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Stabilization of xylanase by random mutagenesis

Akemi Arase[a], Tetsuya Yomo[a], Itaru Urabe[a], Yasuo Hata[b], Yukiteru Katsube[c] and Hirosuke Okada[a,*]

[a]*Department of Biotechnology, Faculty of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565, Japan,* [b]*Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611, Japan* and [c]*Institute for Protein Research, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565, Japan*

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Four heat-resistant mutants of xylanase (N56, N102, N104 and F1) were obtained by random mutagenesis. The mutant genes had the following amino acid changes: N56, Ser-26 to Trp, Gly-38 to Asp and Thr-126 to Ser; N102, Gly-38 to Asp; N104, Gly-38 to Ser and Arg-48 to Lys; F1, Ser-12 to Cys. Kinetic studies showed that N104 is stabilized by an increase in the activation enthalpy, while the other mutants are stabilized by a decrease in the activation entropy.

Xylanase; *Bacillus pumilus*; Random mutagenesis; Enzyme stabilization; Heat-resistant mutant

1. INTRODUCTION

It is generally believed that enzymes have become well-adapted to their physiological environment and are at their optimum state; therefore, most mutations are thought not to improve the properties of enzymes. Accordingly, the improvement of enzymes has been sought by well-designed site-directed mutagenesis rather than by random mutagenesis. With respect to stability, however, enzymes may not be at their optimal state. In fact, we have obtained many stability-increased mutants of glucose dehydrogenase [1,2]. If this is a common feature of enzymes from mesophiles, random mutagenesis should be a strong method for the stabilization of enzymes. In addition, if randomly-occurring stability-increasing mutations are assigned on the three-dimensional structure of an enzyme, they should provide us with valuable information on enzyme stabilization.

Xylanase (EC 3.2.1.8) is a potentially important enzyme for the use of xylan in agricultural wastes. A xylanase gene (*xynA*) was cloned from *Bacillus pumilus* IPO [3], sequenced [4] and the enzyme was crystallized [5], and its structure was determined by X-ray crystallography at 2.2 Å resolution (unpublished results). In this work, the xylanase gene was randomly mutagenized, and four heat-resistant mutants were obtained. The results obtained here clearly show the usefulness of random mutagenesis for the stabilization of enzymes.

Correspondence address: I. Urabe, Department of Biotechnology, Faculty of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565, Japan.

**Present address:* Department of Applied Microbial Technology, The Kumamoto Institute of Technology, 4-22-1 Ikeda, Kumamoto 860, Japan.

2. EXPERIMENTAL

2.1. *Materials*

Restriction endonucleases and other enzymes for DNA manipulation were purchased from Toyobo Co. Ltd. (Osaka). The following bacterial strains, bacteriophages, and plasmids were used: *Escherichia coli* strains JM103 (*Δ(lac-pro) thi strA supE endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q} *lacZ M15*) [6,7] and KP3998 (F$^-$ *hsdS20* (r_B^- m_B^-) *ara-14 proA2 lac I*^{q} *galK2 rpsL20 xyl-5 mlt-1 supE44* λ$^-$) [8]; M13mp18 and mp19 phages; and plasmid pHIX312 [9] containing *xynA*. *E. coli* KP3998 was a generous gift from Dr. T. Miki (Kyushu University).

2.2. *Random mutagenesis*

pHIX312 [9] was digested with *Hin*cII and *Bam*HI, and the 0.75-kb DNA fragment containing the xylanase gene was inserted into M13mp18 and M13mp19. The single-stranded DNA of the hybrid plasmid was treated at 20°C with hydrazine (for 5–20 min), formic acid (5–20 min), or sodium nitrite (1–3 h) by the method of Myers et al. [10]. The chemically mutagenized single-stranded DNA was annealed with P1 primer and made into a duplex form by reverse transcriptase [10]. The duplex DNA was digested with *Bst*EII and *Bam*HI, the resulting 0.72-kb fragment containing the main part of *xynA* (lacking the 30-bp 5′-terminal sequence of *xynA*) was ligated with pHIX312 that had been digested with *Bst*EII and *Bam*HI to remove its wild-type *xynA* sequence, and *E. coli* KP3998 was transformed with the hybrid plasmid.

