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Mutational Analysis of the Autoprocessing Site of Subtilisin YaB-G124A

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The potential residue at the autoprocessing site for improving processing efficiency was evaluated from hydrolysis of 19 cleavage-site-mimicking octapeptides, VTTXQTVP (−4 to +4), by the mature subtilisin YaB and YaB-G124A mutants. Both enzymes cleaved the octapeptides mainly at two sites, X-Q (A-site) and Q-T (B-site), at varied preferences. Based on the results above, Met^{-1} of YaB-G124A was mutated and, as expected, extracellular enzyme production increased with Gln or Ala replacement, but decreased with Ile or Asp substitution. Together with previous structural studies, our results suggest that autoprocessing is dependent on not only the primary structure, but also the peptide flexibility around the processing site. Cleavage at the B-site resulted in a novel YaB mutant lacking the N-terminus Gln 1, which led the mutant enzyme to less enzymatic activity by 80% and less thermal stability by 20°C, perhaps due to its ligation to the high-affinity calcium ion.

Key Words: **subtilisin YaB; autoprocessing; protein engineering; calcium binding.**

The alkaline serine protease subtilisin, secreted from a wide variety of *Bacillus* species, is an important industrial enzyme as well as a model system for protein engineering. More than 50% of amino acid residues, mainly on subtilisin BPN′ from *B. amyloliquefaciens*, and subtilisin E from *B. subtilis*, have been mutated and characterized (1, 2). More than 90 crystal structures of various subtilisins including mutants and enzyme-inhibitor complexes have been determined at high resolution, in particular, the *B. lentus* subtilisin at 0.78-Å ultrahigh resolution (3).

The subtilisin YaB from alkalophilic *Bacillus* YaB displays high sequence homology to other subtilisins, e.g., 83, 55, and 54% identity to *B. lentus* subtilisin, subtilisin BPN′, and subtilisin E, respectively (3–9). Although it has a P1 substrate preference for small amino acids, such as Ala, it still retains the ability to bind and cleave the substrates with large side chains, revealing that the substrate specificity is still not sufficiently strict. To further restrict the enzyme specificity, Gly 124, located near the waist of the S1-site, has been changed into Ala (YaB-G124A) or Val, and both mutants seemed restricted to Ala and Gly (10).

As other subtilisins, the mature YaB of 265 residues is processed from its precursor consisting of a 27-residue pre peptide for protein secretion and an 83-residue pro domain for folding of the active enzyme (9, 11). Removal of the pro domain by autocleavage during processing is crucial for the secretion and production of subtilisins. Recently, Takahashi *et al.* (12) showed that the autoprocessing efficiency of a subtilisin E mutant with altered specificity for acid residues was improved by substituting the autoprocessing site Tyr^{-1} with Asp or Glu. Therefore, in order to approach the residue preference at the −1 site for improving autoprocessing efficiency with a higher yield of active enzyme, hydrolysis of 19 synthetic octapeptides, VTTXQTVP, that mimic the cleavage sites −4 to +4, by YaB and the YaB-G124A mutant were first studied. Based on the results, Met^{-1} was mutated in YaB-G124A, and the autoprocessing rate, the enzyme production, and the autocleavage site of the mutants were investigated. Together with previous structural studies, the effect of the protein sequence around the processing site on the processing efficiency is discussed. A novel YaB mutant lacking the N terminus, was analyzed to address the functional role of Gln 1.

MATERIALS AND METHODS

Materials. Fmoc-protected amino acids, and all other reagents, required for amino acid synthesis on the Applied Biosystems 431A (ABI) peptide synthesizer were purchased from Sigma (U.S.A.). The

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ECL immunoblotting kit, and PVDF membrane were purchased from Pharmacia Biotech (Sweden). The host cell *Bacillus subtilis* DB104, the plasmid pEXG124A, and all other chemicals were as previously described (10).

Synthesis and hydrolysis of octapeptides. Octapeptides were synthesized on an ABI 431A synthesizer using the solid-phase peptide synthesis method (13), and purified by HPLC on a Vydac C_{18} column (4.6 × 250 mm, Phenomenex Co.) with greater than 99% purity. The target peptide and the enzyme were mixed at a 1×10^{-5} molar ratio in 50 mM carbonate buffer (pH 10.5) with 1 mM $CaCl_2$, and incubated at 37°C for 10 min. Nine volumes of 0.1% trifluoroacetic acid was then added to stop the reaction and the products were analyzed by HPLC on a Vydac C_{18} column.

