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A Cold-Adapted Protease Engineered by Experimental Evolution System[1]

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A new cold-adapted protease subtilisin BPN′ mutant, termed m-51, was successfully isolated by use of an evolutionary program consisting of two-step *in vitro* random mutagenesis, which we developed for the screening of mutant subtilisins with increased activity at low temperature. The m-51 mutant showed 70% higher catalytic efficiency, expressed by the k_{cat}/K_m value, than the wild-type at 10°C against *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as a synthetic substrate. This cold-adaptation was achieved mainly by the increase in the k_{cat} value in a temperature-dependent manner. Genetic analysis revealed that m-51 had three mutations, Ala→Thr at position −31 (A-31T) in the prodomain, Ala→Val at position 88 (A88V), and Ala→Thr at position 98 (A98T). From kinetic parameters of the purified mutant enzymes, it was found that the A98T mutation led to 30% activity increase, which was enhanced up to 70% by the accompanying neutral mutation A88V. The A-31T mutation severely constrained the autoprocessing-mediated maturation of the pro-subtilisin in the *Escherichia coli* expression system, thus probably causing an activity-non-detectable mutation in the first step of mutagenesis. No distinct change was observed in the thermal stability of any mutant or in the substrate specificity for m-51. In the molecular models of the two single mutants (A88V and A98T), relatively large displacements of alpha carbon atoms were found around the mutation points. In the model of the double mutant (A88V/A98T), on the other hand, the structural changes around the mutation point counterbalanced each other, and thus no crucial displacements occurred. This mutual effect may be related to the enhanced activity of the double mutant.

Key words: cold-adaptation, evolutionary engineering, kinetic properties, subtilisin BPN′, thermal stability.

Cold-active enzymes performing at high catalytic rates at low temperatures have many potential uses in bio-processes conducted at a reduced energy cost in the cold or in environments where heating is impractical. Predictable applications of cold-active enzymes include food processing, washing, biomass conversion, or environmental bioremediation. The orthodox strategy for obtaining cold-active enzymes is routinely initiated from the screening of naturally occurring psychrophilic isolates for enzymatic activities of interest (*1-4*). In constrast to this approach, we have been attempting to create cold-adapted forms from mesophilic enzymes by artificial evolution, called "evolutionary engineering," based on a Darwinian sequential program of mutagenesis and selection (*5-7*).

To date, we have studied the cold-adaptation of subtilisin BPN′, a mesophilic and industrially useful alkaline serine protease, as a good model enzyme to which protein engineering can be also applied for alteration of its properties (*8*). Our screening program consists of random mutagenesis, for obtaining proteases with enhanced activities at low temperature, *via* multistep mutations with a combination of primary mutation causing activity loss and secondary mutation causing recovery of the activity (*5, 7*).

Here we describe the characterization on enzymatic and molecular modeling bases of a cold-adapted subtilisin which exhibits 70% higher activity than the wild-type enzyme at 10°C.

MATERIALS AND METHODS

Bacterial Strains and Expression Systems—Escherichia coli JM109 (*9*) was used as the host strain for the screening of subtilisin mutants on proteolytic activity assay plates (2% skim-milk, 1% lactose, 1% yeast extract, and 50 μg/ml ampicillin) established by Tange *et al.* (*5*). The recombinant subtilisin gene on the plasmid pUC18 (*9*) was expressed under the original promoter of subtilisin and the

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lac promoter in *E. coli*. For secretory overproduction of the recombinant subtilisin, the host strain *Bacillus subtilis* UOT0999 lacking multiple protease genes was cultivated in liquid Luria-Bertani (LB) medium (*10*) containing 20 μg/ml tetracycline.

In Vitro Random Mutagenesis—Random G(C) to A(T) mutations were performed for the whole pUC18 plasmid harboring the wild-type subtilisin gene (termed pUΔS16-1) by treatment with hydroxylamine at 65°C for 2 h in 0.1 M sodium phosphate buffer (pH 6.0) containing 1 mM EDTA (*5*). The mutagenized plasmid DNA was redissolved in 10 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA. The mutation points were analyzed by dideoxynucleotide chain-termination sequencing using a BcaBEST kit (Takara Shuzo). Six sequencing primers were synthesized by the solid-phase phosphoamidite method with an Applied Biosystems 381A DNA synthesizer (*11*).