2.3. *Purification of xylanases*

E. coli KP3998 cells harboring a plasmid containing the wild-type or each mutant gene of xylanase were grown on LB medium [11]. (1 liter) as described previously [9]. The cells were suspended in 50 mM potassium phosphate buffer (pH 6.5) containing DNase (3 U/ml) and RNase (0.1 U/ml), disrupted with a French pressure cell, and the supernatant was obtained by centrifugation. Each xylanase was purified from the supernatant by stepwise column chromatographies of DEAE-Sepharose CL-6B and CM-Sephadex C-50 as described previously [12]. Xylanase activity was measured at 40°C as described previously [9,12], using oat spelt xylan (Sigma) as a substrate. The wild-type xylanase corresponds to the M-wild xylanase in the previous paper [9]; this enzyme has a methionine residue before the mature sequence, i.e. at the position of −1.

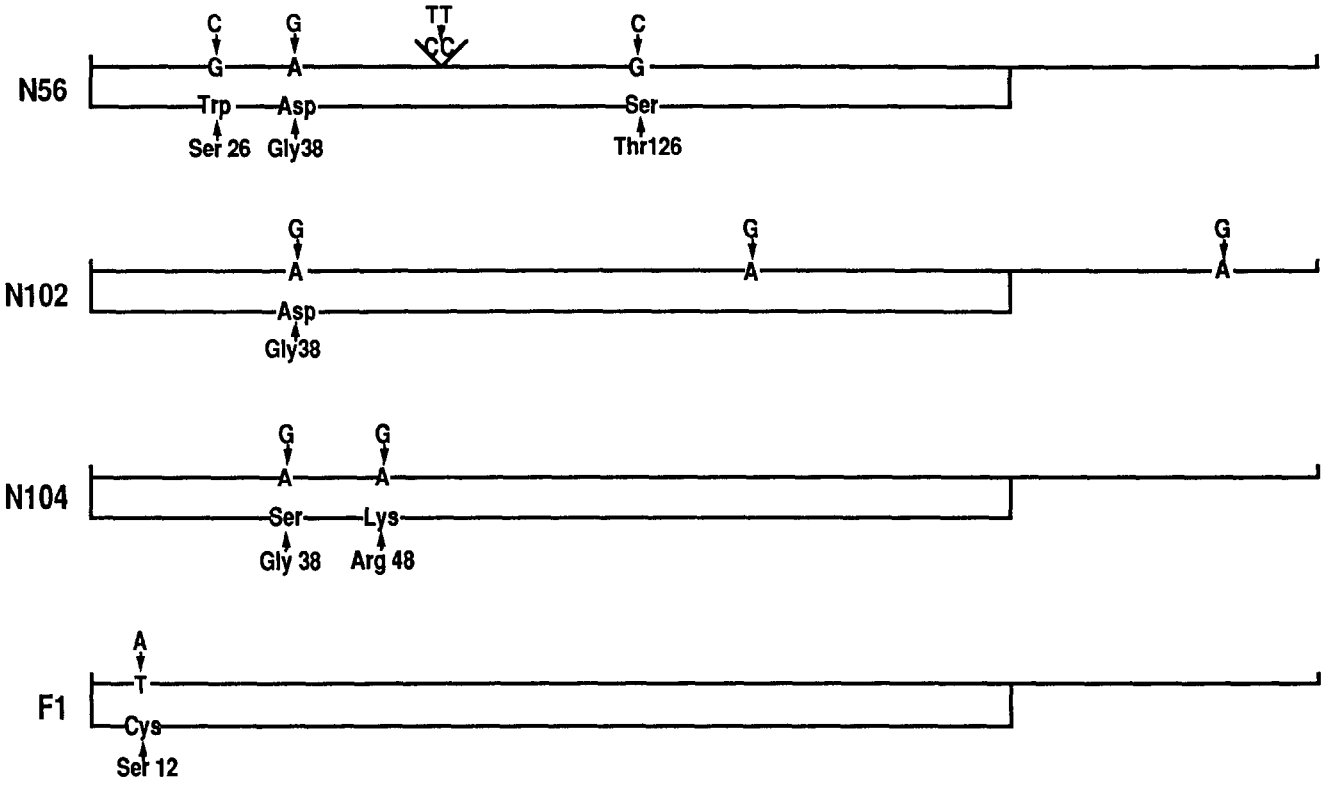

Fig. 1. Base changes and deduced amino acid substitutions in the mutant genes coding for heat-resistant xylanases. The box represents the structural gene of xylanase. We found the following errors in the previous sequence of the wild-type xylanase [4]: adenine at position 336 should be cytosine, adenine at 367 should be guanine, and cytosine at 615 should be thymine; the second error changes the deduced amino acid sequence from Ser-76 to Gly-76.

