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EFFECT OF THE WEAK Ca^{2+}-BINDING SITE OF SUBTILISIN J BY SITE-DIRECTED MUTAGENESIS ON HEAT STABILITY

Jeong Su Jang, Kwang Hee Bae, and Si Myung Byun*

Department of Life Science,
Korea Advanced Institute of Science and Technology,
373-1 Kusong-Dong, Yusung-Gu, Taejon 305-701, Korea

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SUMMARY: The functional role of the negatively charged amino acid residue in subtilisin J from *Bacillus stearothermophilus* has been investigated by site-directed mutagenesis. Glu-195 located at the weak Ca^{2+}-binding site was replaced with Gln to examine the role of Glu-195 in the heat stability of subtilisin J. Mutant enzyme was expressed in *Bacillus subtilis* and was purified from the culture supernatant. When the mutant enzyme was expressed at 37°C in the presence of 2mM calcium chloride, the pattern of enzyme production was quite different from that of wild-type. The purified Gln-195 mutant enzyme was analyzed with respect to optimal temperature, optimal pH, and heat stability. The mutation was found to decrease the heat stability but not catalytic efficiency (k_{cat}/K_m) and optimal pH. These results demonstrate the important role of the negatively charged side chains at the weak Ca^{2+}-binding site in the heat stability of subtilisin.

Ca^{2+} is one of the most common metal ions found associated with proteins playing a more or less well-defined functional role in their biological activity (1, 2). A subtle and less-obvious involvement of Ca^{2+} has been observed with some protease (3, 4), where the cation is neither directly engaged in the catalytic mechanism nor in the substrate-recognition process, but has some influence on enzyme activity. In the proteases, the presence of Ca^{2+} slows down autolysis, and enhances thermal stability.

Subtilisin, an alkaline serine protease produced by a variety of *Bacillus* species (5), has two Ca^{2+} ion binding sites (6, 7). One (site A) binds Ca^{2+} with high affinity and is located near the N-terminus. The other (site B) appears to bind Ca^{2+} and other cations much more weakly and is located 32 Å away.

We previously cloned and expressed the gene of *B. stearothermophilus* subtilisin J in *B. subtilis* (8). This paper describes the role of the Glu-195 at the weak Ca^{2+} ion binding site in the heat stability.

*To whom correspondence should be addressed.

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MATERIALS AND METHODS

The site-directed Glu-195 → Gln mutation was introduced into the *aprJ* gene from *B. stearothermophilus* using PCR (9), and the mutation was confirmed by dideoxy chain-termination sequencing (10) on the M13mp19. The mutation of Glu-195 to Gln-195 was performed with a oligonucleotide primer having the sequences 5'-GCAGGTTCTC*AGCTG*GATGTGATG-3' (asterisks show the location of mismatches and the underlined sequence shows the position of a new *Pvu*II site). The mutant gene was expressed in the plasmid pZ124(Km^r) and the mutant protein purified from the culture supernatant as described previously (8). The enzyme was assayed in a solution containing 0.3 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide and 0.1 M Tris-HCl (pH 8.6) at 25°C. The assay measured the increase in absorbance at 410 *nm* per min due to hydrolysis and release of the *p*-nitroaniline (ε_{410} = 8,480 $M^{-1}cm^{-1}$) (11).

RESULTS AND DISCUSSION

Glu-195 located at the weak Ca^{2+} binding site is highly conserved amino acid residue between subtilisin family, especially for the subtilisins from *Bacillus* sp. (Fig. 1). To investigate the role of Glu-195 in the heat stability, Glu-195 was replaced with Gln using PCR. Mutant subtilisin gene was expressed in, and subtilisin was secreted from the *B. subtilis* strain DB104 (Fig. 2). As shown in Fig. 2, the hallow formed by Gln-195 mutant subtilisin was nearly same as the wild-type subtilisin.

To investigate the effect of Ca^{2+} ion on the protease production, *B. subtilis* DB104 carrying mutant subtilisin gene was cultivated at 37°C in the presence of 2 mM, 5 mM, and 10 mM calcium chloride, respectively. Figure 3 shows the enzyme production patterns of the enzyme per unit cell. In the presence of 2 mM $CaCl_2$, the Gln-195 mutant shows the bell-shaped form while the wild-type appears to be increased with time. This may be due to autolysis. But the enzyme production increases with time in the presence of 5 mM or 10 mM $CaCl_2$ as wild-type subtilisin does.

