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Role of Proline Residues in Conferring Thermostability on Aqualysin I

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To understand the molecular basis of the thermostability of a thermophilic serine protease aqualysin I from *Thermus aquaticus* YT-1, we introduced mutations at Pro5, Pro7, Pro240 and Pro268, which are located on the surface loops of aqualysin I, by changing these amino acid residues into those found at the corresponding locations in VPR, a psychrophilic serine protease from *Vibrio* sp. PA-44. All mutants were expressed stably and exhibited essentially the same specific activity as wild-type aqualysin I at 40°C. The P240N mutant protein had similar thermostability to wild-type aqualysin I, but P5N and P268T showed lower thermostability, with a half-life at 90°C of 15 and 30 min, respectively, as compared to 45 min for the wild-type enzyme. The thermostability of P7I was decreased even more markedly, and the mutant protein was rapidly inactivated at 80°C and even at 70°C, with half-lives of 10 and 60 min, respectively. Differential scanning calorimetry analysis showed that the transition temperatures of wild-type enzyme, P5N, P7I, P240N and P268T were 93.99°C, 83.45°C, 75.66°C, 91.78°C and 86.49°C, respectively. These results underscore the importance of the proline residues in the N- and C-terminal regions of aqualysin I in maintaining the integrity of the overall protein structure at elevated temperatures.

Key words: proline, serine protease, subtilase, thermostability.

Abbreviations: AAPF, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; DSC, differential scanning calorimetry; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; MES, 2-(*N*-morphlino)ethane sulfonic acid; MOE, The Molecular Operating Environment; ODA, oligonucleotide-directed dual amber; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; VPR, proteinase from *Vibrio* PA-44.

Elucidating the molecular basis of protein thermostability is important from both basic and practical standpoints, since it would eventually enable us to construct genetically engineered proteins that could work at much higher temperatures than their naturally occurring counterparts.

Members of the subtilisin-like serine protease family seem promising for studies of this issue. Proteases in this family are closely related proteins in which an active serine residue is intimately involved in the catalytic cycle (*1*). They show 35–40% identity in overall amino acid sequence and remarkable conservation of the arrangement of amino acids in the active site. Moreover, they share a similar three-dimensional structure with an α/β protein scaffold. Despite their structural similarity, the temperature-stability of serine proteases varies widely, with the enzymes being psychrophilic, mesophilic, thermophilic or hyperthermophilic according to the characteristics of the organisms from which they are obtained. For instance, aqualysin I from *Thermus aquaticus* YT-1, which is an extremely thermophilic Gram-negative bacterium, shows high homology with subtilisins (37–39%) and proteinase K (43%), and is optimally active at about 80°C (*2, 3*). In contrast, *Vibrio* proteinase from psychrotrophic *Vibrio* PA-44 (VPR), which is homologous to psychrophilic proteinase from *V. alginolyticus* (86% similarity) (*4*), is also homologous to aqualysin I (60% similarity), but its optimum temperature is about 45°C (*5*). Comparison of the amino acid sequences of these related enzymes would provide us with useful information about the roles of specific amino acid residues in conferring thermostability on a protein.

The aqualysin I gene was cloned from the Gram-negative thermophilic bacterium *T. aquaticus* YT-1 and expressed in *Escherichia coli* (*6*). The mature protein is processed from a precursor through cleavage of both N- and C-terminal peptide fragments, and the location of the N- and C-terminal aqualysin I pro-sequences as well as their function in producing the mature protein have been analysed (*7–9*). Aqualysin I is classified into the proteinase K family, which consists of a group of Gram-negative-bacteria-derived proteinases within the subtilase superfamily, based on analysis of its sequence homology with more than 100 subtilases (*10*). Aqualysin I has four cysteine (Cys) residues, Cys66, Cys99, Cys163 and Cys194, that participate in 2 disulfide bonds, Cys66–Cys99 and Cys163–Cys194, and is active up to about 80°C (*11, 12*). Introduction of disulfide bonds

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into subtilisin and other related serine proteases at the positions where they are found in aqualysin I improved the thermostability of subtilisin (*13–15*). It was also noted that a serine protease from the extremely thermostable eubacterium *Aquifex pyrophilus* has eight Cys residues, as compared to four in aqualysin I, in the putative mature form (*16*). Based on these observations, it was once assumed that disulfide bonds at appropriate locations play an important role in determining the thermostability of serine proteases by contributing to maintaining the properly folded structure.

