro-sequence. In Escherichia coli cells harboring recombinant plasmid carrying the aqualysin I gene, proteolytic activity is obtained on treatment at 65°C and mature enzyme is detected. In the case of mutant genes containing partial deletions in the NH₂-terminal pro-sequence, no proteolytic activity was detected and the precursor protein was found to be unstable in E. coli. These results indicate that the NH₂-terminal pro-sequence is required to produce the active enzyme by stabilizing the precursor structure. Amino acid substitutions in the conserved sequence of the NH₂-terminal pro-sequence found among subtilisin-type proteases made the processing faster compared with the wild type.

Aqualysin I is a heat-stable serine protease (28-kDa protein) which is secreted into the culture medium by Thermus aquaticus YT-1, an extreme thermophile.1-4) Aqualysin I is a subtilisin-type protease, the primary structure of aqualysin I being about 40% identical with subtilisins.3) T. aquaticus YT-1 is a Gram-negative bacterium, and the cells are enveloped by two kinds of membranes, a cytoplasmic and an outer membrane.5) Aqualysin I is, therefore, secreted by translocation across the two membranes. Aqualysin I is produced as a large precursor (51-kDa protein) consisting of four structurally distinguishable domains; an NH₂-terminal signal peptide (14 amino acid residues), an NH₂-terminal pro-sequence (113 residues), the protease domain (281 residues), and a COOH-terminal pro-sequence (105 residues).6)

We constructed an expression plasmid for the gene encoding the aqualysin I precursor (aqul) in Escherichia coli, the aqul gene being expressed under the control of the tac promoter.6) When the aqul gene is expressed, a 38-kDa protein (a precursor of the mature enzyme with the COOH-terminal pro-sequence) is found in the membrane fraction, probably being bound to the outer membrane. On treatment at 65°C, the 38-kDa protein is processed into and solubilized as 28-kDa mature aqualysin I. Processing of the NH₂- and COOH-terminal pro-sequences is done by the proteolytic activity of aqualysin I itself, in that order.6)

Recently, the pro-sequence of subtilisin E has been shown to be necessary for the production of active protease and to be involved in the protein folding.7-9) The domain structure of the precursor of subtilisin E is different from that of aqualysin I; signal peptide and pro-sequence are present in the NH₂-terminal side of subtilisin E domain.10,11) Accordingly, the former contains only the NH₂-terminal pro-sequence and the latter

* Present address: Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama, kanagawa 227, Japan.
both NH$_2$- and COOH-terminal pro-sequences in the pro-enzyme. It would be interesting to know the respective functions of the two pro-sequences of aqualysin I and whether the NH$_2$-terminal pro-sequence of aqualysin I corresponds to that of subtilisin E in their function. In this study, we describe how the NH$_2$-terminal pro-sequence of aqualysin I is essential to produce enzymatically active protease like that of subtilisin E.

Materials and Methods

Bacterial strains and growth conditions. E. coli MV1184 was used as a host for expression of aqualysin I and propagation of phage M13. For efficient expression of aqualysin I, E. coli MV11 harboring recombinant plasmids was aerobically grown at 30°C overnight in the medium (pH 7.2) containing 1% Tryptone, 0.2% yeast extract, 0.5% NaCl, 1% glucose, and 100 µg/ml of ampicillin. The resultant culture was inoculated (3%) into the same medium and cultured at 30°C. After harvesting at $A_{660nm}$=0.8, the cells were suspended in modified 2H medium (2% Tryptone and 0.8% NaCl, pH 7.2) containing thiamine (5 µg/ml), ampicillin (100 µg/ml), and 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and then cultured at 37°C for 3 hr to induce the production of aqualysin I.

Gene engineering. Most of the methods used for gene engineering were based on those of Maniatis et al. DNA sequencing was done by the dideoxy chain termination method. Site-directed mutagenesis. The oligodeoxynucleotide for site-directed mutagenesis was synthesized with an Applied Biosystems 381A DNA synthesizer and monomers for its synthesis were purchased from Applied Biosystems, Inc. Site-directed mutagenesis was done with M13mpl9, using a Muta-Gene in vitro mutagenesis kit (Bio-Rad), as described by Kunkel et al. The entire region of the DNA fragment was sequenced to prove that only the mutation expected had occurred.

