Increased Thermal Resistance and Modification of the Catalytic Properties of a β-Glucosidase by Random Mutagenesis and in Vitro Recombination*

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The bglB gene from Paenibacillus polymyxa was subjected to random mutagenesis mediated by error prone polymerase chain reaction amplification and DNA shuffling. After this treatment, mutant variants of the encoded β-glucosidase with enhanced thermal resistance were selected. We identified five amino acid substitutions at four different positions of the sequence that increased the resistance of the enzyme to heat denaturation. Four of the mutations, H62R, M319V, M319I, and M361I, did not change the kinetic parameters of the enzyme. However, mutant N223Y, which caused only a marginal increase in thermoresistance, showed an 8-fold decrease in Kₘ. Copies of the bglB gene carrying each one of the individual mutations were recombined in vitro by DNA shuffling. As a result, we obtained an enzyme that simultaneously exhibited a 20-fold increase in heat resistance and an 8-fold increase in the catalytic efficiency. The structural basis of the properties conferred by the mutations was analyzed using homology-based structural models. The four mutations causing a more pronounced effect on thermoresistance were located in loops, on the periphery of the (α/β)₈ barrel that conforms the structure of the protein. Mutation N223Y, which modifies the catalytic properties of the enzyme, was on one of the barrel β-strands that shape the active center.

Protein engineering unfolds as a fundamental tool for biochemistry and biotechnology. It is used to answer specific questions about protein structure and function, and at the same time it can render modified protein structures better suited to perform specific industrial roles. Protein engineering work has been canalized through two main approaches. The first approach (rational design) makes use of the available information about a given protein to predict specific amino acid changes that would result in a specific functional modification. The second approach is based on the generation of random sequence changes and selection of those that cause a desired modification. The iterative application of random mutagenesis and selection, in a process that mimics Darwinian evolution and that has been designated “directed evolution,” has proved very useful in different instances (1–4). A critical step of the directed evolution is to have an efficient procedure to generate a suitable repertoire of mutations. This has been greatly helped by the development of a method designated DNA shuffling that combines PCR-mediated random mutagenesis and recombination (5, 6).

The modification of an enzyme to achieve increased thermal resistance is a good practical example of protein engineering. A scheme of directed evolution can be easily applied if the enzyme has an activity easy to assay for. This is the case of two β-glucosidases (BglA and BglB) encoded by bglA and bglB genes from Paenibacillus (Bacillus) polymyxa. The primary sequences of both proteins are 448 residues long, and both proteins exhibit 47% identity at the amino acid level (7). The two enzymes present different quaternary structures; BglA is an octamer of about 400 kDa, whereas BglB is a monomer of about 51 kDa (8, 9). They belong to the family 1 of glycosyl hydrolases (10–12). Because it is characteristic of family 1 glycosyl hydrolases, the determination of the structure of BglA by x-ray diffraction revealed a (β/α)₈ barrel conformation with the catalytic pair of residues, the acid/base and the nucleophile in β-strands 4 and 7, respectively (13). The structure of other members of the same family has also been determined. The core of the (β/α)₈ barrel is highly homologous in all these structures, and the main regions of structural diversity are found in four loops connecting the C terminus of β-strands of the barrel with the N-end of the α-helices (14–17). Family 1 glycosidases catalyze the hydrolysis of β-glycosidic linkages by a double displacement mechanism involving the formation of a covalent glycosyl-enzyme intermediate, resulting in the net retention of the anomeric configuration of the substrate (18, 19).

Mutant BglA enzymes have previously been characterized with increased structural and cellular stabilities (20, 21). In the present study, we extend this analysis to the BglB enzyme. We subjected P. polymyxa bglB gene to random mutagenesis and DNA shuffling-mediated recombination and screened for mutants encoding proteins with enhanced thermal resistance. We isolated and characterized different mutants with this property. One of the recovered mutations caused a remarkable increase in the catalytic efficiency of the enzyme. The structural basis of the effect caused by these mutations was analyzed using homology based structural models of BglB.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Plasmids—Escherichia coli DH5α was used as host strain for cloning and protein expression. Plasmid pLGBGB derives from pBG4/SC3 (7). It consists of an 1.6-kilobase insert of P. polymyxa DNA that contains the bglB gene, cloned in the polylinker region of vector pUC18 (22). Plasmid pLGBGB was used as template in the error-prone PCR reactions conducted to generate mutations in the...
bglB gene. Plasmid ΔpUC18 was used as the cloning vector for mutagenized bglB preparations. This vector is a modified version of pUC18 that lacks the partial sequence of the lacZ gene (21).