However, cloning and characterization of a subtilisin-like serine protease named VPR from a psychrotrophic *Vibrio* sp. raised serious questions about this hypothesis. The enzyme showed cold-adapted properties, having higher catalytic efficiency under mesophilic conditions and lower thermal stability as compared with the related mesophilic proteinase K from fungus *Tritirachium album* Limber (*5, 17*). Interestingly, VPR also has four Cys residues, and 2 disulfide bonds, Cys67–Cys99 and Cys163–194, that are positioned very similarly to those in aqualysin I and in *V. alginolyticus* proteinase (*4*). Moreover, pyrolysin from hyperthermophilic archaea *Pyrococcus furiosus* and stetterlysin from extremely thermophilic archaea *Thermococcus stetteri* have no Cys residues (*18*). These results suggest that the disulfide bonds in aqualysin I, VPR and other subtilases may have little, if any, relevance to the thermostability of these enzymes.

It was reported recently that when a proline residue located in a conserved region of α-glucosidases from plant species and in plant β-glucanase was substituted by another residue in barley α-glucosidase (*19*) and barley β-glucan endohydrolase (*20*), the enzymes acquired increased thermostability. These observations drew our attention to the role of proline residues in stabilizing the structure of bacterial serine proteases. In this work we compared the amino acid sequences of aqualysin I and VPR, and found four proline residues which are replaced by other amino acid residues in VPR, and are located in putative surface loops of aqualysin I as inferred from three-dimensional homology modelling using proteinase K as a template. This prompted us to investigate whether these surface proline residues contributed to eliciting and/or preserving the activity of aqualysin at high temperature.

MATERIALS AND METHODS

Bacterial Strains and Growth Media—E. coli TG1 {*supE, hsd*Δ5, *thi*, Δ(*lac-proAB*)/F′ [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]} and MV1184 {*ara*, Δ(*lac-proAB*), *rpsL*, *thi*(Φ80*lacZ*ΔM15), Δ(*srl-recA*) 306::Tn10 (*tet*^{r})/F′ [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15] were used as an expression host and a gene-engineering host, respectively.

LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, pH 7.0) was used for pre-culture and growth of bacteria. Solid medium contained bacto-agar (1.5%). Ampicillin (50–100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (50 μg/ml) were added to the medium when needed.

Construction of Expression Plasmid and Mutant Plasmids—pAQNΔC105, with an insert coding for aqualysin I, with a signal peptide and an N-terminal prosequence, but without a *C*-terminal prosequence, was a kind gift from Prof. H. Matsuzawa at the Department of Bioscience and Biotechnology, Aomori University. Part of the 5′-terminal sequence of the aqualysin I gene was modified by site-directed mutagenesis with the ODA–PCR method (Mutan®-Super express Km; TaKaRa), using pAQNΔC105 as a template and an oligonucleotide 5′-ACA ACC ACC CAA AAC CAT **GAA TTC** GAA AAG CGC CAT CA-3′, as a primer, where mismatched bases are underlined and letters in boldface represent an *Eco* RI recognition site. The *Eco* RI-*Hin* dIII fragment of the PCR product was inserted into pMAL vector (New England Biolabs, Inc.), and an expression vector, pMAQ-c2Δ, was constructed to produce a fusion protein with maltose binding protein.

To construct plasmids with a mutated aqualysin I gene, site-directed mutagenesis was carried out using pMAQ-c2Δ as a template according to the same procedure explained earlier. Oligonucleotide primers used for site-directed mutagenesis are shown below with mismatched bases underlined:

P5N; 5′-GCTACCCAGAGCAACGCTCCTTG-3′,
P7I; 5′-GCCCGGCTATTTGGGGCCTG-3′,
P240N; 5′-GTATCTAGAGCAAAATAACTCGGCTACGC CGGCC-3′,
P268T; 5′-GAGACGGTTGGTGGACCCCG-3′.

Fragments with an appropriate mutation, P5N, P7I, P240N and P268T, were trimmed at both ends with restriction enzymes, *Eco* RI-*Bam* HI, *Eco* RI-*Hin* dIII, *Eco* RI-*Hin* dIII and *Bam* HI-*Hin* dIII, respectively, and reinserted into an expression vector, pMAQ-c2Δ, to give plasmids with mutant aqualysin I genes. These plasmids were designated pMAQ-P5N, pMAQ-P7I pMAQ-P240N and pMAQ-P268T, respectively. The nucleotide sequences around the mutation site as well as other parts of the gene were confirmed by DNA sequencing.

Gene Engineering and Chemical Reagents—Gene engineering experiments were carried out essentially according to Sambrook *et al*. (*21*). Enzymes for gene engineering were purchased from TaKaRa and Boehringer-Mannheim, and used according to the manufacturer's instructions. Other reagents used were of the highest quality available from Wako Pure Chemicals (Tokyo) and Sigma Chemicals (St. Louis).