Screening Procedure—A mixture of the *Eco*RI-*Hin*dIII fragments including the mutagenized subtilisin gene was religated into the pUC18 plasmid to generate a mutant library. Because the protease subtilisin is excreted from the cell, active mutants were identified as colonies that cleared a skim milk plate at various temperatures. When *E. coli* JM109 was transformed with the recombinant plasmid and cultivated on the skim-milk plate at 37°C overnight to form transformant colonies, detectable cleared zones, caused by proteolysis of the skim-milk, appeared around the colonies after a further 2 days of incubation at 10°C. The change in proteolytic activity of mutant subtilisins was judged on the basis of the formation velocity of the cleared zone at the initial experimental stage. For precise estimation of the catalytic properties of the mutant subtilisin, the DNA fragment including the subtilisin gene was subcloned into the *Eco*RI-*Hin*dIII sites of pHY300PLK (*12*), a shuttle expression vector between *E. coli* and *B. subtilis*, and the recombinant subtilisin was overproduced by *B. subtilis* UOT0999.

Preparation of Single and Double Mutant Subtilisins—A-31T single mutant and A88V/A98T double mutant were genetically prepared with wild-type and m-51 subtilisin genes, both of which are placed on the pUΔS16-1, by using the unique *Cla*I site located between two nucleotide positions corresponding to −31 and 88 of subtilisin. For preparing two single mutant subtilisins, A88V and A98T, two mutagenic primers were synthesized as follows: 5′-GCCAAGCG(C→T)ATCACTTT-3′ for A88V (MUT-A88V) and 5′-TTCTCGGT(G → A)CTGACGGT-3′ for A98T (MUT-A98T). The target mutation was introduced using the primer pairs, MUT4 (Takara Shuzo) and mutagenic primers described above (for the first PCR), and M13 primer RV (Takara Shuzo) and M13 primer M4 (Takara Shuzo) (for the first and second PCRs) *via* heteroduplex formation between the two first PCR products (*13*). PCR was carried out using programs of 25 cycles of 94°C for 30 s (denaturation), 55°C for 2 min (annealing), and 72°C for 3 min (elongation) (for the first PCR), and 10 cycles under the same conditions as those for the first PCR (for the second PCR). The single-strand region of the heteroduplex was filled in by the second PCR, followed by double digestion with *Eco*RI and *Hin*dIII. The double-strand DNA fragment carrying the target mutation could, in principle, be selectively digested with both enzymes and subjected to cloning into the same restriction sites of the plasmids, pUC18 and pHY300PLK, respectively.

Purification of Recombinant Enzymes—A recombinant *B. subtilis* harboring the wild-type or mutated subtilisin gene was cultivated in 100 ml of LB medium containing a final concentration of 20 μg/ml tetracycline at 37°C for 24 h. Subtilisin excreted into the medium was recovered by ammonium sulfate precipitation (40% saturation), followed by dialysis against 20 mM sodium phosphate buffer (pH 6.3) for 2 days. The dialysate was subjected to ion-exchange chromatography on a DEAE-cellulose column, and eluted out with 20 mM phosphate buffer. The pass-through fraction was further purified by CM-cellulose column chromatography using a linear gradient of 0-0.2 M NaCl. The active protease fractions were detected by the cleared zone corresponding to caseinolytic activity on the plate containing skim milk. The purified sample was precipitated by adding a fourfold volume of acetone to the fraction containing subtilisin. The purity of the recovered samples was checked by sodium dodecylsulphate (SDS)-polyacrylamide (15%) gel electrophoresis according to the method of Laemmli (*14*).

