

Temperature, organic solvent and pH stabilization of the neutral protease from *Salinivibrio proteolyticus*: significance of the structural calcium

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In order to clarify the impact of Ca-binding sites (Ca1 and 2) on the conformational stability of neutral proteases (NPs), we have analyzed the thermal, pH and organic solvent stability of a NP variant, V189P/A195E/G203D/A268E (Q-mutant), from *Salinivibrio proteolyticus*. This mutant has shown to bind calcium more tightly than the wild-type (WT) at Ca1 and to possess Ca2. Q-mutant was resisted against autolysis, thermoinactivation and pH denaturation in a Ca-dependent manner and exhibited better activity in organic solvents compared to the WT enzyme. These results imply that Ca1 and Ca2 are important for the conformational stability of NPs. [BMB reports 2011; 44(10): 665-668]

INTRODUCTION

Neutral proteases (NPs) are zinc metalloproteases with biotechnological applications such as aspartame and peptide synthesis (1, 2). They contain 1 to 4 calcium ions critically important for their thermostability (3-5); the thermophilic NPs such as thermolysin (TLN) comprise four calcium binding sites (Ca1 to Ca4), while the lower stable ones such as *Pseudomonas aeruginosa* elastase has single calcium binding site (Ca1). Ca1, Ca2 and Ca4 are located in an extended surface region in the vicinity of the active site cleft but Ca3 is far from it. In spite of convincingly clear information about the impact of Ca3 in thermostability of NPs (6, 7), a number of examples exist in which this trend is not followed. For instance, our studies have recently been indicated that four point mutations (Val189Pro/Ala195Glu/Gly203Asp/Ala268Pro), located around Ca1, resulted in the remarkable im-

provements of activity and thermostability of a mesophilic NP from *Salinivibrio proteolyticus* (SVP), which advanced thermostability was due to a high binding affinity of Ca1 to Ca²⁺ and binding of an extra Ca²⁺ to Ca2 (8). Moreover, Ala195Glu, Gly203Asp, Ala268Pro and Ala195Glu/Ala268Pro mutants provided superior activity and stability in water miscible organic solvents (9). Here, thermal and pH stability and organic solvent tolerance of constructed quadruple mutant (Q-mutant) SVP was studied and compared with that of the wild-type (WT) to assess the importance of the extended surface region and its Ca-binding properties in stability. The results showed that improving the calcium binding properties causes a dramatic increase in the thermal stability, organic solvent tolerance and pH stability. Finally, this region was found to be critical for the conformational stability of NPs.

RESULTS AND DISCUSSION

Following preliminary characterization, four substitutions including Val189Pro, Ala195Glu, Gly203Asp and Ala268Pro were combined among a series of amino acid substitutions, and the resulting Q-mutant was analyzed in comparison with the WT SVP (8).

Autolysis has known as the major mechanism responsible for the irreversible inactivation of NPs (10). The stabilizing mutation can therefore prevent local unfolding and consequently hinder the autolysis. Fig. 1 illustrates the mutational stabilization towards autolysis at 70°C. Apparently, after incubation at 70°C for 5 min, the WT enzyme was digested to a much greater extent than the stabilized Q-mutant.

To determine the irreversible denaturation, both enzymes (WT and Q-mutant) were heated for different times at 65 and 70°C and then assayed for residual activity at 60°C. According to the results, the mutant showed a high tendency for retaining its activity at 65°C while the WT enzyme almost completely inactivated after 50 minutes. Data seemed to fit the first order enzyme deactivation model with a rate constant of 0.001 min⁻¹ for Q-mutant and 0.034 min⁻¹ for the WT enzyme.

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Stability divergence was also noticeable at 70°C as inactivation rates of 0.010 min⁻¹ and 0.104 min⁻¹ was obtained for Q-mutant and WT, respectively. Collectively, the thermal inactivation studies are consistent with the autolysis data indicating that limited mutations greatly increased the thermostability of SVP. As clearly indicated in Fig.1b and c, thermoinactivation of the Q-mutant is Ca-dependent, while the inactivation of the WT enzyme is Ca-independent. Thus, further stability of mutant SVP can be explained by the tight calcium binding at Ca1 as well as creation of an extra calcium binding site (Ca2) in the surface region between residues 187-228. Although the activity of thermostabilized enzymes often reduces at low and moderate temperatures, the Q-mutant activity was strikingly higher than the WT at all temperatures (8).