Purification and Activity Measurement of Wild-type and Mutant Aqualysin I—After induction by 0.2 mM IPTG (isopropyl β-D-thiogalactopyranoside) at $OD_{660} = 0.8$, the transformants were cultivated overnight in LB medium. The cells were harvested by centrifugation and sonicated, and the crude extract was subjected to heat treatment (70°C, 1 h), hydrophobic chromatography (Butyl sepharose; Amersham Biotech) with a linear gradient of ammonium sulphate from 40% to 0% saturation, and cation exchange chromatography (Resource S; Amersham Biotech) with a 0–1.0 M NaCl linear gradient. The enzyme was purified to homogeneity through these steps to give a single band on SDS-PAGE

on staining with CBB R-250 (*22*). Enzyme activity was measured at 40°C with *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF) as a substrate in 50 mM HEPES-NaOH (pH 7.5) containing 1 mM $CaCl_2$. The change in absorbance at 410 nm was continuously monitored, and the activity was estimated with $\varepsilon_{410} = 8680\ M^{-1}\ cm^{-1}$ as a molar extinction coefficient. One unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of *p*-nitroaniline from the substrate in 1 min. In cases of using casein as a substrate, 20 μl of enzyme solution at an appropriate concentration was added to 980 μl of 1% casein (Wako) and incubated for an appropriate period of time, and the reaction was stopped by adding 1500 μl of 5% trichloroacetic acid (TCA) solution. The mixture was further incubated for 30 min at room temperature, then centrifuged at 15,000×*g* for 30 min at 4°C, and the proteolytic activity was estimated by measuring the absorbance of the supernatant at 280 nm. One unit of enzyme, using casein as a substrate, was defined as the amount of enzyme that causes an increase of 0.002 absorbance unit at 280 nm/min. Protein concentration was measured with the micro-assay method (BioRad), which is based on the Bradford method (*23*), using bovine serum albumin (BSA) as a standard.

Determination of Temperature Dependence of Proteolytic Activity and Heat Stability of Aqualysin I—To examine the temperature dependence of the enzyme activity, 2450 μl of 1% casein solution (50 mM Tris-Cl, pH 8.5) was pre-incubated at an appropriate temperature for 5 min, and then 50 μl of enzyme solution was added. The enzyme reaction was stopped at appropriate times (0.5–5 min) by transferring a 500-μl aliquot of the reaction mixture into a tube containing 750 μl of 5% TCA solution as stop solution. After a 30 min incubation at room temperature, the mixture was centrifuged and the activity was estimated from A_{280} of the supernatant as described earlier.

To examine the heat stability, the enzyme was diluted with 20 mM MES-NaOH buffer (pH 6.0, 1 mM $CaCl_2$) to give a 50 μg/ml solution. The enzyme solution was incubated for an appropriate time at various temperatures (70 ~ 100°C), and then was cooled quickly. The remaining activity was measured with 3 mM AAPF as a substrate at 40°C as described earlier.

Differential Scanning Calorimetry (DSC) Analysis—For DSC analysis, purified wild-type aqualysin I and mutants were treated with 25 mM phenylmethane sulfonyl fluoride (PMSF) in MeOH for 30 min to prevent autolytic degradation during the calorimetric measurements. After complete inactivation of the protease activity was confirmed, the samples were dialysed overnight against 20 mM phosphate buffer (pH 7.4) containing 1 mM $CaCl_2$ and filtered through a nitrocellulose filter (0.45 μm pore size). DSC measurements were kindly performed at Nihon SiberHegner K.K. using VP-Capillary DSC Platform system (MicroCal) at 0.5–1.0 mg/ml protein concentration under the condition as follows: Scan Rate, 250°C/hr; Scan Temp, 25-110°C; Feedback Mode/Gain, High; Filter Period, 2 sec; Pre-scan Thermostat, 15 min.

RESULTS

Identification of Surface Proline Residues Present in Aqualysin I but not in Vibrio Proteinase—Figure 1 shows the alignment of the amino acid sequence of aqualysin I with those of five related serine proteases in the proteinase K family. Comparison of the amino acid sequences between aqualysin I and the psychrophilic *Vibrio* proteinase revealed that 5 out of 11 proline residues in the former are replaced by other amino acid residues in the latter. A homology modelling study with 'The Molecular Operating Environment' (MOE; Chemical Computing Group Inc.) using proteinase K as a template told us that four of these, namely Pro5, Pro7, Pro240 and Pro268, are located on putative surface loops of aqualysin I. To examine whether these surface proline residues in aqualysin I contributed to eliciting and/or preserving its activity at high temperature, we constructed 4 mutants, P5N, P7I, P240N and P268T, in which proline residues at position 5, 7, 240 and 268, respectively, in aqualysin I were replaced by amino acid residues at the corresponding positions in the *Vibrio* proteinase. All the mutants examined were expressed in *E. coli* and were stable in the bacterial cells (data not shown).