Protease Activity Assay—Wild-type and mutant subtilisin activities were measured at various temperatures by monitoring the release of *p*-nitroaniline at 410 nm due to enzymatic hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe (or Leu)-*p*-nitroanilide, abbreviated AAPF or AAPL, (0.02-0.8 mM) in 100 mM Tris/HCl buffer (pH 8.6) containing 2 mM $CaCl_2$ (*5*). A 10-μl aliquot of 4-8 μM purified subtilisins or culture supernatant subtilisins was mixed rapidly with 990 μl of the above substrate solution to give a final reaction volume of 1 ml. The apparent concentration of subtilisin was determined spectrophotometrically using an absorbance coefficient of $E^{1\%}_{280nm}=11.7$ (*15*) and molecular weight of 27,500, to permit calculation of k_{cat} from the relationship $k_{cat}=V_{max}/[enzyme]$. The precise quantification of each purified active subtilisin was performed by active-site titration with the specific proteinaceous inhibitor, *Streptomyces* subtilisin inhibitor (*16*). The SSI concentration was determined spectrophotometrically at pH 7.0 using A_{276} (1 mg/ml) = 0.829 (*5*). The estimated value was used to correct the value of specific activity and the kinetic constant, k_{cat}. Preincubation times before addition of enzyme were 30 to 60 min. An EYELA Uni Cool type UC-55N (Japan) was used as a cooling unit for control of the proteolysis reaction. For tracing the proteolytic activity-temperature profile of the wild-type and m-51 mutant, proteolytic activity was assayed at four temperatures, 10, 25, 37, and 50°C, using 50 mM synthetic substrates, AAPF and AAPL.

Thermal Stability of Subtilisin—A 1-ml aliquot of 1 μM purified subtilisin was incubated at 60°C, and samples of 50 μl were taken at various times, and immediately cooled on ice. The residual subtilisin activity was measured using AAPF as the substrate as described previously (*5*).

Molecular Modeling—The refined tertiary structure of subtilisin BPN′ (Protein Data Bank-ID No. 2SIC) was used as a data source for computational analysis (*17*). Distances among mutation points in m-51 are presented in Fig. 1. In the topallh22x force field of CHARMM (*18, 19*), using the program X-PLOR 3.851 (*20*), all the hydrogen atoms were generated and added to the coordinates of subtilisin BPN′ from 2SIC, and the resultant wild-type structure was optimized by the energy minimization. Subsequently,

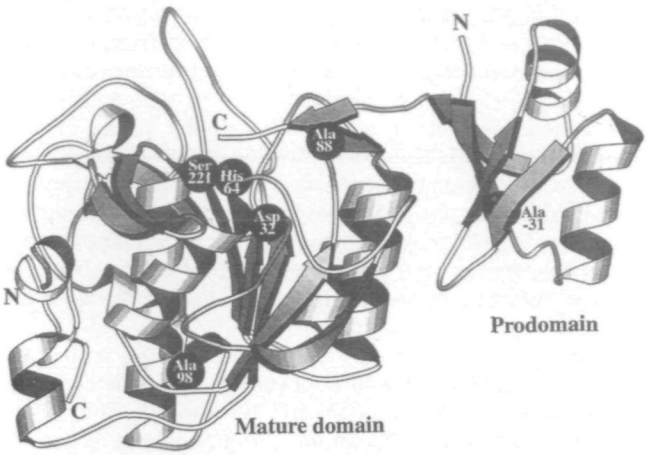

Fig. 1. **Mapping of the mutations on tertiary structure of prodomain and mature domain in subtilisin BPN′.** The catalytic triad residues, Ser-221, His-64, and Asp-32, are indicated by closed circles. Cold-adapted mutant subtilisin, m-51, possesses three mutations, A-31T, A88V, and A98T.

structural models of the A88V and the A98T single mutants and the A88V/A98T double mutant were constructed from the wild-type structure by using the mutation function provided in QUANTA97. In addition, the structure of each model was optimized again. Superposition of the structural models and calculation of the hydrogen bond positions were done by use of QUANTA97.