To characterize the enzymatic properties of Gln-195 subtilisin, the mature enzyme was purified from the culture supernatant to a single band by SDS-polyacrylamide gel electrophoresis. The wild-type enzyme was also purified to homogeneity from *B. subtilis* harboring plasmid pZS101 and examined as a control. Kinetic constants, k_{cat} and K_m, were determined from the initial rate measurements for the hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Table I). As shown in Table I, the replacement of Glu-195 with Gln-195 was found to slightly increase the catalytic rate constant. And the catalytic efficiency, k_{cat}/K_m of Gln-195 mutant subtilisin J is about 1.5 times higher than that of wild-type subtilisin J. This result indicates that the side-chain of Gln-195 mutant located near the surface of the molecule apart from the substrate binding pocket has little effect on the catalytic function of the enzyme.

The thermostabilities of the purified enzymes are shown in Fig. 4. In the case of the wild-type subtilisin J, increasing [Ca^{2+}] was found to decrease the rate of irreversible thermal

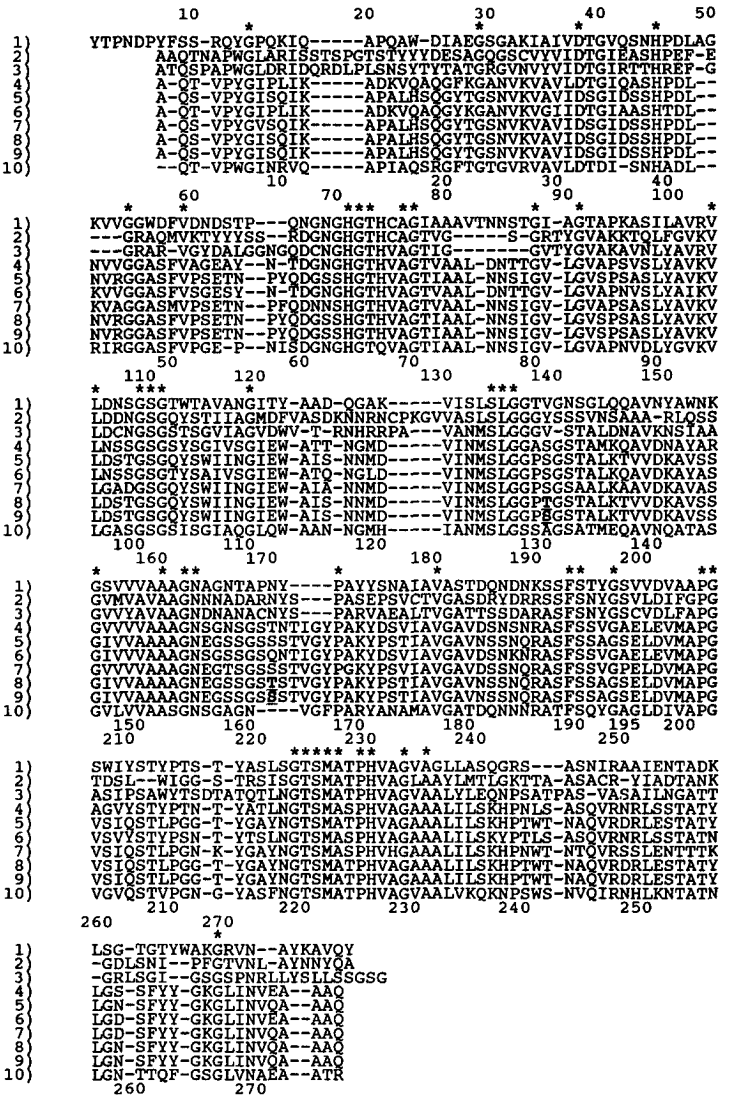
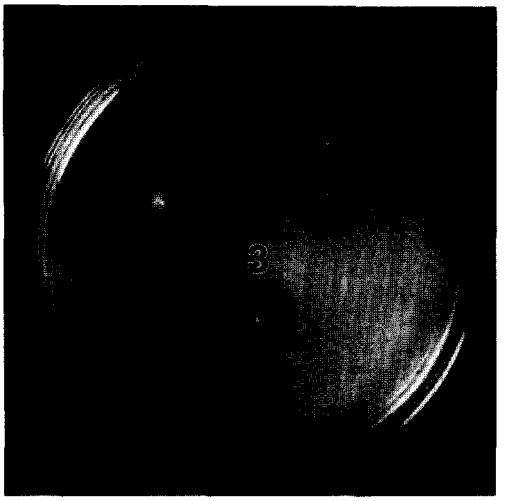