Purification of Wild-type Aqualysin I and its Mutants—Wild-type aqualysin I was purified as described under 'Materials and Methods'. Mutant aqualysin I-constructs, pMAQ-P5N, pMAQ-P7I, pMAQ-P240N and pMAQ-P268T, which were derived from pMAQ-c2Δ, were expressed and the protein products were purified to homogeneity in essentially the same way as wild-type aqualysin I, except that P7I and P240N required longer heat-treatment (2 h) than the wild-type enzyme (1 h) for the processing of their N-terminal prosequences (Fig. 2).

Activity of Wild-type Aqualysin I and its Mutants with AAPF as a Substrate—The activity of wild-type aqualysin I and its mutants was measured with 3 mM AAPF as a substrate at 40°C. The specific activity of these enzymes ranged from 0.13 to 0.15 U/μg, showing that all the mutants had almost the same activity as wild-type aqualysin I at 40°C. These results indicate that the substitution of these Pro residues in aqualysin I did not affect the specific activity of the enzyme at 40°C and strongly suggest that the proper conformation of the active site is conserved in the mutant enzymes.

Kinetic Parameters of Wild-type Aqualysin I and its Mutants—The kinetic parameters of wild-type aqualysin I and its mutants with AAPF as a substrate are shown in Table 1. While the K_m values of wild-type aqualysin I and all the mutants were almost the same, the k_{cat} values of the P5N and P7I mutants were smaller than the others, and were about 70% of that of wild-type aqualysin I.

Temperature-dependence of the Activity of Wild-type Aqualysin I and its Mutants—Figure 3 compares the temperature-dependence of the activity of wild-type aqualysin I and its mutants. Wild-type aqualysin I showed full activity of casein hydrolysis in the range of 80–100°C. The P5N and P7I mutants exhibited maximum activity at 80°C, and the activity was gradually decreased at temperatures higher than that. The optimal temperature for these mutants was