**Random Mutagenesis and Screening for Thermoresistant β-Glucosidase Mutants**—Random mutagenesis of the bglB gene of *P. polymyxa* was carried out by error-prone PCR amplification (23, 24), by DNA shuffling (5, 6), or by a combination of both procedures. Error prone PCR amplification was performed basically as described by González-Blasco et al. (21). The oligonucleotides used as primers for the amplification of bglB were JP27 5′-TGTGACGTCTGATCCGAAAG-GACG-3′ in the direct orientation and AC25 5′-GGGTGACAGATCCC-CCCTTTTTTATTAAAAA-3′ in the reverse orientation. DNA shuffling reactions were carried out as described by Stemmer (5, 6) with some modifications. The substrate for the Dnase treatment was obtained by either standard or error-prone PCR amplification of the bglB gene. Fragments between 50–150 base pairs were purified with a Qiagen gel extraction kit (Qiagen, Germany) and reassembled in a PCR reaction without primers in a 50-μl volume for 20–40 cycles of amplification. The reassembly product was diluted 1:40 or 1:100 and used as template in an additional PCR run in a final volume of either 50 or 100 μl, that used primers JP27 and AC25 and 15–30 cycles of amplification. Mutagenized preparations of the bglB gene obtained after error-prone PCR or DNA shuffling reactions were ligated into the ΔpUC18 vector. This material was used to transform *E. coli*, selecting for ampicillin resistance. Transformant colonies with thermoresistant pUC18 vector. This material was used to transform *E. coli*, selecting for ampicillin resistance. Transformant colonies with thermoresistant β-glucosidase activity were identified by the plate assay described by López-Camacho and Polaina (25). Plates containing transformant colonies were incubated at temperatures that cause inactivation of wild type BglB and then assayed for residual activity. To distinguish thermoresistant mutants from the wild type, the plates with the transformants were incubated for 1 h at 55 °C. To screen for highly thermoresistant clones resulting from in vitro recombination of point mutations, the plates were incubated for 40 min at 70 °C.

**β-Glucosidase B Purification and Activity Assay**—Expression and purification of wild type and mutant BglB glucosidases from *E. coli* was carried out following the same procedure described for BglA (26). The cells from a bacterial culture expressing the bglB gene were collected and disrupted by sonication. The crude enzyme preparations or purified samples, was measured as described by López-Camacho et al. (27). Enzymatic inactivation was followed as a first order kinetic process, and the half-life (t1/2) of the enzyme was determined from the first order rate constants of inactivation, corresponding to the slopes of the lines obtained by plotting ln (percentage of residual activity) versus incubation time. Kinetic parameters (Km and kcat) were determined by fitting hyperbolic Michaelis-Menten curves with the program SigmaPlot version 4.00 (Jandel Scientific). The substrate concentration used in the determination of kinetic parameters were 0.2–15 mM and 6.3–12.6 mM, respectively.

The effect of pH on the thermal resistance of the enzyme was determined as follows. Enzyme samples were added to prewarmed tubes containing 50 mM phosphate buffer at the corresponding pH (pH 7 or 4) and incubated at 47 °C. Aliquots were taken at different times and kept on ice for 4 h to allow the recovery of reversibly denatured enzyme. After this treatment, all the samples were adjusted at the same pH (7.0) and ionic strength. Residual β-glucosidase activity was measured at 37 °C with 5 mM p-nitrophenyl-β-D-glucopyranoside as the substrate (27). Irreversible thermal inactivation of crude enzyme preparations or purified samples, was measured as described by López-Camacho et al. (27). Enzyme inactivation was measured as the decrease in the enzymatic activity (ΔA) following the method described by López-Camacho et al. (27). The reaction was stopped with 1 M sodium bicarbonate, and the released nitrophenol was quantified by determining absorbance at 400 nm. To determine the effect of reducing agents on the thermal resistance of the enzyme, parallel thermal inactivation assays were carried out with or without dithiothreitol (10 mM) in the assay buffer.