Characterization of the YaB-G124A mutants. Site-directed mutagenesis, isolation, and enzymatic assay of YaB-G124A mutants, were carried out according to the previous procedures (10). Distribution of the mutants outside and inside the host cells was assayed by immunoblotting. Proteins in the medium were precipitated by 10% (v/v) trichloroacetic acid, separated on a 12% SDS–PAGE gels, and transferred to a PVDF membrane. Anti-subtilisin YaB from rabbit serum (diluted 1:5000) was used as the first antibody, and the ECL system (Pharmacia) for development of the hybridization signal.

RESULTS AND DISCUSSION

Hydrolysis of 19 Octapeptides by YaB and YaB-G124A

Various amounts of four kinds of products in the octapeptide hydrolysis were detected by HPLC analysis, demonstrating that both enzymes cleaved the octapeptides mainly at two sites, X-Q (VTTX and QTVP, A-site) and Q-T (VTTXQ and TVP, B-site) with varied preferences. The relative hydrolysis ratios, shown in Table 1, were calculated from the peak areas of the hydrolysis product QTVP (A-site) or TVP (B-site) from each octapeptide divided by that of the QTVP peptide from the peptide, VTTMQTVP.

Hydrolysis of 19 octapeptides by YaB revealed that the P1 preference is roughly His, Phe, Met, Leu, Asn, Tyr > Lys, Gln, Arg, Trp, Ala > Thr ⩾ Val, Ile, Pro, Gly, Asp, Glu, when TTV are the P2–P4 residues. The P2 preference is roughly Ile > Val, Pro, Gly, Lys, > Ala, Ser, Thr > Met, His, Arg, Leu ⩾ Phe, Tyr, Trp, Gln, Asn, Asp, Glu, when QTT are the P1, P3–P4 residues. On the other hand, hydrolysis analysis by YaB-G124A revealed that the P1 preference is only restricted to Ala, Gln, Met, and Ser, demonstrating Gly 124 has a clear influence on the P1 specificity (10, 14). The P2 preference is roughly Ile, Met > Lys, Val > Thr, Ser, Leu > His, Ala, Pro, Arg, Gly > Gln, Phe, Tyr, Asn ⩾ Trp, Asp, Glu. Since YaB-G124A has more P1 restriction and prefers Gln at the P1-site, it cleaves most octapeptides at the B-site.

Because of 83% sequence identity, the atomic model of YaB could be predicted based on the crystal structure of *B. lentus* subtilisin (3), and compared with those of other subtilisins. Our structural analysis suggested that the octapeptides would bind at the S4–S4′ or S5–S3′ subsites of YaB, resulting cleavage at A- or B-sites, respectively. And the broad substrate specificity of YaB is the results of broad binding subsites, and more than five hydrogen bonds between the backbones of the substrate and the enzyme, as are other subtilisins (2, 15). In addition, structural comparison revealed that the substrate-binding sites undergo a substantial shrinkage upon different inhibitor binding (16), suggesting the structural plasticity enhances the broad substrate preference.

TABLE 1

Octapeptide Hydrolysis by YaB

A site B site
↓ ↓
VTTX-Q-TVP

	YaB		YaB-G124A	
X	A site	B site	A site	B site
	Relative hydrolysis ratio[a]			
Met	1.0[a]	0.3	1.0	2.4
Gln	0.6	0	1.1	0.2
His	1.1	0.2	0	0.5
Arg	0.5	0.1	0	0.4
Leu	0.9	0.1	0	0.6
Asn	0.9	0	0	0.1
Phe	1.0	0	0	0.2
Tyr	0.8	0	0	0.2
Trp	0.5	0	0	0
Ala	0.4	0.4	1.2	0.5
Ser	0.7	0.4	0.8	0.7
Thr	0.2	0.4	0	0.8
Lys	0.7	0.6	0	1.5
Val	0	0.7	0	1.0
Ile	0	1.1	0	2.5
Pro	0	0.6	0	0.5
Gly	0	0.6	0	0.4
Asp	0	0	0	0
Glu	0	0	0	0

[a] HPLC peak area of the hydrolysis product QTVP (A site) or TVP (B site) from each peptide divided by that of the product QTVP from the VTTMQTVP peptide (X = Met).