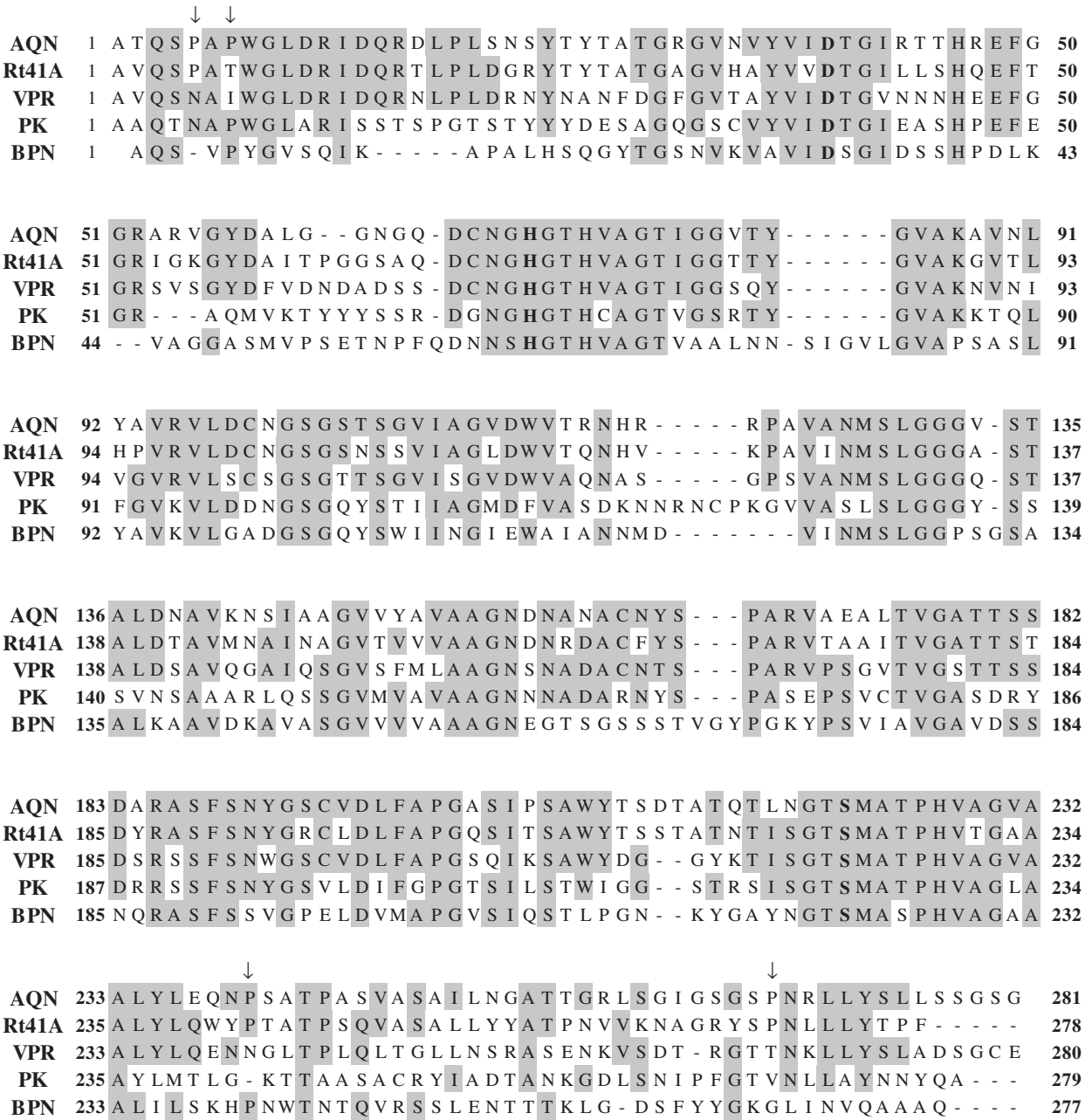

Fig. 1. **Alignment of amino acid sequences of related serine proteases in the proteinase K family.** AQN, aqualysin I; Rt41A, serine protease from *Thermus* sp. Rt41A; VPR, protease from *Vibrio* sp. PA-44; PK, proteinase K from fungus *Tritirachium album* Limber; BPN, subtilisin BPN′ from *Bacillus amyloliquefaciens*. Catalytic residues, Asn, His, and Ser, are shown in boldface. Residues occupying positions corresponding to Pro5, Pro7, Pro240 and Pro268 are indicated by arrows (↓). The optimal temperatures for AQN, Rt41A, VPR and PK are 80°C, 90°C, 45°C and 55°C, respectively.

definitely lower than that for wild-type aqualysin I, but was still much higher than that of VPR (45°C). The caseinolytic activity of P240N and P268T mutants showed rather complicated temperature dependences which are difficult to interpret at present, but they were as active as the wild-type enzyme even at 100°C. The activity of P7I was much lower than those of the wild-type enzyme and other mutants at temperatures above 80°C.

Thermostability of Wild-type Aqualysin I and its Mutants—The activity of wild-type aqualysin I and mutants remaining after heat treatment at temperatures ranging from 70 to 100°C was determined with AAPF as a substrate. Figure 4 shows that wild-type aqualysin I and P240N displayed very similar thermostability. They retained about 60% of the initial activity even after 120 min treatments at 70°C, but at 90°C and 100°C they were inactivated with a half-life of about

Table 1. **Kinetic parameters of wild-type aqualysin I and its mutants.**[a]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
Wild type	98.1 ± 2.75	0.69 ± 0.04	141.6 ± 9.34
P5N	72.1 ± 2.86	0.75 ± 0.06	96.1 ± 9.69
P7I	66.5 ± 2.03	0.74 ± 0.03	91.3 ± 2.78
P240N	85.2 ± 3.76	0.79 ± 0.05	107.6 ± 7.74
P268T	88.7 ± 5.91	0.72 ± 0.06	128.8 ± 12.83

[a]Parameters were determined at 40°C with AAPF as substrate.

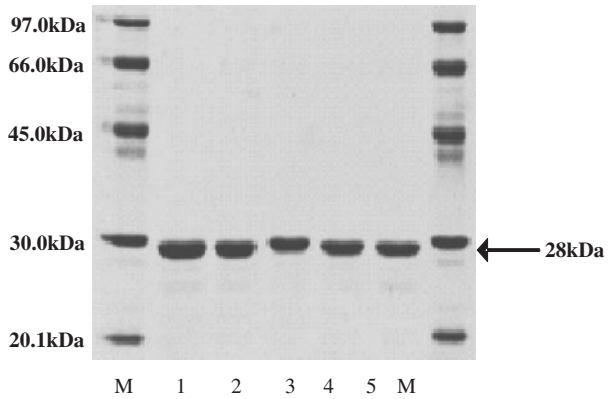

Fig. 2. **SDS-PAGE of wild-type aqualysin I and its mutants.** Wild-type and mutant enzymes purified as described in the text were pre-treated with a 100-fold molar excess of PMSF for 1.5 h at room temperature and analysed by SDS-polyacrylamide gel electrophoresis according to Laemmli (*22*) using a 12% polyacrylamide gel. Lane 1, wild-type aqualysin I; lane 2, P5N; lane 3, P7I; lane 4, P240N; lane 5, P268T; lane M, Low Molecular Weight Marker (molecular mass; 20.1, 30.0, 45.0, 66.0, 97.0 kDa, Amersham Biotech).