Enzyme activity measurements. The proteolytic activity of aqualysin I was assayed at 70°C by the same method as described previously. 2mm diisopropyl fluorophosphate at room temperature for 30 min and then fractionated by SDS-PAGE. Immunoblotting analysis of aqualysin I and its precursors was done with an antibody against aqualysin I, as described previously.

Pulse-chase experiments. Cells were grown at 30°C overnight with shaking in M9 minimal medium12) containing 0.5% glucose, 1 µg/ml thiamine, and 100 µg/ml ampicillin. The resultant culture was inoculated (2%) into the same medium containing 0.5% glycerol and cultured at 30°C until $A_{660nm}$=0.8, and then the expression of the aqul gene was induced by addition of 0.2 mM IPTG at 37°C for 1 hr. The cells were labeled with 30 µCi/ml of [35S]methionine (>1,000 Ci/mmol; Amersham) for 3 min. After a 0.5-ml sample was removed, radioactive incorporation was stopped by adding 200 µg/ml of unlabeled methionine. Sample (0.5 ml) were removed at various times during the chase. The reaction was stopped by mixing the samples with an equal volume of 10% ice-cold trichloroacetic acid and put at 0°C for more than 20 min.

Antibody precipitation. After pulse-chase experiments, [35S]methionine-labeled proteins cross-reacting with the anti-aqualysin I antibody were solubilized and precipitated by the method of Ito et al. For precipitating the cross-reacting proteins, formamid-fixed, heat-treated Staphylococcus aureus cells (Enzyme Center, Inc.) were used. The proteins were fractionated by SDS-PAGE, and the radioactivity was detected by fluorography.

Results

Construction of plasmids carrying mutant aqualysin I genes

To investigate the functions of the NH$_2$-terminal pro-sequence (113 amino acid residues) of aqualysin I precursor, plasmids carrying deletions in the pro-sequence-coding regions of pAQN were constructed with restriction sites and by site-directed mutagenesis. pAQNJM74 was constructed using Stul sites to delete 74 amino acid residues (amino acid residues -94 to -21 in the NH$_2$-terminal pro-sequence).

In the NH$_2$-terminal pro-sequences of subtilisin-type proteases, there is a conserved sequence consisting of six amino-acid residues, Tyr-Ile-Val-Xaa-Phe-Lys. A homologous sequence, Tyr-Ile-Val-Val-Phe-Lys (amino acid residues -73 to -68), is also found in the NH$_2$-terminal pro-sequence of aqualysin I. pAQNJM6 has a deletion of the conserved...
Involvement of N-Pro-sequence in Aqualysin I Production

Fig. 1. Construction of Mutants in the NH$_2$-Terminal Pro-sequence of Aqualysin I Precursor.

PAQN is a derivative of pAQI. In the coding region of aqualysin I precursor (the *aql* gene), new restriction sites, AccI (nucleotide 141 from initiation codon; ref. 6), BamHI (nucleotide 503), and BglII (nucleotide 966), were introduced, and XmaIII and KpnI sites became unique by removing another site (nucleotide 531 and nucleotide 592, respectively) present in pAQI, by site-directed mutagenesis. These mutations were done so as not to cause any change of the encoded amino acid residues. PAQN has pUC ori and ampicillin-resistance gene. The *aql* gene is under the control of the tac promoter and lacP'. pAQI was constructed using Stul sites (nucleotides 98 and 320) to delete the coding sequence for amino acid residues -94 to -21 in the NH$_2$-terminal pro-sequence.