**Homology Modeling of the BglB Protein Structure**—The three-dimensional structures of wild type and mutant BglB proteins were modeled with the program SWISS-MODEL version 3.51 at the Expsys server (28–30). The amino acid sequence of BglB that was subjected to modeling was aligned with the residue positions with respect to the sequence database (accession number M80211). These differences, H376Q and G377R, were identified during the sequencing done to characterize mutant bglB genes obtained in this work and later found to be present in the wild type bglB gene as well. The resolved structures of five different family 1 glycosidases were used as modeling templates. The Brookhaven Protein Data Bank entries of these proteins are 1BGA, 1CBG, 1PBG, 1MYR, and 1GOW. The degree of identity between the templates and the bglB sequence was 47, 39, 31, 38, and 42%, respectively. Visualization and analysis of the modeled structures were done using Swiss-PdbViewer (28).

**RESULTS**

**Isolation of Random Mutations That Increase the Thermal Resistance of β-Glucosidase B**—The bglB gene encoding β-glucosidase B from *P. polymyxa* (BglB), contained in plasmid pLGBGB, was amplified by PCR under conditions that promote the misincorporation of nucleotides by Taq polymerase. The amplified material was ligated into vector ΔpUC18 and used to transform *E. coli* competent cells. Replica plates of the ampicillin resistant transformants were incubated under conditions that would inactivate wild type BglB and then assayed for β-glucosidase activity. Among about 10,000 transformant colonies that were screened, we recovered 10 clones that maintained the capability to hydrolyze the chromogenic substrate p-nitrophenyl-β-D-glucopyranoside. As an alternative procedure to generate mutations, we used DNA shuffling. The bglB gene was first amplified by error-prone PCR. The recovered DNA was digested with Dnase I and subjected to a double-step PCR to reassemble full-size bglB gene molecules. This material, which was visible as a single band on an agarose gel, was recovered from the gel, ligated into ΔpUC18, and subsequently used to transform *E. coli*. Although only a few hundred transformant colonies were obtained, three of them expressed thermoresistant β-glucosidase activity.

The increased thermal resistance of the β-glucosidase expressed by the mutant clones was confirmed by comparing the rate of thermal inactivation of crude preparations of these enzymes and wild type BglB. Among those clones that were more heat-resistant than wild type BglB, there were five that exhibited unique patterns of inactivation, indicating that they might contain independent mutations. These five mutant bglB alleles were sequenced to identify the mutations responsible for their enhanced heat resistance. Of the four mutations obtained by error-prone PCR, three were transitions, and the remaining one was a transversion. They corresponded to the following codon and amino acid substitutions: CAC → CCC (H62R), ATG → ATT (M361I), and ATG → AGT (M319V). The additional mutation, obtained by DNA shuffling, was an ATG → ATT transversion that corresponded to the M361I substitution.

**Characterization of the Thermoresistance and Kinetic Properties of the Mutant Enzymes**—Wild type BglB and the thermoresistant enzymes were purified to homogeneity. The purified enzymes were used to measure their thermal resistance and kinetic parameters. Fig. 1 shows the irreversible thermal inactivation of the different enzymes at 50 and 55 °C. Half-life time values of the enzymes at both temperatures are listed in Table I. The purified N223Y enzyme showed about the same resistance as wild type BglB at both temperatures. Table II shows the kinetic parameters obtained for the different enzymes. A kcat value of 38.9 s−1 and a Km of 6.4 mM were obtained for wild type BglB. Four of the five mutants exhibited no significant differences in their kinetic parameters with respect to the wild type enzyme. However, mutant N223Y presen rico et al. (26). The kcat value of 15.4 s−1 and a Km of 0.7 mM. Thus, N223Y hydrolyses the β-glycosidic linkage at a lower rate than the wild type enzyme, but the overall catalytic efficiency of this mutant, expressed as the kcat/Km ratio, is about 4-fold greater than that of the wild type enzyme.