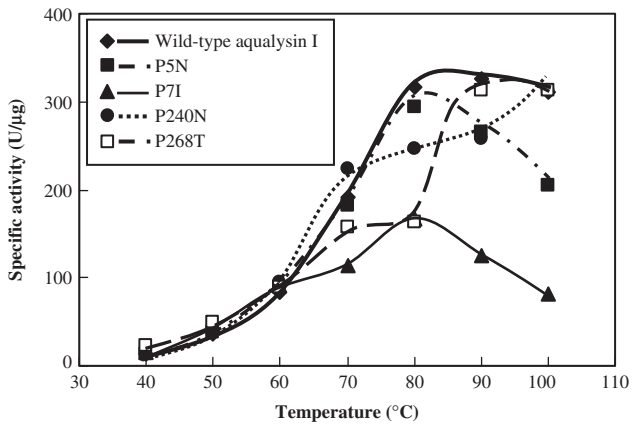

Fig. 3. **Temperature-dependence of activity of wild-type aqualysin I and its mutants.** Temperature-dependence of the activity was measured as described in 'Materials and Methods' with casein as substrate. ◆ —, ■ ---, ▲ — ● ·····, and □ – – represent the activity of wild-type aqualysin I, P5N, P7I, P240N and P268T, respectively.

45 and 5 min, respectively. This indicates that Pro240 does not contribute much to conferring thermostability on aqualysin I, while results on other mutants revealed that Pro268, Pro5 and Pro7 were contributing to the themostability of the enzyme to varying extents as described

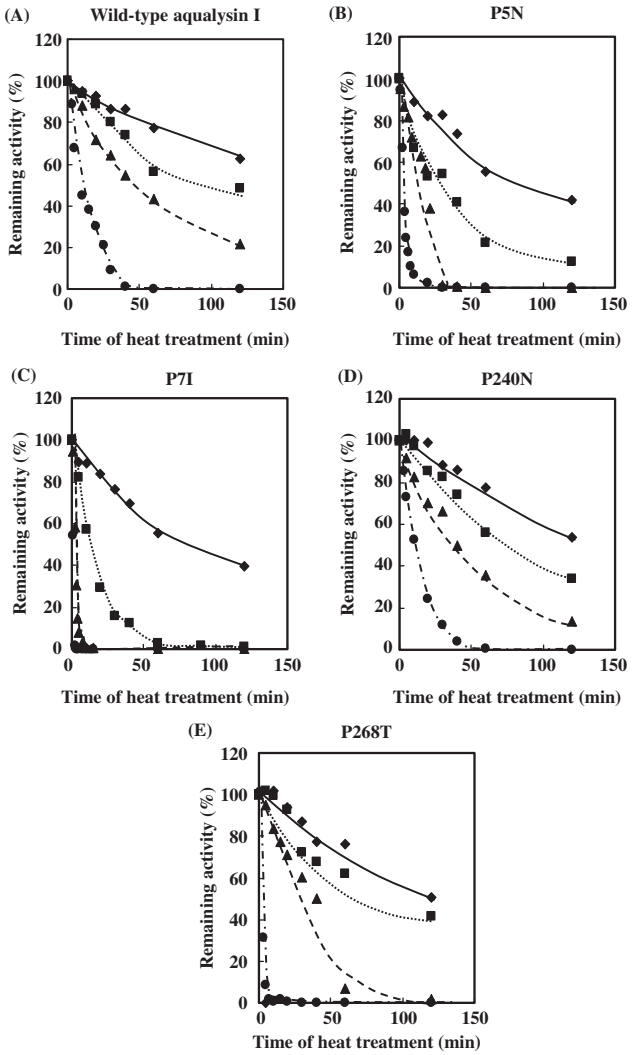

Fig. 4. **Heat stability of wild-type aqualysin I and its mutants.** Heat stability was determined as described in 'Materials and Methods'. Remaining activity after heat treatment at 70°C (◆), 80°C (■), 90°C (▲) and 100°C (●) for the indicated time periods relative to that before heat treatment of wild-type aqualysin I (A), P5N mutant (B), P7I mutant (C), P240N mutant (D) and P268T mutant (E) is plotted.