Deletion mutants in the NH$_2$-terminal pro-sequence of aqualysin I precursor

Induced E. coli cells harboring PAQN, pAQI$_{ΔN74}$, and pAQI$_{ΔN6}$ were treated at 65°C for up to 4 hr. After removal of denatured E. coli proteins by centrifugation, proteolytic activity in the supernatant was measured (Fig. 2). Remarkable proteolytic activity was observed only for PAQN after heat treatment for 2 hr and thereafter, and no significant proteolytic activity was detected for pAQI$_{ΔN74}$ and pAQI$_{ΔN6}$.

In the supernatant examined for proteolytic activity, the 28-kDa protein corresponding to mature aqualysin I purified from the culture medium of *T. aquaticus* was found for PAQN, as reported previously, but not for pAQI$_{ΔN74}$ and pAQI$_{ΔN6}$ (data not shown) by Coomassie Brilliant Blue staining after SDS-PAGE. As shown in Fig. 3, in the induced cells, anti-aqualysin I antibody-reactive 38-kDa protein was detected for PAQN (lane 1), which is pro-aqualysin I with the COOH-terminal pro-sequence as described previously. Mature aqualysin I (28-kDa protein) was not detected by immunoblotting as shown, though the reason is unknown. As for pAQI$_{ΔN74}$ (lane 3) and pAQI$_{ΔN6}$ (lane 2), however, no antibody-reactive proteins were detected at all.

Accordingly, detection of the gene product derived from the NH$_2$-terminal deletion sequence. The wild-type and mutant aqualysin I genes were expressed under the control of the tac promoter in E. coli MV1184 cells, like pAQI as described previously.

Deletion mutants in the NH$_2$-terminal pro-sequence of aqualysin I precursor

Induced E. coli cells harboring pAQI, pAQI$_{ΔN74}$, and pAQI$_{ΔN6}$ were treated at 65°C for up to 4 hr. After removal of denatured E. coli proteins by centrifugation, proteolytic activity in the supernatant was measured (Fig. 2). Remarkable proteolytic activity was observed only for pAQI after heat treatment for 2 hr and thereafter, and no significant proteolytic activity was detected for pAQI$_{ΔN74}$ and pAQI$_{ΔN6}$.

In the supernatant examined for proteolytic activity, the 28-kDa protein corresponding to mature aqualysin I purified from the culture medium of *T. aquaticus* was found for pAQI, as reported previously, but not for pAQI$_{ΔN74}$ and pAQI$_{ΔN6}$ (data not shown) by Coomassie Brilliant Blue staining after SDS-PAGE. As shown in Fig. 3, in the induced cells, anti-aqualysin I antibody-reactive 38-kDa protein was detected for pAQI (lane 1), which is pro-aqualysin I with the COOH-terminal pro-sequence as described previously. Mature aqualysin I (28-kDa protein) was not detected by immunoblotting as shown, though the reason is unknown. As for pAQI$_{ΔN74}$ (lane 3) and pAQI$_{ΔN6}$ (lane 2), however, no antibody-reactive proteins were detected at all.

Accordingly, detection of the gene product derived from the NH$_2$-terminal deletion sequence. The wild-type and mutant aqualysin I genes were expressed under the control of the tac promoter in E. coli MV1184 cells, like pAQI as described previously.
Fig. 2. Effects of Deletion in the NH$_2$-Terminal Pro-sequence on Aqualysin I Production.

_E. coli_ cells harboring pAQN (●), pAQNΔN74 (▲) and pAQNΔN6 (□) were induced by IPTG for 3 hr. Cells were collected by centrifugation, suspended in 50 mM Hepes–NaOH buffer (pH 7.5) containing 10 mM CaCl$_2$, and then treated at 65°C for the indicated times. After removal of denatured _E. coli_ proteins by centrifugation, proteolytic activity in the supernatant was measured at 70°C using casein as substrate, as described previously, and is shown as units per ml of culture.

mutant was attempted by pulse-labeling with [35S]methionine and the stability of the product was investigated by a chase experiment. Figure 4 shows that the 48-kDa precursor of the active-site Ser mutant (pro-aqualysin I with both NH$_2$- and COOH-terminal pro-sequences) directed by pAQN (S222A) was stable for more than 40 min. This precursor reacts with the anti-aqualysin I antibody as reported previously. pAQN (S222A) directed also the synthesis of 43-kDa protein which was stable during the pulse-chase experiment (Fig. 4). However, this protein can not be detected by immunoblotting. The reason why such a protein was detected by pulse-labeling is unknown.