3. RESULTS

The xylanase gene was chemically mutagenized as described above. The transformants (about 60 000 colonies) harboring the hybrid plasmid containing the mutagenized xylanase gene were analyzed for heat-resistant enzyme activity by heat treatment (60°C for 30 min) followed by Congo red plate assay [13]. The wild-type enzyme is inactivated by the heat treatment and cannot be detected by the plate assay. Among the transformants, we obtained the following four positive clones: N56, N102, N104 and F1. N56, N102, and N104 were obtained by sodium nitrite treatment, and F1 by formic acid treatment.

The nucleotide sequences of the xylanase genes encoding heat-resistant mutant enzymes were identified by a Toyobo Sequence kit (Toyobo Co. Ltd). Fig. 1 shows the base and amino acid substitutions caused by the mutagenesis. The amino acid substitutions cluster in the N-terminal region, although the gene was mutagenized randomly. Especially, the mutation at Gly-38 is observed in all the mutants obtained by the mutagenesis with sodium nitrite. It is also noteworthy that N102 and F1 are stabilized by the following single amino acid substitutions: Gly-38 to Asp and Ser-12 to Cys, respectively. Therefore, Gly-38 and Ser-12 are the main target points for mutations that increase the heat resistance of this enzyme without a large loss of its activity.

The wild type and the four mutants of xylanase (N56, N102, N104, and F1) were purified as described in section 2.3. The homogeneity of the final preparation was checked by SDS-PAGE [14]; the purities of the wild type, N56, N102, N104, and F1 were then quantified with a densitometer to be 100%, 99.9%, 99.9%, 100% and 97.0%, respectively. In addition, these enzymes show a fused single precipitin line with rabbit antiserum against the wild-type enzyme by the double immunodiffusion test [15] (data not shown), thereby confirming the immunological identity of the mutant enzymes.

The effects of the amino acid substitutions in Fig. 1 on the thermostability of xylanase were investigated at various temperatures ranging from 51°C to 61°C, and the results at 57°C are shown in Fig. 2. At the temperatures tested, all the mutant enzymes are more heat-resistant than the wild type, and in the temperature range of less than 59°C, the order is N104 > N56 > N102 > F1. The heat inactivation rate constant (k) was calculated from the results as shown in Fig. 2 assuming first-order kinetics, and the results are shown in Fig. 3. The activation parameters for the heat inactivation were

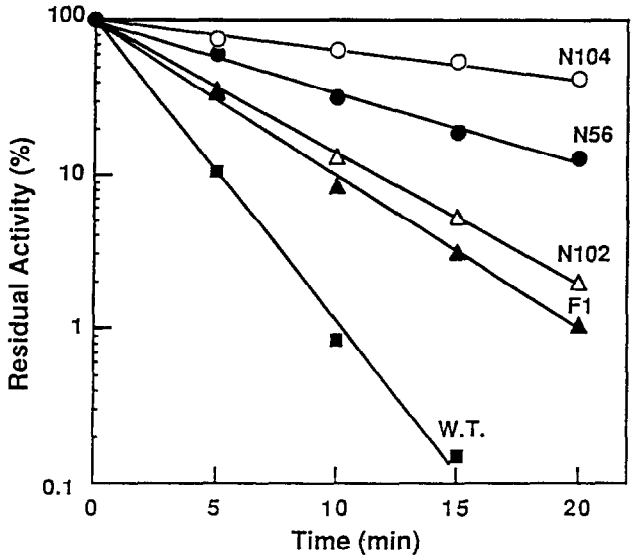

Fig. 2. Time course of heat inactivation of the wild type (W.T.) and mutant xylanases. The enzymes were heated at 57°C in 50 mM potassium phosphate buffer (pH 6.5). The residual activity was measured as described previously [9,12].

estimated from Fig. 3, and the values of the activation enthalpy (ΔH^*) and the activation entropy (ΔS^*) for the mutants are listed in Table I as the difference from those for the wild type. These results show that N104 is stabilized by an increase in ΔH^*, while the other mutants are stabilized by a decrease in ΔS^*. This means that the mechanism of stabilization is different between N104 and the others.