RESULTS AND DISCUSSION

Isolation of Subtilisin Mutants with Higher Activity at Low Temperatures—Subtilisin mutants were screened by using basically the same microbial system (*5*): host-vector systems of *E. coli* for plate assay and *B. subtilis* for secretory recombinant protein production. Random mutagenesis was carried out using hydroxylamine (which leads specifically to C→T or G→A transitions) to enhance the protease activity of subtilisin at a low temperature *via* multi-step mutations, consisting of a primary mutation causing a loss of activity, and a secondary mutation causing activity recovery. This intragenic suppression-type mutation has been technically advantageous in allowing us to obtain, in a positive selection manner, the improved mutant subtilisins from a majority of mutants with reduced activity as well as activity-non-detectable primary mutants.

The mutant subtilisin candidates with higher activities at 10°C were screened on the skim milk plates at a frequency of 2×10^{-6} by measuring the initial rate of cleared zone formation of each transformant colony appearing on the plates. These screened subtilisin genes were subcloned into the *B. subtilis* host-vector system for the secretory overproduction of subtilisins. One of them, a newly isolated mutant subtilisin termed m-51, showed 70% higher proteolytic activity than the wild-type at 10°C using AAPF as a synthetic substrate.

Identification of Mutations in the m-51 Mutant Subtilisin—DNA sequencing revealed that m-51 possessed three mutations: GCT→ACT, GCA→GTA, and GCT→ACT, corresponding to Ala→Thr at position −31 (A-31T), Ala→Val at position 88 (A88V), and Ala→Thr at position 98 (A98T), respectively. The tertiary structure of subtilisin presented in Fig. 1 is available for considering the effects of these amino acid substitutions on cold-adaptation. In our search, two mutations in the mature region, A88V and A98T, were found to be novel in the list of over 450 subtilisin mutants so far isolated (*21*).

Most of the activity-increase and activity-decrease mutations so far obtained (actual positions: 72, 84, 88, 92, 98, 131, and 197) appear to fall in N-terminal half of the subtilisin mature portion (*5-7*). This suggests that this region, in particular the stretch from 80 to 100, might be a hot area for achieving the cold-adapatation of the entire subtilisin consisting of 275 amino acid residues.

Temperature Dependence of Proteolytic Activity of m-51—To characterize the kinetic properties of the m-51 mutant, we purified the recombinant protein from culture supernatant of transformant *B. subtilis* to homogeneity on SDS-polyacrylamide gel (data not shown). No significant change in production level was observed among the mutant subtilisins under the culture conditions employed. Table I shows the temperature dependence of m-51 subtilisin based on kinetic parameters using AAPF as the substrate. The proteolytic activity expressed as k_{cat}/K_m of m-51 subtilisin, increased from 30% higher to 70% higher than that of the wild-type when the temperature was reduced from 50°C to 10°C. In this case, cold-adaptation was achieved mainly by the increase in the k_{cat} value in a temperature-dependent manner.

Analysis of Mutation Effect at Each Position—To analyse the contribution of each mutation to cold-adaptation, we divided the triple mutation in the m-51 subtilisin gene into three single mutations (A-31T, A88V, and A98T) and one double mutation (A88V/A98T) by site-directed mutagenesis and restriction enzyme digestion, as described in "MATERIALS AND METHODS." Plasmids containing the genes for the wild-type subtilisin or one of the mutants were introduced into *E. coli* JM109.

No detectable cleared zone was observed on the skim milk plate for the A-31T single mutant subtilisin-producing *E. coli* JM109. This strongly suggests that A-31T was the primary mutation causing activity-non-detection as far as the *E. coli* host-vector system is concerned. Position −31 corresponds to position 47 of a 77-amino-acid N-terminal prodomain, which is considered to be an intramolecular chaperone that facilitates folding of the subtilisin molecule through a maturation process regulated by an autoprocessing mechanism (*22*). Recently, hydrophobicity at this position was demonstrated to be partly important for forming a defined tertiary structure of the prodomain, as revealed by genetic amino acid replacement (*23*) based on X-ray crystallography of the complex form between prodomain and mature domain, as illustrated in Fig. 1 (*24*). According to the crystal structure of the subtilisin/prodomain complex, the side chain of Ala-31, the 47th residue of the pro-domain, projects into the hydrophobic interior of the pro-domain. And there is little space around the methyl group. Therefore, if the side chain is replaced by a hydrophilic and bigger side chain, the immature protein will become remarkably unstable. This instability is considered to be the reason why the maturation of the A-31T single mutant does not progress well. On the other hand, the maturaion of the A-31T/A88V/A98T triple mutant progresses correctly. The mutation of Ala88 and Ala98 might

