RESEARCH

ENZYME ENGINEERING FOR NONAQUEOUS SOLVENTS: RANDOM MUTAGENESIS TO ENHANCE ACTIVITY OF SUBTILISIN E IN POLAR ORGANIC MEDIA

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Enzyme activity is often dramatically reduced in polar organic solvents, even under conditions where the folded structures are stable. We have utilized random mutagenesis by polymerase chain reaction (PCR) techniques combined with screening for enhanced activity in the presence of dimethylformamide (DMF) to probe mechanisms by which improved enzymes for chemical synthesis in polar organic media might be obtained. Two amino acid substitutions which enhance subtilisin E activity in the presence of DMF, Q103R and D60N, were identified by screening on agar plates containing DMF and casein. The two substitutions are located near the substrate binding pocket or in the active site, and their effects on the catalytic efficiency k_{cat}/K_M for the hydrolysis of a peptide substrate are additive. The effects of D60N are apparent only in the presence of DMF, highlighting the importance of screening in the organic solvent. Protein engineering is an effective approach to enhancing enzyme activity in organic media: the triple mutant D60N+Q103R+N218S is 38 times more active than wild-type subtilisin E in 85% DMF. An evolutionary approach consisting of multiple steps of random mutagenesis and screening in continually higher concentrations of organic solvent should result in enzymes that are substantially more active in organic media.

Although enzymes catalyze a number of chemical transformations important to the chemical and pharmaceutical industries, they enjoy only limited use as industrial catalysts. The limitations stem largely from the narrow range of conditions enzymes can tolerate. Potential applications in chemical synthesis are greatly expanded if enzymes can function in polar organic solvents, where organic substrates are much more soluble. Other potential advantages of enzyme catalysis in organic solvents include novel chemistry and absence of microbial contamination[1,2]. Unfortunately, many potentially useful enzymes exhibit poor catalytic activities in polar organic solvents.

Solvent participates in the forces that determine an enzyme's structural stability as well as its ability to stabilize a reaction transition state, and it is reasonable to assume that the new balance of forces attained when an enzyme is dissolved in a polar organic solvent will be optimal neither for stability nor activity. Protein engineering is a promising approach to creating enzymes that function in polar organic solvents; changes in amino acid sequence may allow the enzyme to recover some of its lost stability and catalytic potential. A set of general design rules for engineering enzymes that are stable in organic solvents[3] is being tested by site-directed mutagenesis of subtilisin E and α-lytic protease[4–6]. The immediate loss of activity that occurs upon transfer to an organic solvent, however, often cannot be attributed to denaturation; enzymes that are quite stable in organic media also exhibit poor activity. Because little is known of the mechanisms by which polar solvents reduce the activity of soluble enzymes, we have relied on a random mutagenesis approach to improve catalytic performance.

Random mutagenesis is most practical when coupled with an efficient screening or selection procedure to identify clones expressing variant enzymes with the properties of interest. We have developed an efficient screen for enhanced activity of a secreted protease based on visual discrimination of halos produced by individual colonies on agar plates containing casein and organic solvent. The serine protease subtilisin E is used in this study because of its potential applications in peptide synthesis and transesterification reactions in organic solvents[7,8]. In addition, the structure and function of this and homologous subtilisins have been well characterized[9–11], which aids interpretation of the random mutagenesis results.

RESULTS

Random mutagenesis and isolation of Q103R and Q103R+D60N subtilisin E. A randomly-mutated mini-DNA library of subtilisin E was constructed by inserting a PCR-amplified gene fragment into *B. subtilis* expression vector pKWC (see Experimental Protocol). Of approximately 300 *B. subtilis* clones tested for expression of active subtilisin on casein plates 72% produced visible halos. Twenty-seven clones which produced halos larger than clones expressing wild-type subtilisin E on casein plates containing DMF were grown in liquid media, and the supernatants were assayed for proteolytic activity on the specific substrate suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (sAAPF-pna). One clone exhibited significantly higher