Inactivation of NPs in organic solvents restricts their industrial applications (11). However, Q-mutant seems to overcome this limitation. Activity measurements in the presence of 40% (V/V) of dimethylformamide (DMF) and isopropanol in-

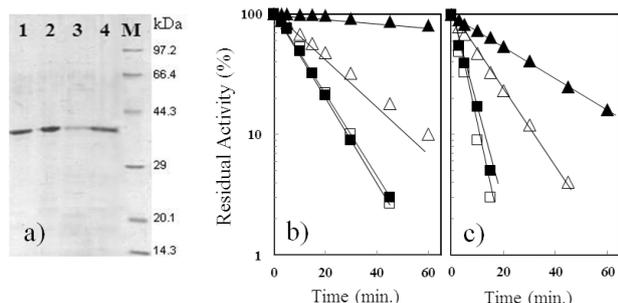


Fig. 1. Thermoinactivation of WT and Q-mutant SVPs. (a) Autolysis of wild-type and quadruple mutant SVP at 70°C. Autolysis investigated by incubation of enzymes (0.2 mg/ml) in 20 mM Tris-HCl buffer, pH 8.5 containing 10 mM CaCl₂. After 5 minutes incubation at 70°C, an aliquot of the proteolysis mixture was analyzed by SDS-PAGE. The molecular weights of the fragments corresponding to digestion of enzymes and marker (M) are indicated. (1) WT enzyme (0 min.), (2) WT enzyme (5 min.), (3) Q-mutant (0 min.), (4) Q-mutant (5 min.). (b and c) thermoinactivation rates of the enzyme variants at 65°C (b) and 70°C (c). WT enzyme in the absence of calcium (□), and in the presence of 10 mM CaCl₂ (■), Q-mutant in the absence of calcium (△), and in the presence of 10 mM CaCl₂ (▲). Standards deviations were within 5% of experimental values.

dicated that Q-mutant can tolerate high concentrations of organic solvents (Fig. 2). These results demonstrate that the mutant is even more active than its industrial counterpart, TLN. Moreover, relative activities increased in the presence of calcium especially for Q-mutant (Fig. 2). Thus the integrity of Q-mutant's active site Ca-dependently preserved at higher concentration of organic solvents. For both enzymes, the loss of enzymatic activity in the presence of organic solvents was accompanied by a decrease in the amount of intact protein due to autolysis (data not shown). Solvent concentrations in which 50% of enzyme activity remains (C₅₀) are summarized in Table 1. Furthermore, these data elucidate that the Q-mutant can provide superior Ca-dependent activity in organic solvents compared to that of the WT and TLN. Stability of each enzyme in organic solvents was estimated by measuring the remaining activity after incubation in 40% (V/V) DMF and isopropanol at 50°C. As expected, the Q-mutant half-life was remarkably higher than the WT especially in the presence of calcium (Table 1).

pH stability further illustrated the potential of the engineered SVP. After 60 minutes incubation at room temperature, the remaining activities at 60°C are shown in Fig. 3 in which the activity measured at pH 8.0 is taken as 100%. It was discovered that amino acid substitutions do not alter the optimal pH of the

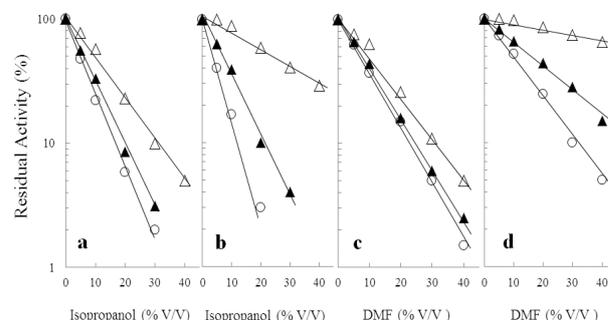


Fig. 2. Effect of calcium on the activity of the WT and Q-mutant SVPs and TLN in 0 to 40% (V/V) of organic solvents. The activities of WT SVP (△), Q-mutant SVP (▲) and TLN (○) at 50°C and pH 8.5 were measured in the absence of CaCl₂ (a and c), and in the presence of 10 mM CaCl₂ (b and d). Standard deviations were within 6% of experimental values.

Table 1. Stability and C₅₀ values of enzyme variants in organic solvents. Half-lives (min) of WT and Q-mutant SVPs and TLN were calculated in 40% (V/V) of organic solvents. C₅₀ values* of WT and Q-mutant SVPs and TLN were indicated in parentheses

Organic solvent	WT		Q-mutant		TLN	
	No Ca	10 mM CaCl ₂	No Ca	10 mM CaCl ₂	No Ca	10 mM CaCl ₂
DMF	8 (8)	15 (16)	10 (15)	73 (65)	ND (8)	ND (12)
Isopropanol	6 (7)	7 (7)	10 (9)	25 (25)	ND (5)	ND (6)

*C₅₀ is the value of the solvent concentration where 50% of enzyme activity remains. Relative C₅₀ were determined as ((C₅₀ value of mutant - C₅₀ value of SVP)/C₅₀ value of SVP) × 100. ND: not determined.