As for pAQNΔN74, 43-kDa protein was synthesized in the induced cells, and the protein was almost completely degraded in approximately 10 min during the chase (Fig. 4). The molecular mass, 43 kDa, is close to that after the deletion of 74 amino acid residues from the 51-kDa entire large precursor on calculation. These results indicate that the deletion mutants in the NH$_2$-terminal pro-sequence of aqualysin...
Involvement of N-Pro-sequence in Aqualysin I Production

I precursor are unstable in E. coli cells and easily degraded by E. coli proteases, so that no proteolytic activity is detected.

Effects of amino acid replacements in the conserved sequence of the NH₂-terminal pro-sequence of aqualysin I

The results described above for pAQNΔN6 suggest that the amino acid sequence of the conserved region is important to produce active aqualysin I. Therefore, five mutants containing single amino-acid replacements in the conserved sequence were constructed by site-directed mutagenesis (Fig. 1). Proteolytic activity was observed for every mutant after treatment at 65 °C, like wild-type (Fig. 5). While 2-hr heat treatment was required for the wild type, 30-min or 1-hr incubation was enough for the mutants to obtain high activity, especially V-70T, K-68R, and K-68E mutants being promptly activated.

Discussion

No proteolytic activities were observed for the deletion mutants in the NH₂-terminal pro-sequence (Fig. 2). The precursor of the deletion mutant was detected by pulse-labeling with [³⁵S]methionine, but the labeled precursor was degraded soon during the chase with cold methionine (Fig. 4). These results indicate that the NH₂-terminal pro-sequence of aqualysin I is essential for the production of enzymatically active aqualysin I and functions to stabilize the precursor structure. Probably the NH₂-terminal pro-sequence of aqualysin I precursor is required to guide the folding of a stable conformation against digestion by proteases. Subtilisin E⁷⁻⁹ and α-lytic protease¹⁹ also required the NH₂-terminal pro-sequence to produce active protease. The function of the pro-sequences would be similar in these proteases.

There is only one conserved sequence among the NH₂-terminal pro-sequences of subtilisin-type proteases, the sequences being Tyr-Ile-Val-Val-Phe-Lys for pro-aqualysin I,⁶ Tyr-Ile-Val-Gly-Phe-Lys for pro-subtilisins,¹⁷ and Tyr-Ile-Val-Lys-Phe-Lys for pro-proteinase K.¹⁸ The deletion of the conserved sequence of pro-aqualysin I resulted in the complete loss of the production of active aqualysin I (Fig. 2), and the detection of its precursor by pulse-labeling experiment was unsuccessful (data not shown). On the other hand, the replacements of not only a non-conserved residue (Val) but also a conserved residue (Lys) did not affect the production of active enzyme, or rather made the processing faster (Fig. 5). These results suggest that the conserved sequence is involved in the production of a stable precursor conformation and the processing of the precursor.

In the case of subtilisin E, the pro-sequence is shown to guide folding of the enzymatically active conformation in vivo⁷¹ and in vitro⁸⁻⁹ at normal temperatures. In the case of aqualysin
I, enzymatically active aqualysin I was obtained on treating the induced E. coli cells at 65°C for at least 2 hr (Fig. 2). This indicates that the conformation of the protease domain in the stable aqualysin I precursor produced at 37°C is different from the enzymatically active conformation. The inability of the protease domain to form an active conformation at 37°C is independent of the presence of the COOH-terminal pro-sequence, since the heat treatment was also required for the complete deletion mutant of the COOH-terminal pro-sequence to obtain active aqualysin I (unpublished data). It would be intriguing to know what are the conformational differences between the protease domain of the inactive precursor and the active protease.

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to H. M.

References