subsequently. The P268T mutant was almost as stable as the wild-type aqualysin I at 70°C and 80°C, but considerably more labile than that at higher temperatures, and its half-life at 90°C and 100°C was ~30 min and less than a few minutes, respectively. P5N was significantly more thermolabile than the wild-type aqualysin I at every temperature tested. At 80°C and 90°C, the P5N mutant was inactivated much faster than the P268T mutant, with a half-life of about 25 and 15 min, respectively. The P7I mutant was the most labile among the mutants examined in this study, and an extensive loss of its activity within 120 min was noted even at 70°C (half-life at 70°C; about 60 min). The half-life of P7I at 80°C, at which other mutants were as stable as the wild-type aqualysin I was only about 10 min. These results strongly suggest that

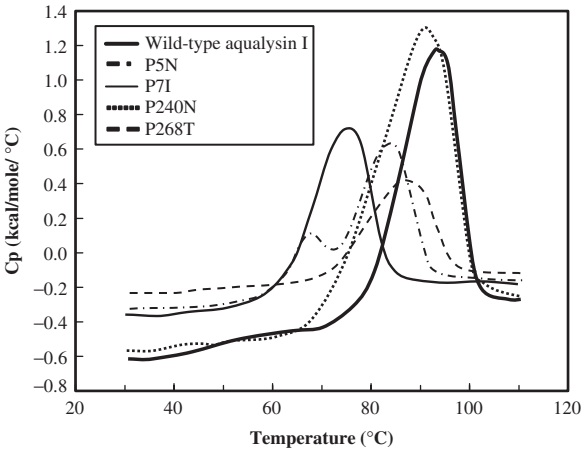

Fig. 5. **DSC analysis of wild-type aqualysin I and its mutants.** The experimental conditions are described in 'Materials and Methods'. Wild-type aqualysin I (—), P5N mutant (-··-), P7I mutant (—), P240N mutant (·····) and P268T mutant (- - ·).

the Pro7 residue was of prime importance for the thermostability of aqualysin I, the importance followed by Pro5 and Pro268 in decreasing order.

Differential scanning calorimetry was carried out on wild-type aqualysin I and mutants in the presence of 1 mM $CaCl_2$ after treatment with PMSF to prevent their autolysis (Fig. 5). The transition temperature of P240N (91.78°C) was only slightly lower than that of wild-type enzyme (93.99°C), while the values for P268I (86.49°C) and P5N (83.45°C) were considerably lower than that for the wild-type enzyme, indicating a significant contribution of these residues to the thermostability, More importantly, the transition temperature of P7I (75.66°C) was markedly lower than that of the wild-type aqualysin I, again emphasizing the importance of Pro 7 in the thermostability of aqualysin I.

DISCUSSION

Serine proteases show widely different thermostability, with psychrophilic, mesophilic, thermophilic and hyperthermophilic enzymes found among them. Although serine proteases have different optimal temperatures and thermostability, their three-dimensional structures are very similar to each other. Thermostable enzymes that are optimally active at much higher temperatures than mesophilic and psychrophilic enzymes must have structural elements that make them more resistant to unfolding at elevated temperatures. Structural elements determining the stability of a given enzyme must be found in its specific amino acid sequence. Proline residues in a surface loop of a protein may be among the factors that contribute to protect the protein from thermal unfolding by limiting the flexibility of the loop structure. They would render the loop more rigid by restricting the flexibility of the peptide backbone, and contribute to maintaining the proper enzyme structure at elevated temperature. Recent reports on plant glucosidases seem to support this notion (*19, 20*).

Alignment of the amino acid sequence of aqualysin I with that of VPR revealed that mature VPR had 6 proline residues, all of which were conserved in aqualysin I, while aqualysin I had five additional proline residues that were not present in VPR. We selected four of these five residues to analyse the possible function of proline residues in stabilizing the structure of the thermophilic serine protease, and constructed mutants P5N, P7I, P240N and P268T in which a proline in aqualysin I was replaced by the amino acid located at the corresponding position in VPR. These proline residues are found in a putative surface loop, not in the catalytic site, based on the predicted three-dimensional structure of aqualysin I.

All the mutants examined were stably expressed in *E. coli*, and, after the removal of the N-terminal prosequences by heat treatment for appropriate time periods, were almost as active as wild-type aqualysin I at 40°C with AAPF as a substrate. This indicates that the replacement of proline at these positions did not profoundly affect the three-dimensional structure of the enzyme at 40°C. However, the various mutant enzymes showed distinct behaviours at higher temperatures. In particular, the P7I mutant was quite unstable at higher temperatures, so that even at 80°C, at which the other mutants as well as wild-type aqualysin I maintained half activity up to 60 min, the activity of this mutant was rapidly decreased. This result agrees with the results of DSC analysis that gave a transition temperature of 75.66°C for P7I, which is 18.33°C lower than that for wild-type aqualysin I. The P5N mutant was also considerably less stable than the wild-type enzyme, and significant inactivation was noted at 80°C, at which the wild-type enzyme was stable. The P268T mutation had an even smaller but still definite effect on the thermostability of the enzyme, in that it was inactivated more rapidly than the wild-type enzyme at temperatures above 90°C. The thermostability of the P240N mutant was practically indistinguishable from that of wild-type aqualysin I. These results underscore the importance of Pro7 in rendering aqualysin I thermostable, although possible labilization due to substitution of a hydrophilic amino acid residue by a hydrophobic one may not be dismissed. Pro 5 also makes a smaller, and Pro268 an even much smaller, contribution to the stability of the enzyme.