**Recombination of the Different Mutations by DNA Shuffling**—Mutations that cause increased thermostability often show additive or cooperative effects when present in the same sequence (31–34). Several reports in the literature describe the construction of proteins with remarkably increased thermore-
Random Mutations Increasing Enzyme Thermoresistance

To test whether the thermoresistance of BglB could be enhanced by the combined action of the characterized mutations, we subjected the mutant bglB genes to DNA shuffling, a process that promotes recombination. A DNA pool containing the five bglB genes carrying each one of the different thermoresistant mutations was used as starting material. The gene pool was amplified by PCR using standard (nonmutagenic) conditions, digested with DNase I, and reassembled following the shuffling procedure. The DNA recovered after the final PCR amplification, presumably composed of recombinant versions of the bglB gene, was cloned in a vector and used to transform E. coli. The transformant colonies were subjected to a thermal treatment that would cause the inactivation of any of the characterized BglB mutants and then assayed for residual β-glucosidase activity. Seven clones, of about 2,000 colonies screened, were identified that expressed β-glucosidase activity resistant to these conditions. The bglB allele present in the clone that exhibited the greatest thermoresistance was sequenced. It corresponded to the triple amino acid replacement H62R/N223Y/M319I. The triple mutant enzyme was purified and further characterized. As shown in Table II, its catalytic properties were very similar to those of the N223Y single mutant. Additionally, it exhibited a considerably greater thermoresistance than any of the single mutants. After 54 min of incubation at 55 °C, pure preparations of this enzyme still maintained half of the initial activity (Fig. 1 and Table I). It was remarkable that the most thermoresistant version of BglB, isolated by shuffling of the mutations, did not correspond to an enzyme that combined all the single mutations. To find out whether this was due to a shortcoming of the shuffling procedure, we used site-directed mutagenesis to construct a copy of the bglB gene that carried thermoresistant mutations in all four identified loci.

**Table I**

| Enzyme                  | t<sub>m</sub> 50 °C | Relative t<sub>m</sub> 50 °C | t<sub>m</sub> 55 °C | Relative t<sub>m</sub> 55 °C |
|-------------------------|--------------------|------------------------------|--------------------|------------------------------|
| BglB wild type          | 10.4               | 1.0                          | 2.7                | 1.0                          |
| H62R                    | 31.0               | 3.0                          | 10.9               | 4.1                          |
| N223Y                   | 9.7                | 0.9                          | 2.8                | 1.1                          |
| M319I                   | 19.6               | 1.9                          | 7.4                | 2.8                          |
| M319V                   | 14.4               | 1.4                          | 2.8                | 1.1                          |
| M361I                   | 25.3               | 2.4                          | 5.3                | 2.0                          |
| H62R/N223Y/M319I        |                    |                              | 53.9               | 20.2                         |
| H62R/N223Y/M319I/M361I  |                    |                              | 13.4               | 4.9                          |

**Table II**

| Enzyme                  | k<sub>cat</sub> | K<sub>m</sub> | k<sub>cat</sub>/K<sub>m</sub> | Relative efficiency |
|-------------------------|-----------------|---------------|-------------------------------|---------------------|
| BglB wild type          | 38.89           | 6.4           | 6.08 × 10<sup>3</sup>         | 1                   |
| H62R                    | 36.63           | 5.7           | 5.25 × 10<sup>3</sup>         | 1.06                |
| N223Y                   | 15.42           | 0.7           | 22.02 × 10<sup>3</sup>        | 3.68                |
| M319I                   | 34.05           | 6.3           | 5.25 × 10<sup>3</sup>         | 0.86                |
| M319V                   | 35.50           | 5.5           | 6.45 × 10<sup>3</sup>         | 1.06                |
| M361I                   | 37.16           | 5.9           | 6.30 × 10<sup>3</sup>         | 1.04                |
| H62R/N223Y/M319I        | 14.86           | 0.3           | 53.86 × 10<sup>3</sup>        | 8.86                |

**Fig. 1.** Thermal inactivation of wild type BglB and mutant enzymes. Shown is the time course of irreversible thermal inactivation at 50 °C (A) and 55 °C (B) of wild type and mutant forms of Bgl: •, BglB wild type; ○, H62R; □, M361I; ■, M319V; □, M319I; ▲, N223Y; ●, H62R/M319I/N223Y. Enzyme aliquots were diluted in prewarmed assay buffer. Samples were taken at different times and assayed for activity (26).
which contained the H62R/N223Y/M319/M361I combination, was purified. We found that its thermal resistance (Table I) was four times lower than that of the triple mutant selected after the shuffling procedure.