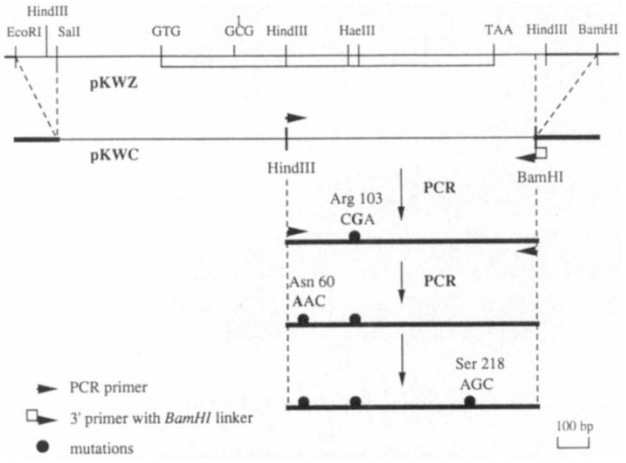

FIGURE 1 General scheme for construction of plasmid pKWC and random mutagenesis of subtilisin E, whose coding region is framed in pKWZ. Two regions of pKWZ deleted to generate pKWC are indicated by dashed lines. Base substitutions obtained after random mutagenesis by PCR and for the N218S mutation are indicated on the PCR-targeted HindIII-BamHI fragments.

proteolytic activity than wild-type in both aqueous buffer and in the presence of 10% DMF. DNA sequencing of the subtilisin E gene from this clone and the wild-type gene from pKWC showed that two base substitutions had occurred in the coding region. One was a silent mutation (replacement of A by C) leading to no change in the amino acid sequence, while the substitution of A by G at bp 762 of the subtilisin E gene sequence[12] led to the replacement of Gln by Arg at amino acid position 103 (Fig. 1). This Q103R gene was used as the template for a second round of mutagenesis by PCR. The resulting variants were screened by the procedure described above, and a variant exhibiting activity similar to that of Q103R in aqueous buffer, but higher activity in the presence of DMF, was selected. Sequencing of the gene from this clone determined that a third base substitution had occurred at bp 632 (replacement of G by A), leading to the replacement of Asp 60 by Asn.

Effects of the mutations on amidase activity. Kinetic parameters k_{cat} and K_M for sAAPF-pna hydrolysis were determined for the purified variant and wild-type subtilisins E. As shown in Table 1, the catalytic efficiency k_{cat}/K_M of Q103R is about three times that of wild-type subtilisin E in aqueous buffer and in the presence of 10% DMF, due to a corresponding decrease in K_M. The double mutant Q103R+D60N exhibits enhanced activity mainly in the mixed solvent: while the mutation at position 60 has little effect on catalysis in aqueous buffer (compared to Q103R), it increases k_{cat} by a factor of 2 in the presence of DMF. Q103R+D60N is nearly 9 times more efficient than wild-type subtilisin E in the presence of 20% DMF and is twice as efficient as Q103R.

A triple mutant is more active and more stable in DMF. A triple mutant, D60N+Q103R+N218S, was created by inserting into the random mutant Q103R+D60N a third mutation, N218S, known to increase the stability and catalytic activity of subtilisin E in mixed solvents[5]. This triple mutant is approximately 30 times more efficient than the wild-type enzyme at hydrolyzing sAAPF-pna in 20% DMF and 10 times better in aqueous buffer (Table 1). Kinetic parameters (k_{cat}/K_M) for the hydrolysis of a second peptide substrate suc-Ala-Ala-Pro-Met-p-nitroanilide (sAAPM-pna) by wild-type subtilisin E and the triple mutant were determined over a range of DMF concentrations up to 85 (v/v)%. As shown in Figure 2, DMF drastically reduces the activity of both enzymes: 85% DMF reduces k_{cat}/K_M by four orders of magnitude. The enhanced activity of the triple mutant observed in low concentrations of DMF is also observed in 85% DMF, where the variant is 38 times more efficient than the wild-type enzyme. D60N+Q103R+N218S is as active in 85% DMF as the wild-type enzyme in 50% DMF.

The deactivation rates of D60N+Q103R+N218S and wild-type subtilisin E were determined in 40% DMF at 50°C. As shown in Figure 3, the triple mutant is more stable than wild-type subtilisin E, with a half-life of approximately 9 hours, compared to 6 hours for wild-type enzyme.