Fig. 1. Comparison of the amino acid sequence of subtilisin J with those of other subtilisin-type serine proteases. The sequence of subtilisin J(9) is shown compared with those of thermitase(1), proteinase K(2), aqualysin I(3), subtilisin Carlsberg(4), subtilisin amylosacchariticus(5), subtilisin DY(6), subtilisin BPN′(7), subtilisin E(8), and alkaline elastase YaB(10). The numbering above the sequences refers to thermitase, and that below the sequences to subtilisin J. Identical amino acids between subtilisin J and others are asterisked. The different amino acid sequences between subtilisin J and subtilisin E are underlined.

inactivation at 60°C as shown in Fig. 4A. Moreover the wild-type enzyme still retained about 50% of the initial activity when kept at 60°C for 30 minutes in the presence of 2 mM $CaCl_2$. But in the case of the Gln-195 mutant subtilisin J, the presence of $CaCl_2$ did not increase the thermostability (Fig. 4B). As shown in Fig. 4B, the Gln-195 mutant was completely inactivated even in the presence of 10 mM $CaCl_2$. On the other hand, Mg^{2+} (2 mM) had no



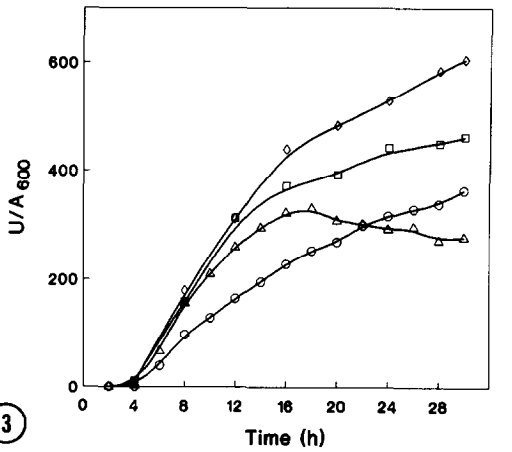


Fig. 2. Protease assay on a tryptose blood agar base-1% skim milk plate (Km; final 5μg/ml). Number 1 refers to *B. subtilis* DB104(pZ124), 2 refers to *B. subtilis* DB104 carrying wild-type *aprJ* gene, and 3 refers to *B. subtilis* DB104 carrying Gln-195 mutant *aprJ* gene.

Fig. 3. Time course of protease activity per A_{600}. *B. subtilis* DB104 carrying wild-type plasmid in the presence of 2 mM $CaCl_2$ (O) was grown at 37°C. *B. subtilis* DB104 carrying Gln-195 mutant plasmid was grown at 37°C in the presence of 2 mM $CaCl_2$ (Δ), 5 mM $CaCl_2$ (□), and 10 mM $CaCl_2$ (◊).

effect on the heat stability of wild-type and Gln-195 mutant subtilisin J (data not shown). From these results, one can conclude that the Gln-195 mutant subtilisin was markedly thermolabile compared with the wild-type subtilisin. This thermolabile characteristic due to the Gln substitution also influences the temperature dependence of the specific activity of the mutant subtilisin (Fig. 5). As shown in Fig. 5B, Ca^{2+} had little effect on the optimal temperature of the Gln-195 mutant subtilisin compared with the wild-type subtilisin (Fig. 5A). But the Gln-195 mutant subtilisin has the same optimal pH (9.0) as the wild-type subtilisin. This may come from the fact that the lower charge density near the weak Ca^{2+} binding site compared with the wild-type subtilisin decrease the binding affinity of this site for Ca^{2+}. The

Table I. Kinetic constants of the wild-type subtilisin J and Gln-195 mutant subtilisin J for the hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide. Assays were performed in 0.1 M Tris-HCl, pH 8.6, at 25°C.

Amino acid residue at position 195	k_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
wild-type Glu	0.9	6.0	6.7
mutant Gln	1.0	10.4	10.4