Table I also shows the specific activities of the mutant xylanases relative to that of the wild-type enzyme. The activity of N104 is about 20% of that of the wild type, but those of the other mutants are similar to or even

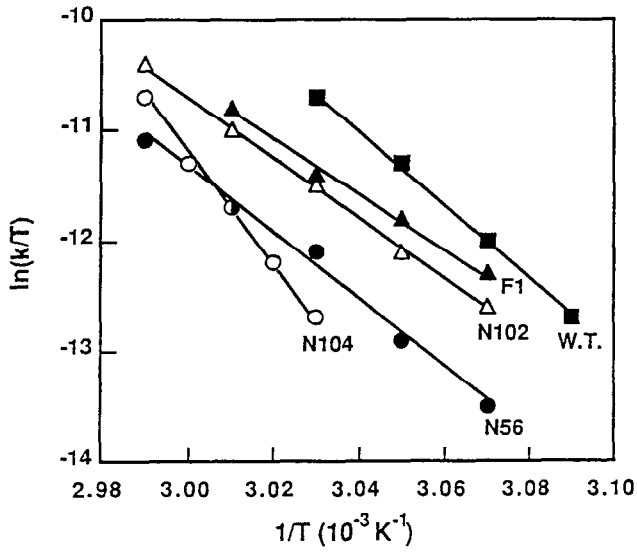

Fig. 3. Arrhenius plot for heat inactivation of the wild-type (W.T.) and mutant xylanases.

higher (N102) than that of the wild-type enzyme. Thus, xylanase was able to be stabilized by random mutagenesis without loosing the catalytic activity.

4. DISCUSSION

Four heat-resistant mutants of xylanase were selected from 60 000 mutant genes prepared by random mutagenesis, and Gly-38 and Ser-12 were assigned as the main target points for stability-increasing mutations. By substituting other amino acid residues at these positions, we will be able to optimize the mutations at these points for the stabilization of this enzyme. It is also important to investigate the additivity of the effects of these mutations including positions other than Gly-38 and Ser-12. In fact, N56 is more heat resistant than N102 owing to the presence of the following two additional mutations: Ser-26 to Trp and Thr-126 to Ser. Thus, random mutagenesis provides us with not only some desired mutants but also many promising strategies for further improvement of the mutants.

Fig. 4 illustrates the positions of the amino acid substitutions found in the heat-resistant mutants on the tertiary structure of xylanase. It is confirmed by computer-graphic simulation that all these substitutions can be accommodated without changing the wild-type conformation. For example, Ser-26 and Gly-38 are on the outer anti-parallel β-sheet, and the substituted residues at these positions protrude from the molecular surface into the solvent without obstructing the motion of the neighboring residues. In addition, it is suggested by the simulation that the carboxyl group of Asp-38 and the hydroxyl group of Ser-189 may form a hydrogen bond in the structures of N56 and N102.

How can we explain the effects of these mutations? For N102, a possible strengthening of the hydrogen bond described above may contribute to the stabilization by increasing ΔH^*. However, the experimental results show that ΔH^* is decreased by the mutation of Gly-38 to Asp, and N102 is stabilized by the decrease

Table I

Specific activity and activation parameters for heat inactivation of mutant xylanases

Enzyme	$\Delta\Delta H^*$ (kJ/mol)	$\Delta\Delta S^*$ (J/mol/K)	Specific activity (%)
N104	+120	+370	19
N56	−30	−100	100
N102	−50	−160	180
F1	−80	−230	100

The activation parameters were calculated from the results shown in Fig. 3, and expressed as the increase (+) or decrease (−) from those of the wild-type enzyme. The values for the wild-type are ΔH^* = 280 kJ/mol and ΔS^* = 560 J/mol/K. The specific activities of the mutants are expressed relative to that of the wild-type enzyme (100% = 241 U/mg).