Hydrolysis of various substrates by YaB against octapeptides (peptidase), succinyl-$(Ala)_3$-*p*-nitroanilide (sAAA*p*NA, amidase), and carbobenzoxy-(amino acid)-O-*p*-nitrophenyl ester (esterase) demonstrated some varied results regarding the substrate specificity. For example, Gly is a good P1 substrate using ester substrates (10), but very poor using peptide substrates here. Therefore, subtilisins may hydrolyze various substrates with distinct enzymatic mechanisms. For example, the active-site Ser mutant with Cys of substilisin BPN′ was shown to barely hydrolyze peptide bonds, but hydrolyze certain ester substrates well (17). Since octapeptides are closer to the true enzyme substrate than the ester and amide substrates, our results may reflect more substrate specificity at P1 and P2 positions.

Design and Characterization of YaB-G124A Mutants

A higher hydrolysis rate at the A-site and a lower rate at the B-site in Table 1 suggested that Gln

	+1
YaB	EVTTM*QTVP*
YaB-G124A	EVTTM*QTVP*
G124A-Q	EVTTQ*QTVP*
G124A-A	EVTTA*QTVP*
G124A-G	EVTTG*QTVP*
	+1
G124A-I	EVTTI*QTVP*
G124A-D	EVTTD*QTVP*
G124A-DD	EVTTDD*TVP*

FIG. 1. Sequences around the autoprocessing site of YaB-G124A mutants. The N-terminal residues determined by a Model 494 microsequencer (Applied Biosystems) are shown in italic and the predicted cleavage residues in bold.

(G124A-Q) and Ala (G124A-A) are better than Met as a P1 residue for the YaB-G124A enzyme, which may improve the autoprocessing rate and the enzyme production. On the other hand, the highest hydrolysis rate at the B-site implied that replacement of Met^{-1} with Ile (G124A-I) of YaB-G124A may lead to a different cleavage site and result in a novel YaB mutant lacking the N-terminus Gln1, because Ile is a good P2 residue but a poor P1 residue. Since Asp is poor for both P1 and P2 positions, substitution of Met^{-1} with Asp (G124A-D) may decrease or even block autoprocessing. Double mutation of Met^{-1} and Gln^{+1} into Asp (G124A-DD) may lead to another novel cleavage site, for example at Thr 2-Val 3. Met^{-1} was also mutated into Gly (G124A-G) because the activity assays with different substrates showed varied results for Gly being P1 residue. Substitution of Met with Asp or Gly was also carried in YaB-G124A in order to compare results in the same enzyme. Therefore, six YaB-G124A mutants listed in Fig. 1, were generated by site-directed mutagenesis, transformed, and expressed in *B. subtilis* DB104.

The autoprocessing rate and the extracellular enzyme production of the mutants were first analyzed. The elastolysis activity of the mutants on an elastin agar plate showed that a clear zone around G124A-Q appeared first, then G124A-A, G124A, and G124A-G during 18–24 h incubation, whereas the zone around G124A-I appeared between 36-48 h, and G124A-D around 72 h. The zone sizes were G124A-Q > G124A-A > G124A > G124A-G ⩾ G124A-I > G124A-DD (Fig. 2). The enzyme production was further confirmed by cultivation in 2× SG medium. The extracellular enzyme activities were assayed using elastin–orcein or sAAA*p*NA as substrates with the similar results shown in Fig. 3. The total enzyme yields of G124A-Q, G124A-A, G124A-G, G124A-I, G124A-D, and G124A-DD were 148, 112, 89, 50, 40, and 33% of that of YaB-G124A at 48 h, respectively. Distribution of the YaB-G124A mutants outside and inside the host cells was also assayed by using immunoblotting, shown in Fig. 4. Production of the mature G124A-Q was detected in the medium at 24 h, and almost reached optimal yield at 48 h. The pro form in the cells of G124A-Q was detected at 24 h, and decreased with the culture time. No significant amount of the G124A-Q pro form was detected after 72 h. On the other hand, production of the mature G124A-D was detected in the medium at 48 h, and the G124A-D pro form produced at 48 h, and accumulated with the culture time. The total amount of the mature and pro-form of G124A-D seemed to be lower than that of G124A-Q.

Results from these three methods above were consistent with each other. As expected, the processing rate and the enzyme production increased when Met^{-1} was replaced with Gln and Ala, and decreased with mutation into Ile, and Asp. Therefore, we concluded that introduction of a proper residue at the processing site is effective for obtaining a higher protein yield, as observed by Takahashi *et al.* (12), and analysis of octapeptide hydrolysis is a well-evaluated system for approaching the proper residues.