The effects of all three mutations are additive. To study the effects of the individual mutations and the extent to which they are additive, the single mutant D60N and the double mutant D60N+N218S were constructed. Kinetic parameters for these variants are reported in Table 1. The single mutation D60N improves k_{cat} in the presence of DMF, while having no significant effect on K_M. The incremental free energies of transition state stabilization ($\Delta\Delta G^{\ddagger}$) for the hydrolysis of sAAPF-pna are also summarized in Table 1 for each of the single, double and triple variants. The free energy contribution of N218S to hydrolysis of this substrate has been reported previously (0.61 kcal mol^{-1} in 20% DMF)[5]. The $\Delta\Delta G^{\ddagger}$ contributions are additive in each of the double variants Q103R+D60N and D60N+N218S. The three individual mutations also show near-perfect additivity in their $\Delta\Delta G^{\ddagger}$ contributions in the triple mutant, both in aqueous media and 20% DMF, where 2.15 kcal mol^{-1} of transition state stabilization is achieved. The three mutations contribute a comparable $\Delta\Delta G^{\ddagger}$ (2.17 kcal mol^{-1}) for the hydrolysis of sAAPM-pna in 85% DMF (Fig. 2).

DISCUSSION

Modified PCR is a simple and effective method for generating randomly-mutated gene libraries. Previous studies indicated that 25 cycles of modified PCR would produce point mutations with a frequency of approxi-

TABLE 1 Kinetic constants and incremental free energies of transition state stabilization ($\Delta\Delta G^{\ddagger}$) for hydrolysis of sAAPFpna by wild-type and variant subtilisins E. Conditions are 0.1 M Tris-HCl, 10 mM $CaCl_2$, pH 8.0 and stated amount of DMF. Errors in reported K_M and k_{cat} are ±10%. The effects of amino acid substitutions on transition state stabilization energies $\Delta\Delta G^{\ddagger}$ for the hydrolysis reaction were determined from the specificity constants k_{cat}/K_M: $\Delta\Delta G^{\ddagger} = -RT \ln (k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild\text{-}type}$

	0% DMF				10% DMF				20% DMF	
	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M M^{-1} s^{-1} × 10^{-3}	$\Delta\Delta G^{\ddagger}$ kcal mol^{-1}	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M M^{-1} s^{-1} × 10^{-3}	$\Delta\Delta G^{\ddagger}$ kcal mol^{-1}	k_{cat}/K_M M^{-1} s^{-1} × 10^{-3}	$\Delta\Delta G^{\ddagger}$ kcal mol^{-1}
WT	21	0.56	38	-	18	2.8	6.3	-	1.4	-
Q103R	31	0.25	124	−0.73	32	1.4	23	−0.80	6.8	−0.98
D60N	22	0.53	42	−0.06	30	3.2	9.6	−0.26	3.1	−0.51
Q103R+D60N	26	0.20	130	−0.76	43	1.2	36	−1.07	12	−1.36
D60N+N218S	37	0.46	80	−0.46	54	2.7	20	−0.71	9.2	−1.18
Q103R+D60N+N218S	40	0.11	360	−1.39	71	0.70	110	−1.76	45	−2.15

mately 2%, and both transition and transversion base substitution could be generated[13]. In the current study, PCR was carried out for 25 cycles on an 800 bp DNA template. Sequencing six of the PCR products revealed 1–2 base substitutions per DNA fragment. Both transition and transversion mutations were found. Twenty-eight percent of the clones did not express functional subtilisin, indicating that the mutation(s) rendered the enzyme inactive, unstable, or incapable of being processed. Because it is limited to creating single base substitutions, random mutagenesis by PCR is unable to provide a truly random sampling of all possible amino acid substitutions. This was not deemed a problem in this study because only a minute fraction of the astronomical number of possible random variants can be screened.

A sensitive and efficient screening method is needed to identify mutants with enhanced catalytic activity in organic solvents. Several screening steps, starting on casein plates without DMF and progressing to plates and liquid cultures with DMF, were used to isolate the interesting variants. The final screen for hydrolysis of sAAPF-pna highlights those variants that are more active towards the particular peptide substrate. To isolate mutants that exhibit increases in k_{cat} rather than enhanced affinity for the particular substrate (lower K_M), the final screen should include more than one substrate or a mixture of substrates (e.g. casein)[14]. The Q103R variant identified using this approach exhibits enhanced activity towards the hydrolysis of sAAPF-pna in both aqueous media and in the mixed solvent, a result of tighter substrate binding reflected in the reduced K_M. Position 103 is on the protein surface, near the substrate binding pocket. Neighboring Tyr 104 is directly involved in substrate binding[15], and the replacement of Gln 103 by the larger Arg side chain may influence Tyr 104-substrate interactions. Varying ionic strength and replacing the succinyl protecting group with an unprotected amino terminus indicate that the positively-charged Arg 103 favors binding to the negatively-charged succinyl group of the substrate[14].