Structural Analysis of Wild Type BglB and the Mutant Enzymes—In the absence of an x-ray-based structure of BglB, we produced homology-based models of this enzyme using the Swiss Model program (28, 30). To build the models we considered five family 1 proteins of known structure, whose amino acid sequences have extensive homology to that of BglB. These proteins are: β-glucosidase A from *P. polymyxa* (BglA), the cyanogenic β-glucosidase from *Trifolium repens*, 6-β-galactosidase from *Lactococcus lactis*, myrosinase from *Sinapis alba*, and β-glycosidase from *Sulfolobus solfataricus*. The degree of sequence identity between BglB and these proteins is: 47, 39, 31, 38, and 42%, respectively. A series of models of BglB were obtained using as templates either each one of the homologous proteins individually or different combinations of them. All the considered models provided a similar solution for the backbone structure of BglB. The α-carbon coordinates provided by the different models differed by only 0.6–1.2 Å, as would be expected given the homology of the sequences used as templates (28, 38). It is characteristic of retaining glycosyl hydrolases that the average distance between the carboxylic oxygen atoms of the two catalytic residues is about 5 Å, whereas in inverting enzymes this distance is greater: about 9 Å (19). Models based on multiple templates were less reliable than the ones based on single templates, because they failed in predicting the distance between catalytic residues Glu167 and Glu356 consistent with the observed effect of the mutation on the rate of catalysis and *Km*.

Residue His62 is located in loop 1, which connects the C-end of the barrel’s β-strand 1 to the N-end of α-helix 1. All models, except the one based on BglA alone, predict that the H62R substitution places the positive charge of Arg62 at a distance of 3.2 Å from the negative charge of Glu429, allowing the formation of a salt bridge (Fig. 3). Additionally, the models predict a novel H-bond between these two residues. To test whether the predicted salt bridge was the cause of the increased thermoresistance caused by the H62R mutation, we compared the effect of pH on the thermal resistance of wild type and mutant enzymes. As shown in Fig. 4, at pH 7 the H62R mutant exhibits much greater thermal resistance than the wild type, whereas at pH 4 the thermal resistance of both enzymes is very similar. This result indicates that the resistance conferred by the H62R mutation is due to an electrostatic interaction, in accordance with the prediction of the structural model.

In three instances: M319V, M319I, and M361I, the replacement of a methionine led to an increase in thermal resistance. Given the susceptibility of methionine to oxidation (39), it was likely that the stabilization provided by these mutations would be due to protection of the enzyme against oxidation during incubation at high temperatures. Residues Met319 and Met361 are located in loops 6 and 7, respectively. In all BglB models these residues are on the surface of the protein, exposed to the solvent and therefore prone to oxidation. To test this hypothesis, we carried out parallel thermal inactivation assays in the presence or absence of 10 mM dithiothreitol in the assay buffer. The inactivation rate of the wild type enzyme in the presence of dithiothreitol was about four times slower than the rate observed in the absence of the reducing agent, whereas in the case of the M319I and M361I mutants the ratio of inactivation rates was only 1.5. These results indicate that the wild type is more sensitive than the mutants to oxidative inactivation and support the idea of the protective effect of the mutations against this effect. An additional effect associated to the M319V, M319I, and M361I mutations is due to the hydrophobic nature of the side chains of isoleucine and valine, which could contribute to better packing of the protein. Indeed, M361I replacement seems to fill up a depression on the surface of the protein, yielding a more compact structure. On the other hand, valine and isoleucine residues at position 319 fill up a cavity between the side chains of Val317, Ile302, and Glu321. Isoleucine fills up this cavity to a greater extent, which is consistent with the higher thermoresistance observed for mutant M319I with respect to that of mutant M319V.

The different models predict that residue Asn223 is located at the C-end of β-strand 5, at the core of the catalytic center of the enzyme. Replacement of this residue by tyrosine places the aromatic ring of tyrosine in close proximity to Glu321, which participates in the hydrolysis of the substrate as the acid/base catalyst. Thus, the position and nature of this replacement seems to fill up a depression on the surface of the enzyme. However, they proved useful for exploring possible amino acid side chain dispositions that would explain new interactions responsible for the phenotype of the mutants.

Fig. 2 represents the structural model obtained for the BglB monomer based on BglA, displaying four of the characterized mutations. Mutations H62R, M319I, M319V (not shown), and M361I are located in loops that surround the cavity in which the active center is located. In contrast, mutation N223Y is in the middle of the barrel, at the C-end of β-strand 5, in close proximity to the catalytic center of the enzyme.

We have used a random mutagenesis scheme to engineer a β-glucosidase. This approach allowed us to recover an enzyme variant with a 20-fold increase in the half-life of the enzyme at 55 °C and an 8-fold increase in catalytic efficiency.