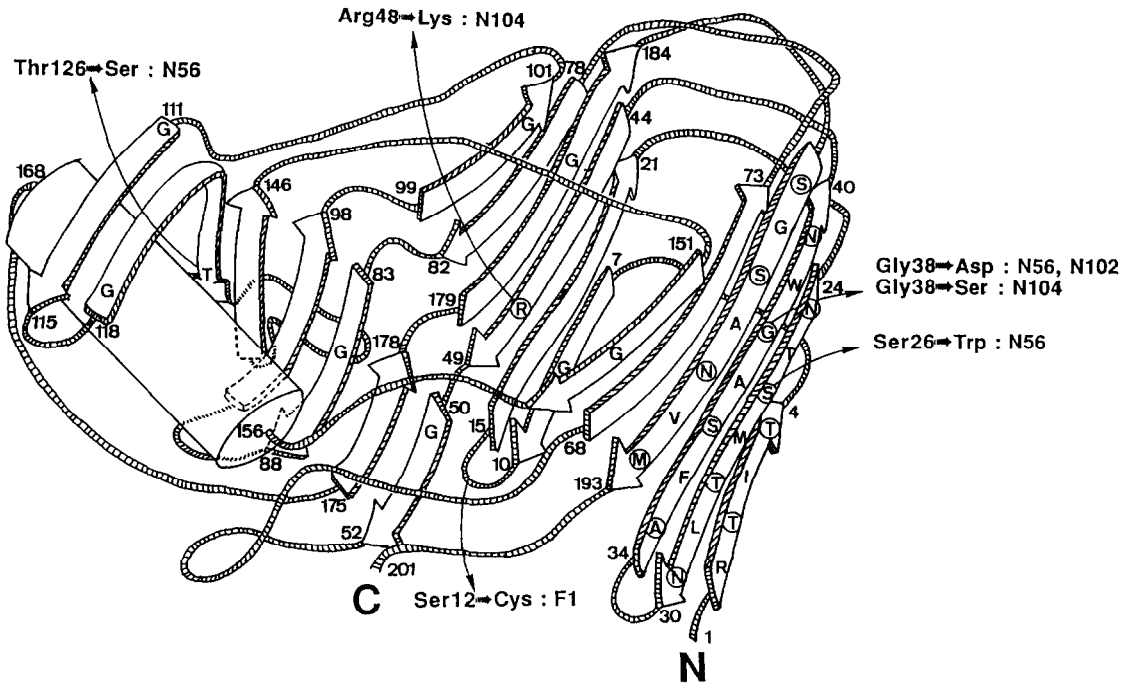

Fig. 4. Schematic illustration of the tertiary structure of xylanase showing the positions of the amino acid substitutions observed in the heat resistant mutants. All the glycine residues on the β-sheets are also shown. The amino acid residues on the β-sheets and protruding from the molecular surface are enclosed with circles.

in ΔS^* (Table I). Therefore, this explanation does not hold true here. On the other hand, Matthews et al. proposed that by replacing glycines at sites that do not interfere with the three-dimensional structure of a protein, it should be possible to decrease the entropy of unfolding of a protein and thereby increase its stability [16,17], and they demonstrated that such substitutions stabilize T4 lysozyme toward reversible and irreversible thermal denaturation [17]. The stabilization of xylanase by the mutation of Gly-38 to Asp (N102) could be explained by this theory, as ΔS^* for N102 is smaller than that for the wild type (Table I). However, the stabilization observed for N104, which contains the mutation of Gly-38 to Ser, is not entropic, and to the contrary, ΔS^* for N104 is larger than that for the wild-type enzyme (Table I). Thus, it is difficult to give a consistent explanation for the stabilizing effects of these mutations, and it is impossible to design these mutations by using the tertiary structure of this enzyme and available theories of stabilization.

The effects of the mutations on the activity of xylanase are also interesting. While the mutation of Ser-12 to Cys (F1) does not affect the activity, that of Gly-38 to Asp (N102) caused an 80% increase. The increased activity is then decreased to the original level by the additional mutations of Ser-26 to Trp and Thr-126 to Ser (N56). As Ser-26 and Gly-38 are neighboring residues on the outer β-sheet, the mutation at Ser-26 may compensate for the effects of the Gly-38 mutation, or as Thr-126 is near the active-site region (including Glu-93 and Glu-182) proposed before [9], the mutation of Thr-126 to Ser may affect the conformation of the region, thereby decreasing the activity. On the other hand, the activity of N104 is only 19% of that of the wild-type enzyme. This decrease in the activity may be mainly due to the mutation of Arg-48 to Lys rather than that of Gly-38 to Ser, because Arg-48 is in the active-site cleft described above.

Although it is difficult to explain the stabilizing effects of these mutations, they may provide us with a general strategy for enzyme stabilization, because there should be some implicit reasons for these four mutants being selected from 60 000 kinds of genes. For example, Gly-38 was selected as a target point out of 24 glycine residues of the xylanase molecule. In addition, this is only one glycine residue that satisfies the following points: it is on a β-sheet, and if it is replaced, the substituted residue protrudes from the molecular surface into the solvent. Therefore, glycine residues that satisfy the above points could be good target residues for site-directed mutagenesis for stabilizing enzymes.

The fact that stability-increasing mutants were obtained by random mutagenesis from a monomeric enzyme of xylanase as well as from a tetrameric glucose dehydrogenase [1] indicates that these enzymes are not optimized in nature for their stability. As this seems to be a common feature of enzymes from mesophiles, there should be much room for stabilizing enzymes by ran-

dom mutagenesis. In addition, this method does not require a knowledge of the tertiary structure of a target enzyme. Thus, random mutagenesis is a good, practical, and generally applicable method for improving the stability and other properties of enzymes.

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