Screening in the organic solvent would be superfluous if the behavior of a variant in the presence of DMF always mirrors its behavior in aqueous media. The second random variant Q103R+D60N, produced by random mutagenesis of Q103R, showed higher activity only in the mixed solvent; it is clear that there exist mechanisms for altering activity that are apparent only during screening in the presence of the organic solvent. Residue 60 is located in the active site, close to His 64 of the catalytic triad. This mutation involves the replacement of a negatively-charged residue by a neutral one, which will alter the charge environment of the active site and His 64 in particular. Further characterization of this variant may lend insight into mechanisms for enhancing k_{cat} in polar organic media.

To reduce the number of uninteresting variants that must be screened, it is useful to limit the random mutagenesis to a particular set of positions. Amino acid substitutions near or in the enzyme's active site and substrate binding pocket are most likely to affect catalysis. Thus far, we have not isolated any interesting variants containing mutations far from the active site or substrate pocket. The random mutations found in this study indicate that effective substitutions can occur at amino acid positions that are highly conserved as well as those that are not. Gln 103 is not conserved; this position is occupied by Ser in subtilisin Carlsberg[10] and Thr in thermitase[16]. Asp 60, on the other hand, is highly conserved; this residue is replaced by another amino acid (Asn) only in thermitase. Since these enzymes have evolved to function in aqueous environments, even highly conserved residues in the active site are reasonable targets for substitution to enhance performance in organic solvents.

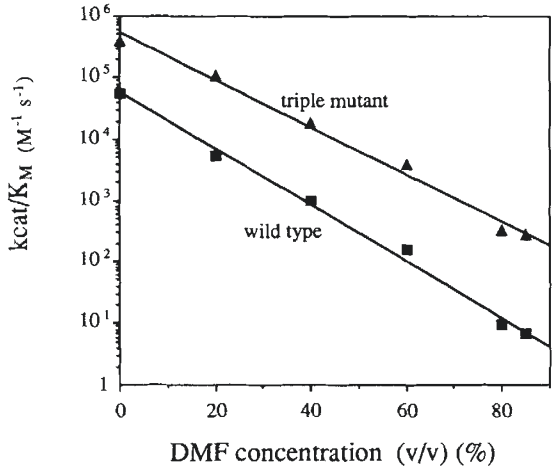

FIGURE 2 Catalytic efficiency for hydrolysis of sAAPM-pna by wild-type subtilisin E (■) and triple mutant Q103R+D60N+N218S (▲). Specificity constants k_{cat}/K_M were determined from initial rate experiments at low substrate concentration (0.01–0.1 K_M) in 0.1 M Tris-HCl, 10 mM $CaCl_2$, pH 8.0 with stated amounts (v/v %) of DMF.

The screening for enhanced activity in organic solvent was carried out in relatively low concentrations of DMF (10–35%). Screening in high solvent concentrations, where the activity is greatly reduced, is considerably more difficult and requires high concentrations of enzyme and of the expensive substrate (whose solubility is limited). This study has shown that the effects of amino acid substitutions on relative catalytic activity can be apparent in only 10–20% DMF. This strategy of screening in low solvent concentrations may miss some useful mutations, however, since some mechanisms for activation may operate only in high concentrations of the organic component. This was found to be the case for altering the stability of subtilisin E by surface charge substitutions: replacing a charged surface residue stabilizes the enzyme in 80% DMF, but not in 40%[6]. Since the catalytic activity improves with the accumulation of multiple mutations, the concentration of solvent used during screening can be steadily increased in subsequent steps. For example, if the triple mutant is used as the template for the next round of mutagenesis, screening could be carried out in 60% DMF rather than 30%.

Engineering an enzyme that is truly optimized to function in a polar organic solvent will probably require the accumulation of multiple mutations. If the effects of individual mutations are additive, however, they can be identified in separate mutagenesis and screening experiments and subsequently combined. A triple mutant was constructed by combining the double random variant D60N+Q103R with N218S, a mutation that was discovered by random mutagenesis of subtilisin BPN' and screening for enhanced thermostability[17]. In water, the triple variant is 7 times more efficient towards the hydrolysis of sAAPM-pna than wild-type subtilisin E; it is 38 times more active in the presence of 85% DMF (Fig. 2). As shown in Table 1, Q103R+D60N+N218S stabilizes the transition state by an incremental free energy that is essentially the sum of all the contributions from each of the single mutants. The additivity of these mutations is likely due to the fact that positions 103 and 218 are located at opposite ends of the substrate binding pocket and are also distant from residue 60 (more than 17 Å).