Discovery and Characterization of a Novel YaB Mutant, G124A(-Q)

The N-terminal analysis of the isolated mutant mature protein demonstrated that YaB-G124A, G124A-Q, G124A-A, and G124A-G enzymes were processed mainly at the Met^{-1} site (A-site), as was YaB. Whereas G124A-I, G124A-D, and G124A-DD enzymes were processed in majority at the Gln^{+1} site (B-site), resulting in a novel YaB mutant [G124A (-Q)] lacking the Gln 1 residue (Fig. 1).

The kinetic parameters of purified YaB-G124A and YaB-G124A(-Q) were determined using sAAA*p*NA as substrate. The YaB-G124A(-Q) enzyme was purified from the 60-h culture broth of G124A-D. The K_m and k_{cat} values were 0.29 mM^{-1} and 37.4 s^{-1} for YaB-G124A and 0.58 mM^{-1} and 13.8 s^{-1} for G124A(-Q), respectively. The k_{cat}/K_m value of the YaB-G124A(-Q) mutant was 24 mM^{-1} s^{-1} compared to that of 128 mM^{-1} s^{-1} for YaB-G124A.

For studies of the thermal stability, the residual catalytic activity toward sAAA*p*NA was measured at various temperatures. As shown in Fig. 5, the residual activity of YaB-G124A (-Q) fell to 21% at 40°C, whereas that of YaB-G124A fell to 22% at 60°C. The melting temperature, at which half of the activity was lost, was around 55°C for YaB-G124A, but 35°C for YaB-G124A (-Q). As far as we know, this is the first identified mutant, in which the enzyme activity reduced by 80% and the melting temperature decreased by 20°C upon removal of the N-terminus residue.

Most subtilisins contain the high-affinity calcium-binding site, termed Ca^1 site, which consists principally of a nine-residue loop linking the N terminus with three hydrogen bonds (18). The Ca^1 in YaB is coordinated by side chain oxygens of Gln 1, Asp 39, and Asn 74, and by main chain oxygens of Leu 72, Ile 76,

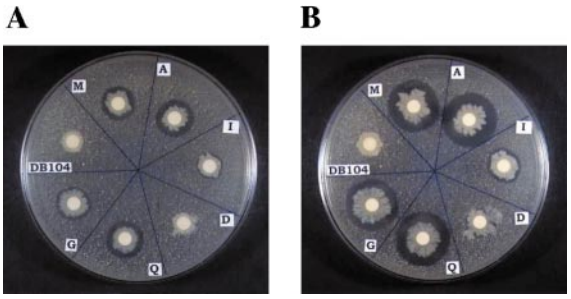

FIG. 2. The elastolytic activity of the YaB-G124A mutants in a LB agar plate containing 1% elastin. The DB104 transformants harboring pHY300PLK vector (DB104), G124A (M), G124A-Q (Q), G124A-A (A), G124A-G (G), G124A-I (I), and G124A-D (D) were cultured at 37°C for 48 h (A) and 72 h (B).

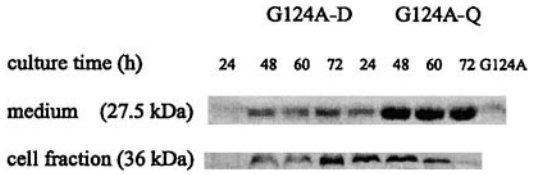

FIG. 4. Distribution of G124A-D and G124A-Q mutants by immunoblot analysis with various culture times: the mature form in the culture medium, and the pro-form in the cell fraction. The mutant *B. subtilis* DB104 was grown at 37°C in 2× SG medium, and the culture portions were collected at 24, 48, 60, and 72 h. The total cellular and medium proteins were separated by SDS–PAGE and detected by an anti-YaB antibody.

and Val 78 (10). Calcium ion binding is shown to slow down the thermal autolysis and to enhance the thermal stability (19). Deletion of the Ca^1-binding loop reduces the thermal stability but accelerates the folding process without the pro domain, revealing that the kinetic barrier for folding subtilisin involves the formation of the Ca^1 site (20). In addition, an N76D substitution in subtilisin E improved the stability, presumably by strengthening the Ca^1 binding (21). Moreover, the crystal structure of a highly thermostable protease from Bacillus Ak.1, demonstrated the calcium-mediated thermostability (18). In short, previous studies showed the Ca^1 binding makes a dominant contribution to conformational stability. Therefore, lack of Gln 1 in G124A(-Q) would cause a weak Ca^1 binding and the flexible conformation around the Ca^1 site, resulting less thermostability and enzyme activity. In addition, the calcium-dependent (or independent) stability has been studied in many mutants (1, 22) because stability has been most amenable for the enhancement of subtilisin, yet perhaps least understood. Therefore, the novel YaB-G124A(-Q) mutant not only reveals the functional role of Gln 1, but also is a good candidate for the study of calcium-relative stability.