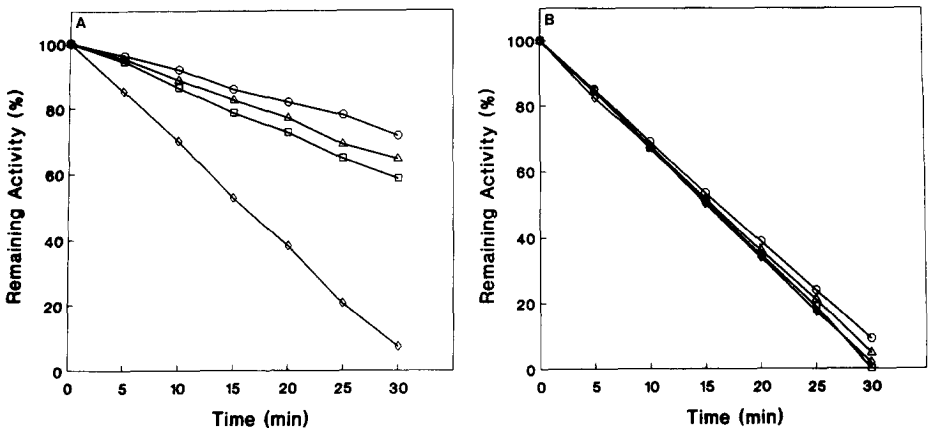


Fig. 4. Thermostability of wild-type (A) and Gln-195 mutant (B) subtilisin J. Remaining activity after heating at 60°C in the absence (◊) and in the presence of 2 mM $CaCl_2$ (□), 5 mM $CaCl_2$ (Δ), and 10 mM $CaCl_2$ (O) was determined using *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as substrate at 25°C and was expressed as a percentage of the original activity.

region of subtilisin BPN′ around the weak Ca^{2+} binding site has a high charge density with Glu-195, Glu-251, Asp-197, Arg-247, and Lys-170 within an 8 Å radius (12). So the replacement of negatively charged Glu-195 with the Gln-195 may slightly lower this charge density to the point that no Ca^{2+} binding takes place. Indeed, from the Ca^{2+} ion binding titration curves obtained from the rates of thermal inactivation of wild-type and Gln-195 mutant subtilisin as a function of -log $[Ca^{2+}]$, the binding affinity of Ca^{2+} for the Gln-195 mutant subtilisin decreased about 10 times than that of wild-type subtilisin (data not shown). This low binding affinity of the Gln-195 mutant subtilisin can contribute to the different

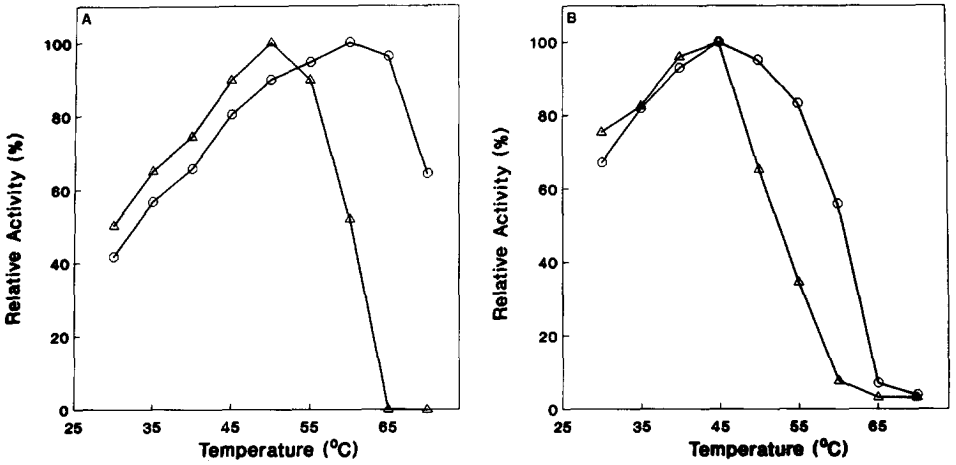


Fig. 5. Effect of temperature on the wild-type subtilisin J (A) and Gln-195 mutant subtilisin J (B). The enzyme activity was assayed at the indicated temperature in the presence (O) and absence (Δ) of 2 mM $CaCl_2$.

protease production in the presence of 2 mM $CaCl_2$ and decrease the heat stability but not its catalytic efficiency (k_{cat}/K_m) and optimal pH.

Previously, Pantoliano *et al.* (7) reported that the binding affinity of Ca^{2+} for the subtilisin BPN′ can be altered by the replacement of Gly-131, Pro-172, and Asp-197 to Asp-131, Asp-172, Glu-172, and Glu-197, respectively. In their study, the replacement of Asp-197 to a much larger Glu was found to decrease the affinity of this site for Ca^{2+}. The Glu-195 and Asp-197 are the highly conserved amino acid residues along with Glu-251 among subtilisin BPN′, subtilisin E, and subtilisin J. So, the replacement of these residues can alter the binding affinity for Ca^{2+}. In this regard, our results clearly demonstrate the presence of the weak Ca^{2+} binding site surrounded with high charge density. It will be of interest to determine the three-dimensional structure of the Gln-195 mutant subtilisin to elucidate the side chain effect at the weak Ca^{2+} binding site.

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