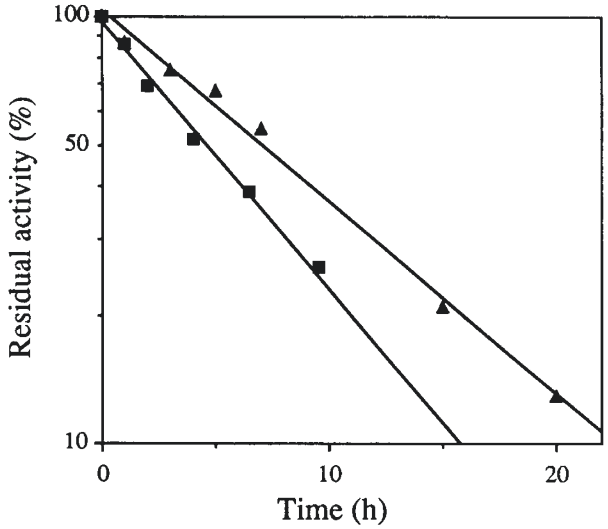

FIGURE 3 Deactivation of wild-type (■) and Q103R+D60N+N218S (▲) subtilisin E in 40% DMF, 50°C.

These substitutions apparently do not cause more than very localized perturbations in the enzyme's structure. Even if mutations begin to interact in the confined space of the active site, effective amino acid substitutions can be accumulated by the "evolutionary" process of sequential mutagenesis in which variant DNA is used as template for generating the next mutation. By screening in higher and higher organic solvent concentrations, this evolutionary approach may lead to dramatically improved enzyme catalysts.

The mutations identified by screening for enhanced activity in the presence of DMF destabilize subtilisin E slightly (data not shown). This loss of stability is more than compensated for by the stabilizing effect of N218S (Fig. 3). As a result, the triple variant which exhibits much higher catalytic activity than wild-type subtilisin E in DMF is also slightly more stable.

EXPERIMENTAL PROTOCOL

Construction of the *B. subtilis* expression vector pKWC. A DNA fragment containing a HindIII site was removed from the upstream region of the subtilisin promoter of vector pKWZ (kindly provided by R. Doi, UC Davis)[18] by double digestion with EcoRI and SalI. The sticky ends of the plasmid were filled using Klenow fragment and ligated using T4 DNA ligase. A HindIII site downstream of the subtilisin gene was also removed using a BamHI linker attached to the PCR 3′ primer to generate pKWC (Fig. 1).

Random mutagenesis using modified PCR. The HindIII-BamHI DNA fragment encoding mature subtilisin E from amino acid residue 49 to the C-terminus[14] was chosen as the target for random mutagenesis. Two oligonucleotides, 5′ GCGGAG-CAAGCTTCGTAC 3′ and 5′ CGGGATCCTGCAGGATTCAA-CATGCGGAG 3′, were used as 5′ and 3′ PCR primers, respectively. PstI and BamHI restriction sites were added to the 3′ primer for convenience in the subsequent cloning steps. The PCR was carried out essentially as described previously[19,13] on 50 ng single-stranded template DNA. A 100 µl reaction mixture contained 10 mM Tris-HCl, pH 8.0, 20 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 10 mM β-mercaptoethanol, 10 µl DMSO, 1 mM each dGTP, dCTP and dTTP, 0.2 mM dATP, 1.5 µl each of the 5′ and 3′ primers (0.4 mg/ml), and 0.5 µl *AmpliTaq*™ DNA polymerase (5U/µl) (Perkin Elmer-Cetus). PCR was carried out by melting the template DNA at 94°C for 1 min and annealing with primer at 42°C for 2 min. Chain extension was initiated with DNA polymerase at 72°C for 3 min, and a total of 25 cycles were performed. After the last cycle, the polymerization at 72°C was extended for an additional 7 min. The size and yield of the amplified DNA fragments were determined by agarose gel electrophoresis. To construct a randomly mutated mini-DNA library, plasmid pKWC and the PCR-generated subtilisin DNA fragments were digested with HindIII and BamHI. Appropriate vector and insert fragments were separated by agarose gel electrophoresis, purified and ligated. These were used to transform a subtilisin-deficient *B. subtilis* strain, DB428 (kindly provided by R. Doi, UC Davis), using a modification of the procedure of Dubnau and coworkers[20,21].