A Broad Substrate Preference with a Specific Autoprocessing Site

Secretion of the mature YaB-G124A mutants (Fig. 1) suggested that autoprocessing mainly occurred at the −1 site, unless the −1 residue was replaced by residues such as Ile and Asp, which severely disturb the P1 specificity. In this case, the flexibility of the cleavage site will allow processing to occur one residue downstream at the Gln^{+1} site at a much slower rate. The processing still occurred at the Gln^{+1} site in the G124A-DD double mutant, even Asp is very unfavorable for both S1 and S2 subsites. Together with previ-

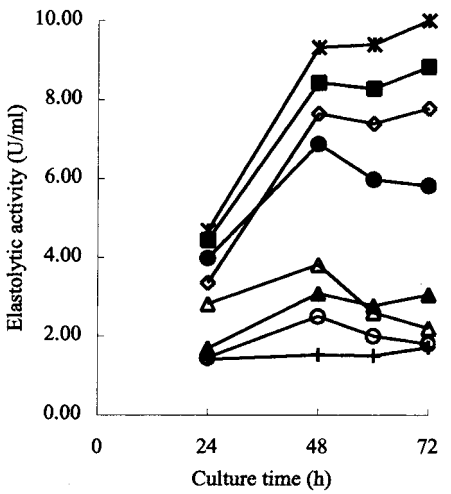

FIG. 3. Time course of the total elastolytic activity of the extracellular YaB-G124A mutants per milliliter of medium. The transformants were cultured in 2× SG medium at 37°C for the indicated time, then the elastolytic activity was measured as described under Materials and Methods. *, G124A-Q; ■, G124A-A; ◇, YaB-G124A; ●, G124A-G; ▲, G124A-D; △, G124A-I; ○, G124A-DD; +, pHY300PLK (vector).

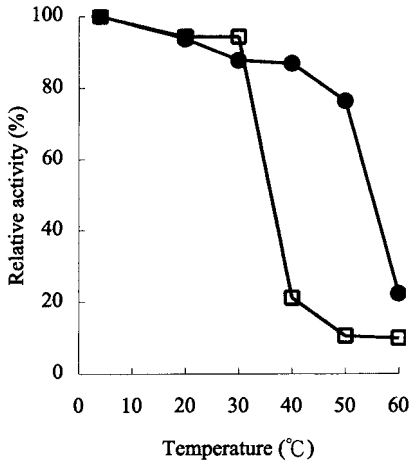

FIG. 5. The thermostability of YaB-G124A (●), and G124A(-Q) (□). The purified enzyme was incubated at the indicated temperature for 1 h, and the residual activity was then determined using sAAA*p*NA as substrate.

ous mutant studies (23, 24), our results here suggested that the primary structure around the processing site could only affect the processing efficiency and shift the cleavage site at most by one residue downstream. Therefore, the specific processing site of pro-subtilisin is more likely to be dependent on the interactions between the pro domain and the mature enzyme.

The crystal structure of the subtilisin BPN′ complexed with the pro domain reveals that the pro domain folds into a single compact domain and interacts with a potential folding nucleus, two parallel helices, of the mature enzyme (25). Then the pro domain assists the pro-enzyme folding into an autocleavable conformation, in which the interdomain linker lies in the active site, with the cleavage site directly adjacent to the catalytic triad. Introduction of different deletions at the junction between the pro domain and the mature subtilisin Carlsberg abolished the autocatalytic processing (23), perhaps due to disruption of the domain interaction and resulting in a non-autocleavable conformation. The interdomain linker might flap away the active site in the non-autocleavable conformation and could be cleaved by the exogenous mature enzyme. If deletions were large enough to damage the pro domain folding, the mature enzyme would not properly fold. In short, mutual assistant folding of the pro domain and the mature domain leading to the interdomain linker into the active site may be how subtilisins with broad substrate preference cleave at a specific autoprocessing site.

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