The results with P7I and P5N mutants strongly suggest that the maintenance of the integrity of the N-terminal structures is important for the proper conformation of aqualysin I, and that the rigidity of the N-terminal loop conferred by Pro7 and, to a lesser extent by Pro5, plays an important role in this conformational stabilization. It was reported recently that replacement of Pro5 in subtilisin BPN′, which corresponds to Pro7 in aqualysin I, by a serine residue led to a 1.2-fold increase in the half-life of subtilisin BPN′ at 60°C. Almog *et al*. (*24*) pointed out that Ser5 forms five hydrogen bonds, two of them involving the side chain hydroxyl group, one to the main chain amide of Gly7 and the other to the buried side chain of His226. On the other hand, Pro5 in subtilisin BPN′ is able to contribute only one hydrogen bond to protein folding. It is likely that the new hydrogen bonds generated as a result of the mutation are among

the primary factors contributing to the increased thermostability of subtilisin BPN′ (*24*). Substitution of proline by hydrophilic amino acids rather than hydrophobic ones may compensate for the disadvantage caused by proline substitution because of the capacity of hydrophilic amino acid residues to make favourable hydrogen bonds with their surroundings. It is interesting that Rt41A, a thermophilic serine protease from *Thermus* sp. Rt41A, has a threonine residue at position 7 instead of proline (Fig. 1) (*25, 26*).

Three-dimensional structure modelling analysis suggested that the mutations at Pro7 and Pro5 resulted in shortening of an α-helical structure in the N-terminal region as compared with that of wild-type aqualysin I, and in addition, that three hydrogen bonds (G156-S189, N157-189, and S203-N219) were lost in the structure of P7I (data not shown). These alterations might have led to a reduction in the ability to maintain a proper enzyme structure at higher temperatures and to a low thermostability and decreased activity of the mutant enzyme at elevated temperatures.

Frenken *et al*. (*27*) proposed that substitution of amino acid residues by proline increased the proteolytic resistance (proline concept), and Markert *et al*. (*28*) indicated that the replacement of Ala20 and Ser21 in a protease-sensitive loop of bovine pancreatic ribonuclease A for Pro rendered the loop protease-resistant by three orders of magnitude. In the case of aqualysin I the replacement of Pro7 by Ile did not affect proteolysis susceptibility greatly. SDS-PAGE analysis revealed that P7I mutant was definitely more susceptible to autolysis than wild-type enzyme at both 70°C and 80°C, but the susceptibility was not more than 2–3-fold different (data not shown). It seems more important that DSC analysis demonstrated that P7I mutant had a transition temperature of 75.66°C that was lower than that of wild-type aqualysin I by 18.33°C. It should be noted that complications due to autolysis were eliminated in this experiment. This clearly indicates that Pro7 is indispensable for the maintenance of active conformation of aqualysin I at elevated temperatures. It may be also added that the wild-type aqualysin I could be refolded to regain full activity after 5h in 6M guanidinium chloride, while P7I mutant recovered only 50% of its initial activity after 2.5 h in 6 M guanidinium chloride at 40°C (data not shown). Conformational stabilization conferred by properly positioned proline residues as revealed through this study may represent an additional facet of the proline concept.

In addition to the importance of stable N-terminal surface loops for stabilization of the protein structure of aqualysin I as discussed earlier, we also observed that P268T substitution resulted in a small but definite destabilization of aqualysin I at higher temperatures. This indicates a significant contribution of the Pro residue located in the C-terminal surface loop to the thermostabilization of the enzyme. In a study on subtilisin ALP1 from alkalophilic *Bacillus*, Yamagata *et al*. (*29*) found that 2 mutations introduced in the C-terminal region of ALP1 were most effective for improving the stability against heat, surfactants and high alkalinity, and they pointed out, on the basis of three-dimensional modelling, that the stabilization of the molecular surface structure was effective for improving the stability of proteolytic enzymes. These observations strongly suggest that stabilization of C-terminal loop structures may as well be important in improving the stability of proteases. The functional importance of the N- and C-terminal loops for stabilizing the overall protein structure of aqualysin I should be carefully examined further in future studies.

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