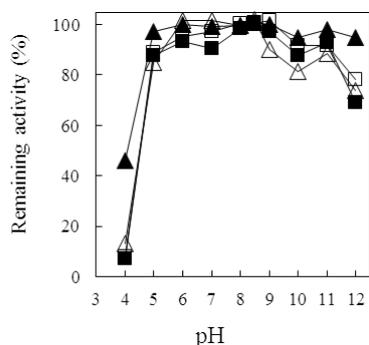


Fig. 3. pH stabilities of WT and Q-mutant SVPs at a broad pH range. Stabilities were measured at room temperature. Purified enzymes were incubated for 60 min at room temperatures, and the remaining activities were determined at 60°C after 25 times dilution in 100 mM Tris buffer, pH 8.5. WT enzyme in the absence of calcium (□), and in the presence of 10 mM CaCl₂ (■), Q-mutant in the absence of calcium (△), and in the presence of 10 mM CaCl₂ (▲). Standards deviations were within 5% of experimental values.

enzyme activity (data not shown). The pH stabilities of the variants were largely identical ranging from 6 to 8.5. However, the stability of Q-mutant at acidic and basic pH values far surpassed that of WT. For instance, the remaining activity of the mutant at pH 4 and 12 increased ~40 and 25% compared to the WT, respectively (Fig. 3). Although calcium did not significantly affect the pH stability of the WT enzyme, it strongly influenced the mutant stability. The remaining activity of the mutant increased ~35 and 20% in the presence of calcium at pH 4 and 12. The Ca-dependent stabilization of Q-mutant at both acidic and basic conditions proposes that calcium contributes to the structural integrity at extreme pHs.

This is the first report on the crucial role of Ca1 and Ca2 in NPs adaptation to different conditions. We evidently indicated that improved calcium binding and creation of a new Ca-binding site not only increase the protein resistance to thermal denaturation, but also improve the active site geometry and protein motions that are required for activity in organic solvents and extreme pHs. This is of practical significance for developing more effective or ideal NPs for industrial applications such as peptides and aspartame precursor synthesis. This work provides new insights into the structural basis underlying the conformational stability of the NPs although a further research is required to assess the role of Ca1 and Ca2.

MATERIALS AND METHODS

Genetics

Cloning, subcloning, and expression were performed as described previously (8). Site-directed mutagenesis was performed by a method described by Fisher and colleagues (12). The nucleotide sequences of mutated SVP gene were verified by MWG, Germany.

Purification of enzymes

Extracellular SVPs were purified as described previously (8). For purification to homogeneity, extracellular SVPs were partially purified in one step on Q-Sepharose column equilibrated with 20 mM Tris-HCl, pH 8.5. Partially purified SVPs were finally loaded on a Sephacryl S-200 gel filtration column, equilibrated with the same buffer containing 0.3 M NaCl and the active fractions were used. All the purification steps were performed at 4°C. Homogeneity of the purified enzymes was confirmed by the single band on SDS-PAGE. Protein concentration was also measured according to the method of Bradford using bovine serum albumin as the standard.

Enzyme activity

The specific activity (product formation/mg enzyme per minute) was determined in 20 mM Tris-HCl (pH 8.5), 0 or 10 mM CaCl₂ and 1.25% (w/v) casein. The assay solution (480 µl) was pre-incubated at specified temperature for one minute, followed by adding 20 µl of suitably diluted enzyme to initiate the reaction. The reaction was terminated after 8 min by adding 500 µl of 10% (w/v) trichloroacetic acid, kept at room temperature for 30 min and then centrifuged at 14,000×g (Hermle 236HK, Germany) for 10 min. The absorbance was measured against a blank at 280 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine in 1 min at 60°C. In organic solvents, protease activity was determined using FAGLA, as a synthetic substrate of NPs as described previously (9).

Determination of thermal stability

For the determination of half-lives and inactivation kinetics, 0.2 mg/ml purified proteases in 20 mM Tris-HCl, pH 8.5 containing 0 or 10 mM CaCl₂ were incubated at 60, 65 and 70°C for differing times, samples were snap-cooled in an ice-water bath and then assayed for residual caseinolytic activity at 60°C. Plots of the log of residual activity versus time were linear, indicating a first-order decay process under these conditions.

Organic solvent stability

Purified proteases in 50 mM Tris-HCl, pH 7, containing 40% (V/V) of organic solvent were incubated at 50°C for different times (9). Samples were removed at various time intervals, and subsequently, half lives were determined as described above.

Determination of pH stability

The pH stabilities of purified enzymes were identified using 50 mM glycine (pH 2.2-3.6 and pH 8.6-10.6), 50 mM succinic acid (pH 3.8-6.0) and phosphate buffer (5.8-8.0 and 9.6-11.8). 200 µl of the enzyme solutions with different pHs incubated for 60 min at room temperatures. The remaining activities were determined at 60°C after 25 times dilution in 100 mM Tris buffer, pH 8.5 by standard method.

All measurements were performed in at least three independent experiments. The deviations from the averages which are

shown in the figures were <5%.

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