compensate for the instability caused by the mutation of Ala-31, although Ala88 and Ala98 are more than 20 Å distant from Ala-31. Therefore, in the *E. coli* host- vector system, normal autoprocessing of A-31T mutant precursor would be hindered by the replacement of Ala with the more hydrophilic Thr. This was supported by the finding that a defective prodomain was generated by double mutaion of A-31T/I-67T in distinct short hydrophobic regions of the prodomain of subtilisin E, a homolog extremely similar to subtilisin BPN′ (*25*). In contrast with the *E. coli* expression system, as described below, A-31T mutant subtilisin was abundantly excreted in the active form by the *B. subtilis* system, suggesting that *B. subtilis* might provide factors that accelerate the autoprocessing of the A-31T mutant subtilisin precursor, while *E. coli* does not.

Using the same purification procedure as for the wild-type subtilisin, three single mutant subtilisins (A-31T, A88V, and A92T) and one double mutant (A88V/A98T) were purified to a high degree from the culture supernatant of each *B. subtilis* transformant. Hydrolytic activity at 10°C of the wild-type, triple mutant (m-51), three single mutants, and one double mutant was compared based on the k_{cat}/K_m value. As shown in Table II, the A88V mutation alone showed no effect, and A98T mutation showed a positive (30% increase) contribution to the subtilisin activity compared to wild-type subtilisin. Interestingly, the combination of A98T and A88V, each of which caused a different change in activity, produced the highest activity (70% increase).

TABLE I. **Kinetic parameters of purified wild-type and m-51 mutant for hydrolysis of AAPF at various temperatures.**

Sample	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($10^3\ s^{-1}\cdot M^{-1}$)	Relative value to wild-type
50°C				
Wild-type	164.9 (±14)	242.2 (±7)	6.8	1.0
m-51	176.3 (±18)	194.0 (±11)	9.1	1.3
37°C				
Wild-type	73.8 (±6)	190.1 (±9)	3.9	1.0
m-51	90.6 (±6)	182.0 (±9)	5.0	1.3
25°C				
Wild-type	36.0 (±3)	141.9 (±6)	2.5	1.0
m-51	50.0 (±4)	115.1 (±5)	4.3	1.7
10°C				
Wild-type	20.6 (±2)	135.3 (±5)	1.5	1.0
m-51	30.4 (±2)	112.8 (±6)	2.7	1.7

Enzyme activity was assayed using acetone precipitated subtilisin samples and *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as the substrate. The protease concentration was estimated by active titration with SSI. Details of the assay are given in "MATERIALS AND METHODS."

TABLE II. **Kinetic parameters of purified wild-type and m-51 mutant, and its derivatives for hydrolysis of AAPF at 10°C.**

Sample	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($10^3\ s^{-1}\cdot M^{-1}$)	Relative value to wild-type
Wild-type	20.6 (±3)	135.3 (±5)	1.5	1.0
m-51	30.4 (±2)	112.8 (±6)	2.7	1.7
A-31T	20.7 (±2)	136.5 (±6)	1.5	1.0
A88V	18.9 (±2)	130.7 (±4)	1.5	1.0
A98T	26.4 (±3)	130.5 (±6)	2.0	1.3
A88V/A98T	29.1 (±1)	110.0 (±2)	2.6	1.7

Enzyme activity assay was carried out as described in "MATERIALS AND METHODS."

Effect of Mutations on Thermal Stability of Subtilisin—In general, cold-active enzymes originating from psychrophilic microorganisms display a higher thermosensitivity than their mesophilic counterparts (*2*). The parameter often used to measure subtilisin stability is the rate of irreversible inactivation and autolysis at elevated temperature (*26*). We examined the thermal stability of mutant subtilisins along with the wild-type subtilisin in terms of autolysis. When the rate of thermally induced inactivation was measured at 60°C in the presence of Ca^{2+}, all of the mutant enzymes showed almost the same half-time as did the wild-type (data not shown).

