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IMPROVEMENT OF THERMAL STABILITY OF SUBTILISIN J BY CHANGING THE PRIMARY AUTOLYSIS SITE

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SUMMARY : The thermostability of subtilisin J, an extracellular serine protease secreted from *Bacillus stearothermophilus*, has been improved by changing the primary autolysis site of the Asp-49 mutant protein. Previously we have shown that the Asp-49 mutant protein has proteolytic activity, but so unstable that it was primarily autolyzed in Tyr-58 – Gln-59 peptide bond during cultivation (Jang et al. Biochim. Biophys. Acta. 1162, 233-235 1993). In the present study, to mitigate the autolytic degradation and increase the thermostability, we deleted the Tyr-58 residue using the Asp-49 mutant as a template. This mutant (Asp-49/ΔTyr-58 mutant) protein showed an improved resistance to heat treatment without changing the catalytic efficiency of the enzyme. These results show that change of primary autolysis site can stabilize the subtilisin.

Subtilisin (EC 3.4.21.14), an alkaline serine protease produced by a variety of *Bacillus* species, has been studied extensively using site-directed mutagenesis in terms of its three-dimensional structure and catalytic properties (1-2). For many years, subtilisin has been used in industrial applications, *e.g.* as an additive to laundry detergents (3). During the practical using process, however, the enzyme meets extreme environments *e.g.* high temperature, detergents, high pH and Ca^{2+}-chelating agents. These factors are known to disrupt the conformational integrity of the enzyme and lead to irreversible autolytic degradation.

Protein engineering by *in vitro* mutagenesis of cloned or synthetic genes has been shown to be a powerful tool in altering enzyme characteristics and in tailoring enzymes for special applications (4). Therefore, protein engineering has been extensively applied to improve the thermal stability of subtilisin (5-6).

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The abbreviations used are : Asp-49/ΔTyr-58 mutant, Asp-49 mutant with deleted Tyr-58 residue; PCR, Polymerase Chain Reaction.

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Table I Comparison of the amino acid sequence around the Ser-49 residue of subtilisin J with those of other subtilisin-type serine proteases

Thermitase	G	W	D	F	V	D	N	D	S	T	P	-	-	-	Q	N
Aqualysin I	R	A	R	-	V	G	Y	D	A	L	G	G	N	G	Q	D
Subtilisin Carlsberg	G	A	S	F	V	A	G	E	A	Y	-	-	-	N	T	D
Subtilisin BPN'	G	A	S	M	V	P	S	E	T	N	P	-	-	F	Q	D
Subtilisin DY	G	A	S	F	V	S	G	E	S	Y	-	-	-	N	T	D
Subtilisin J	G	A	S	F	V	P	S	E	T	N	P	-	-	Y	Q	D
				50												60

The numbering below the sequences refers to Subtilisin J.

Fontana et al. studied the correlation between the sites of chain breakage in limited proteolysis and the segmental mobility in thermolysin, the thermostable neutral protease from *B. thermoproteolyticus* (7). In addition, Braxton & Wells identified the sites of autolysis in subtilisin BPN' at elevated temperature (8). Ala-48 – Ser-49 peptide bond was a one of these sites. It has been also shown that autolysis plays a significant role at the stability limit of subtilisin at elevated temperature (9).

We have previously cloned and expressed the gene of *Bacillus stearothermophilus* subtilisin J in *Bacillus subtilis* (10). Ser-49 in subtilisin J is located in accessible loop structure near the active site and highly conserved residue among subtilisins from *Bacillus* sp.(Table I). In recently, we have also shown that the Asp-49 mutant protein is so unstable that it is primarily autolyzed in Tyr-58 – Gln-59 peptide bond during cultivation (11). However, enzymatic activity of the Asp-49 mutant protein is higher than that of wild type in early growth phase. In the present study, the Tyr-58 residue was deleted using the Asp-49 mutant as a template to mitigate the autolysis and improve the thermostability of subtilisin J. Also, we only deleted the Tyr-58 residue as a control of the Asp-49/ΔTyr-58 mutant protein. We found that the Asp-49/ΔTyr-58 mutant protein has a more improved thermostability than the wild-type protein without a change in its catalytic efficiency.

MATERIALS AND METHODS

The site-directed Tyr-58 deleted mutation was introduced into the wild type gene and the Asp-49 mutant gene using PCR (12), and the mutation was confirmed by dideoxy chain-termination sequencing (13) on the M13mp19. The mutagenesis was performed with a oligonucleotide primer having the sequences 5'-CAAACCCACAGGACGGCA-3'. The mutant gene was expressed in the plasmid pZ124(Km^r) and the mutant protein purified from the culture supernatant as described (10). The enzyme was assayed in a

solution containing 0.3 mM *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide and 100 mM Tris-HCl (pH 8.6) at 25°C. The assays measured the increase in absorbance at 410 *nm* per min due to hydrolysis and release of the *p*-nitroaniline (ε_{410} = 8,480 $M^{-1}cm^{-1}$) (14). The enzyme assays for kinetic constants, k_{cat} and *K*m, were performed with substrate concentrations varying between 0.1 and 3 mM. Reactions were initiated with addition of 10μl of the corresponding enzyme solution (5 μM).

RESULTS AND DISCUSSION

It has been shown that autolysis plays a significant role at the stability limit of subtilisin at elevated temperature. It has been also shown that increased thermal stability reduces autolysis for some serine proteases. Therefore, we investigated the characteristics of subtilisin J in terms of thermal stability and autolysis.