Screening for enhanced subtilisin E activity in DMF. Following transformation, cells were plated onto agar plates containing modified Schaeffer's medium[22] and 1% casein. When the expression of the subtilisin reached a maximal level (determined by the size of the halo), the clones were transferred using nitrocellulose paper to a second set of agar plates containing DMF (27.5–35%) and 1% casein. Under these conditions a clone expressing wild-type subtilisin E does not produce a detectable halo. These plates were incubated at 37°C for 12 to 20 h. A clone expressing wild-type subtilisin E and a clone expressing a variant known to be more active in DMF (N218S) were also plated as controls. Although the transferred clones could not survive on the DMF plates, subtilisin secreted by some of the clones produced halos larger than wild-type and N218S, and these clones were selected for further study. Cells from these positive clones were collected and grown in 2 ml modified Schaeffer's medium at 37°C for 16 h to 20 h. A small volume of supernatant from each culture was used to assay subtilisin activity in 10 mM Tris-HCl, pH 8.0 and 10 mM $CaCl_2$, with and without 10% DMF, as described below.

Sequencing of the mutant subtilisin genes. The PCR-amplified HindIII-BamHI fragment of the mutant subtilisin gene was subcloned into pUC119 and pUC118[23] to obtain different orientations of the insert for sequencing. Single-stranded DNA was isolated from *E. coli* infected with M13 KO7 helper phage[23] for sequencing by the dideoxynucleotide-chain-termination method[24] with T7 DNA polymerase[25]. A DNA template of wild-type subtilisin was used as the control.

Purification of the mutant protein. Purification of wild-type and variant subtilisins E was carried out according to published protocols[26]. The *B. subtilis* were grown at 37°C in 100 ml SG medium for approximately 36 h. Subtilisin was recovered from the medium by ammonium sulfate precipitation followed by two acetone precipitation steps. Following each precipitation, protein was dialysed against 0.01 mM sodium phosphate buffer, pH 6.2. The enzyme was then purified by ion exchange chromatography on CM Sepharose, with a gradient of 0–0.4 M NaCl in 10 mM sodium phosphate buffer, pH 6.2, followed by dialysis against 10 mM tris-HCl, pH 8.0 and 2mM $CaCl_2$. Protein concentrations were determined using the Bio-Rad protein assay, as described by the supplier. The purities of the variant and wild-type subtilisin E preparations were determined by SDS PAGE[27]. A major band with a molecular weight about 27 kD was detected in each protein sample (data not shown), and the purity was estimated to be greater than 95%.

Constructions of D60N, D60N+N218S and Q103R+D60N+N218S subtilisin E. DNA fragments from double mutant, wild-type and single mutant N218S were obtained by double digestion with HindIII and BamHI, followed by partial digestion with HaeIII. D60N was created by ligating the appropriate HindIII-HaeIII DNA fragment from the Q103R+D60N double mutant with the corresponding HaeIII-BamHI DNA fragment from wild-type subtilisin gene. D60N+N218S was created by ligating the appropriate HindIII-HaeIII fragment of D60N with the corresponding HaeIII-BamHI fragment of N218S (Fig. 1). The constructions were confirmed by DNA sequencing. The HindIII-BamHI DNA fragments containing the mutations were reinserted into pKWC and transformed into *B. subtilis* DB428.

Enzyme kinetics. Subtilisin amidase activities were measured on the substrates sAAPF-pna or sAAPM-pna at 37°C in 0.1 M tris-HCl, pH 8.0 and 10 mM $CaCl_2$. Assays were initiated by mixing the enzyme and the substrate and the reaction buffer, and the amount of released *p*-nitroaniline was measured spectrophotometrically at 410 nm as a function of time. For activity measurements in the mixed solvent, the aqueous buffer was replaced by DMF to achieve the indicated percentage (v/v) of DMF. K_M can k_{cat} values for hydrolysis of sAAPFpna by the subtilisin variants were obtained by nonlinear regression of the data using the program Enzfitter (Biosoft). k_{cat} values determined in the presence of DMF were corrected for the effects of DMF on the extinction coefficient of *p*-nitroaniline.

Enzyme stability. Enzyme stabilities at 50°C in 40 (v/v) % DMF were determined as described previously[6]. The enzymes were incubated at 50°C in 10 mM Tris-HCl, pH 8.0, 2mM $CaCl_2$ with 40% DMF. The residual amidase activities were measured over time by diluting not more than 100 µl of the enzyme solution into

a standard 1.5 ml buffer mixture, as described above.

Acknowledgments

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