Substrate Specificity of m-51—When the substrate AAPF was changed to AAPL, specific proteolytic activity-temperature profile was basically unchanged. This result indicates that no drastic conformational change occurs in the substrate binding area. This was also confirmed by computer-aided modeling analysis (described below).

Analysis of Mutation Effects Based on Molecular Modeling—The rms deviation of the A88V single mutant is 0.080 Å from the wild type for the alpha-carbon atoms; that of the A98T single mutant 0.086 Å; and that of the A88V/A98T double mutant 0.050 Å. For the region from the 88th residue through the S3 site (Tyr104) *via* the 98th residue, the secondary contact region (Asp99 and Gly100), the S1 site (Gly102), and the S2 site (Gln103), the rms deviations of the alpha-carbon atoms are plotted as shown in Fig. 2. There are maximums in the vicinity of the mutation point and the S2 site in the A88V single mutant. In the A98T single mutant, the deviation is large overall, and the transition of the region from Val95 through Gly102, which contains the mutation point and the S1 site, is especially large. On the other hand, in the A88V/A98T double mutant, the deviation almost disappears. The most likely explanation for this is that a synergistic effect operates in the A88V/A98T double mutant to counterbalance the deviations introduced by the mutations of Ala88 to Val and Ala98 to Thr. Because the shift in the overall structure of the A88V/A98T double mutant from that of the wild type is the smallest, and little change occurs in the region from the S1 through S3 site, the K_m level of the

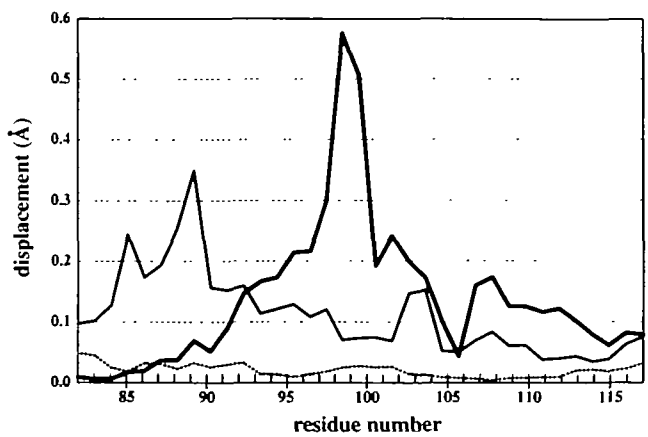

Fig. 2. **Plots of the displacements of alpha carbon positions of the mutants from the wild-type structure.** The displacement of the A98T single mutant is shown by the thick line; the displacement of the A88V single mutant, by the thin line; and the displacement of the A88V/A98T double mutant, by the dotted line.

double mutant must be equal to that of the wild type. The synergistic effect in the double mutant by which the structural change is counterbalanced decreases K_m by a yet unknown mechanisms.

In summary, by evolutionary engineering, we isolated a cold-adapted mutant (m-51) of subtilisin BPN′ with three residue-substitutions (A-31T/A88V/A98T) which shows higher catalytic efficiency than the wild-type enzyme by 70% at 10°C, and showed that the double mutation A88V/A98T is sufficient for this improvement. In contrast to the catalytic efficiencies of the two previous mutant enzymes (M-15 and m-63), which are improved due to a decrease in the K_m value (*6, 7*), the catalytic efficiency of the mutant enzyme m-51 was improved through an increase in the k_{cat} value. Although naturally occurring cold-active enzymes tend to have conformational flexibility (*27, 28*), Arnold *et al.* currently succeeded in acquiring an evolved enzyme with significantly increased thermal stability without cost to its activity at lower temperature (*29*). This suggests that there may be a lot of different patterns for achieving cold-adaptation of enzyme, regardless of whether the two properties are inversely correlated or not correlated at all.

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