Wild-type and mutant subtilisin J were expressed in the *B. subtilis* DB104. The activities of the ΔTyr-58 mutant and the Asp-49/ΔTyr-58 mutant were nearly equivalent to that of the wild-type subtilisin J.

To characterize the enzymatic properties of the ΔTyr-58 mutant and the Asp-49/Δ Tyr-58 mutant subtilisin, the mature enzymes were purified from the culture supernatant to a single band by SDS-polyacylamide gel electrophoresis. The wild-type enzyme was also purified to homogeneity from *B. subtilis* harboring wild-type gene and examined as a control.

The thermostabilites of the purified enzymes are shown in Table II. When kept at 60° C for 30 min in the presence of 2 mM $CaCl_2$, the Asp-49/ΔTyr-58 mutant protein is more resistance to heat treatment than wild-type protein. The wild-type enzyme retained about 50% of the initial activity when kept at 60°C for 30 min in the presence of 2 mM $CaCl_2$. However, the Asp-49/ΔTyr-58 mutant protein has more than 70% of the initial activity at the same conditions. Half-life of irreversible inactivation ($t_{1/2}$) of the wild-type at 60°C in the presence of 2 mM $CaCl_2$ is 34 min. On the other hand, that of the Asp-49/ΔTyr-58 mutant is 49 min at the same condition. Contrast to the deletion of the Tyr-58 residue in

Table II Half-life of wild-type and mutant subtilisin J at various temperatures

Enzyme	Half-life at		
	50°C	60°C	70°C
Wild-type	230 min	34 min	3 min
ΔTyr-58 mutant	205 min	28 min	2 min
Asp-49/ΔTyr-58 mutant	315 min	49 min	6 min

Remaining activity (%) after heating for 30 min at indicated temperatures in the presence of 2 mM $CaCl_2$ was determined at 25°C using *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as the substrate. Enzyme concentrations were adjusted to 50μg/ml.

Table III Kinetic constants of the wild-type subtilisin J, the ΔTyr-58 mutant and the Asp-49/ΔTyr-58 mutant subtilisin J for the hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide

Enzyme	k_{cat}/Km (s^{-1} mM^{-1})	*K*m (mM)	k_{cat}
Wild-type	6.7	0.9	6.0
ΔTyr-58 mutant	6.2	0.9	5.6
Asp-49/ΔTyr-58 mutant	7.6	0.8	6.1

the Asp-49 mutant, the deletion of the Tyr-58 residue in the wild-type was not stabilize the subtilisin. It is supposed that because the primary autolysis sites of the ΔTyr-58 mutant were not changed, the ΔTyr-58 mutant protein was not more stable than wild-type protein.

To investigate the kinetic constants, k_{cat} and *K*m, enzyme assays were carried out at 25°C using the synthetic peptide *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, which is a well-known typical substrate for subtilisin. As shown in Table III, the Asp-49/Δ Tyr-58 mutant protein shows a slight increase in catalytic efficiency relative to the wild-type protein. This result demonstrates that the Asp-49/ΔTyr-58 mutant protein has a more improved thermostability than wild-type without dramatic changing the catalytic efficiency.

In this study, approach to stabilize subtilisin J towards autolytic degradation is changing the amino acid sequence near the primary cleavage site of the Asp-49 mutant protein. So far, this approach has received little attention, although it does not require detailed knowledge of the three-dimensional structure of the enzyme. In the case of subtilisin, the amino acid at position P1 plays a critical role in determining their substrate specificity, and at least six amino acid(designated P4-P'2, (15)) are involved in binding with subtilisin. Especially, when proline residue present at positions P3, P1, P'1 or P'2, it has a negative influence on the binding (*K*m) to the subtilisin binding cleft (16,17). Autolytic degradation of subtilisin is an intermolecular interaction. In the case of the Asp-49 mutant protein, six amino acids, $TNPY^{58}$-$Q^{59}D$ is equivalent to substrate binding site of subtilisin J.

We replaced the primary autolytic cleavage site (Tyr-58 or Gln-59) of the Asp-49 mutant protein with various amino acids according to subtilisin substrate specificity. However none of the mutants was substantially more stable than wild type subtilisin J. (unpublished data) Therefore, we deleted the Tyr-58 residue of the Asp-49 mutant subtilisin J.

In the case of the Asp-49/ΔTyr-58 mutant protein, P1 site and P'1 site is Pro-57, Gln-59, respectively. Deletion mutation of Tyr-58 in the Asp-49 mutant gene result in

considerable reduction in the autolysis rate and increasement of thermal stability. Catalytic efficiency of the Asp-49/ΔTyr-58 mutant protein is similar to that of wild-type protein. Half-life of irreversible inactivation at 60°C is about 1.5 times longer than that of wild-type subtilisin J. It is supposed that; 1) deletion of Tyr-58 residue cause loop structure to compact, so this region is rather inaccessible to subtilisin J, and 2) because P1 site of Asp-49/ΔTyr-58 mutant protein is proline, very unfavorable P1 site residue, autolytic degradation of the Asp-49/ΔTyr-58 mutant protein is reduced.

To elucidate the detailed mechanism of improved thermal stability, X-ray crystallographic study of the Asp-49/ΔTyr-58 mutant protein is now in progress.

Our approach to increase the thermal stability can be applied to various enzymes, especially, in the case of severely autolyzed proteases.

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