The structural analysis of mutant enzymes was hampered by the fact that BglB has proven refractory to crystallization. It is known that proteins sharing 35–50% sequence identity, whose structure has been solved by x-ray diffraction, differ very little
in their α-carbon coordinates, showing a relative mean square deviation of 1–1.5 Å (38). Therefore, to circumvent the problem because of the absence of a crystallographic model of BglB, we considered 10 theoretical models based on solved homologous proteins sharing 30–50% sequence identity with this enzyme, which likely provide a good approximation to the actual structure. In all these models, the residues whose substitutions increase thermal resistance lie on loops connecting the β-strands of the barrel to the adjacent α-helices. This observation is consistent with the fact that loops are the regions of the α/β barrel with higher variability in sequence, where more amino acid replacements could occur without causing a deleterious change of the structure (13). In contrast, residue Asn223, whose mutation causes a change in the catalytic activity of the enzyme, is located in the active center in close spatial proximity to the catalytic residue Glu167.

According to the structural models, the increase of thermoresistance can be explained by known mechanisms. Substitution H62R likely strengthens the interaction between loops number 1 and 8 through an ion pair. Many different studies have implicated this type of interaction in protein stabilization (13, 20, 40–45). Elimination of chemically labile residues like Met or Asn that can undergo oxidation or deamidation at high temperatures is expected to render the enzyme more resistant to irreversible thermal denaturation (46–48). This is the case of substitutions M319I, M319V, and M361I. Additionally, these substitutions might contribute to thermoresistance by another mechanism: improved packing efficiency (49–52). In this case the better packing would be provided by the branched side chains of Ile and Val in an hydrophobic environment. The fact that Ile and Val at position 319 stabilize the enzyme to a different degree strongly supports the packing effect of these substitutions. No significant increase in thermoresistance was observed for N223Y with purified protein preparations. Any benefit obtained from the substitution of the Asn residue seemed to be counterbalanced by other negative interactions. Although proximal to Glu167, Asn223 does not interact directly with the oxygen atoms of the catalytic dyad. Therefore, it is not clear why changing this residue in mutant N223Y would cause a 50% reduction in the catalytic rate. One possibility is that the presence of the bulky side chain of tyrosine in the vicinity of the catalytic dyad might perturb the environment of these residues or result in a less favorable catalytic geometry. On the other hand, tyrosine residues are common at sugar
binding sites of many glycosidic enzymes (19). The decrease in $K_m$ observed for N223Y would be consistent with Tyr$^{223}$ contributing to substrate binding.

Different studies show that the combination of point mutations that affect a given property of a protein results in a considerable enhancement of this property, because of either an additive or cooperative effect (32–36, 53). However, combining different mutations in a single protein molecule by site-directed mutagenesis implies a laborious task. We used DNA shuffling to promote random recombination between the single mutations and obtained an enzyme containing three mutations H62R/N223Y/M319I that exhibited a remarkable increase in thermostability. It was interesting that no novel mutations were found in the shuffled BglB molecules, despite the high mutagenesis rate of this procedure (5). The characterization of the properties of the triple mutant H62R/N223Y/M319I indicated that these substitutions might have a cooperative effect. The half-life of the triple mutant at 55 °C was about 20 times that of wild type BglB. This enhancement was greater than what would be expected from an additive effect. In addition to the increased thermostability, the triple mutant showed a slightly lower $K_m$ value than the single mutant N223Y. This resulted in a further improvement of the catalytic efficiency of the triple mutant with respect to the wild type (about 8-fold). Changes in substrate binding affinity brought about by mutations distant from the binding site have previously been reported (54, 55). Overall, this recombination step allowed us to simultaneously improve the thermal stability and the catalytic efficiency of the enzyme. We tested the thermostability of the quadruple mutant H62R/N223Y/M319I/M361I, and it was lower than that of the triple mutant. This result indicated that even though the M361I substitution improved the thermostability of wild type BglB, it exerted a net negative effect in the presence of the other three substitutions.

Our results provide information about critical properties of an enzyme, such as thermostability and catalytic efficiency, based on a straightforward procedure of isolation, identification, and structural analysis of random mutations. In this scheme, DNA shuffling was a powerful tool to generate new versions of the protein structure, among which recombinants of desired properties could be selected.
