Thermostability Improvement of a *Streptomyces* Xylanase by Introducing Proline and Glutamic Acid Residues

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ABSTRACT

Protein engineering is commonly used to improve the robustness of enzymes for activity and stability at high temperatures. In this study, we identified four residues expected to affect the thermostability of *Streptomyces* sp. S9 xylanase XynAS9 through multiple sequence analysis (MSA) and molecular dynamic simulations (MDS). Site-directed mutagenesis was employed to construct five mutants by replacing these residues with proline or glutamic acid (V81P, G82E, V81P/G82E, D185P/S186E, and V81P/G82E/D185P/S186E), and the mutant and wild-type enzymes were expressed in *Pichia pastoris*. Compared to the wild-type XynAS9, all five mutant enzymes showed improved thermal properties. The activity and stability assays including circular dichroism and differential scanning calorimetry showed that the mutations at positions 81 and 82 increased more the thermal performance than the mutations at positions 185 and 186. The mutants with combined substitutions (V81P/G82E and V81P/G82E/D185P/S186E) showed the most pronounced shifts in temperature optima, about 17°C upward, respectively, and their half-lives for thermal inactivation at 70°C and melting temperatures were increased by >9 times and approximately 7.0°C, respectively. The mutation combination of V81P and G82E in adjacent positions more than doubled the effect of single mutations. Both mutation regions were at the end of long secondary structure elements and probably rigidified the local structure. MDS indicated that a long loop region after positions 81 and 82 located at the end of inner β-barrel was prone to unfold. The rigidified main chain and filling of a groove by the mutations on the bottom of the active site canyon may
stabilize the mutants and thus improve their thermostability.

**Keywords:** Xylanase; thermostability improvement; site-directed mutagenesis

Hemicellulose is the second most abundant natural polysaccharide after cellulose. Xylan is the main carbohydrate found in hemicellulose that constitutes 30–35% of lignocellulosic biomass (1). Efficient utilization of xylan is essential for conversion of hemicellulosic materials to other value-added products. Due to the complex structure of xylan, it requires an enzyme system of several hydrolytic enzymes for complete degradation. Among them, xylanase plays a crucial role in xylan hydrolysis as it catalyzes the hydrolysis of 1,4-β-D-xylosidic linkages in xylan to short xylooligosaccharides. Currently, xylanase has been attracting much attention due to its wide biotechnological applications in the food, animal feed, pulp and paper industries, textile, and in the deconstruction of lignocellulose for biofuels production (2–6). On the basis of the catalytic domains, xylanases are mainly classified into glycoside hydrolase (GH) families 5, 8, 10, 11, 30 and 43 (7), and GH10 and GH11 xylanases are best studied.

High-temperature active/stable enzymes are more favorable than mesophilic counterparts, since high temperatures enhance the mass transfer rate, reduce the substrate viscosity, and reduce the risk of contamination (8, 9). For example, thermophilic xylanases are applied in bioconversion processes and pulp bleaching, during which a variety of high-temperature treatments are used prior or
simultaneously to enzyme treatment. In preparing animal feed, thermostable xylanases are added into the feed before the pelleting process (typically carried out at 70–95°C). To obtain thermostable xylanases, one strategy is to search for novel xylanases from extremophiles. A number of thermostable GH10 xylanases have been reported from thermophilic *Thermotoga maritima* MSB8 (90°C; 10), *Thermotoga petrophila* RKU-1 (90°C; 11), and *Thermobifida alba* UL JB1 (80°C; 12), and acidomesophilic *Bispora* sp. MEY-1 (85°C; 13). The available sequences and structures of these thermophilic xylanases (i.e. 1VBU and 3NIY, 14, 11) provide valuable information for better understanding of the structural and functional characters of thermostable enzymes.

Improving the thermal properties of mesophilic enzymes by protein engineering is the other important strategy to obtain thermostable enzymes. The approaches are generally used including but not limited to replacement of the N-terminus, introduction of disulfide bridges, and increasing the number of salt bridges or hydrogen bonds (15). Many successful protein engineering examples for thermostability improvement have been published. Wintrode et al. (16) employed DNA shuffling to develop a protease mutant with increased melting temperature ($T_m$) of 25°C and increased half-life at 60°C (1200 folds). In combination with computer-aided prediction and rational and random design, this method has been proved to be more efficient. Fenel et al. (17) and Wang et al. (18) specifically introduced a disulphide bridge into the xylanases of *Trichoderma reesei* and *Thermomyces lanuginosus*, shifting their temperature optima at least 10°C higher. Joo
et al. (19) improved the $T_m$ of *Bacillus circulans* xylanase by 4.2ºC based on thermal fluctuation analysis.

XynAS9, a GH10 xylanase XynAS9 from *Streptomyces* sp. S9, has been expressed in *Escherichia coli* and showed superior properties like broad ranges of pH adaptation and stability (20). However, its thermostability is poor at temperatures above 65ºC, the most common industrial temperature. To widen its application spectrum in industries, it’s necessary to improve the thermal properties of XynAS9. Recombinant XynAS9 produced in *Pichia pastoris* showed increased temperature optimum (73ºC), but was still liable at 65ºC and above (data not shown). The objective of the present study was to develop thermostable XynAS9 by employing bioinformatics-driven, rational engineering. By replacing some key residues, the thermostability of XynAS9 was expected to be improved significantly.

**MATERIALS AND METHODS**

**Strains, plasmids, media, and chemicals.** *Streptomyces* sp. S9 was the donor strain of the GH10 xylanase gene *xynAS9* (EU153378) (20). *Escherichia coli* Trans I-T1 (Transgen, Beijing, China) was used for plasmid amplification. *P. pastoris* GS115 (Invitrogen, Carlsbad, CA) was used as the expression host. Plasmids pEASY-T3 (Tiangen, Beijing, China) and pPIC9 (Invitrogen) were used for cloning and expression, respectively. Pfu DNA polymerase and restriction endonucleases and T4 DNA ligase were from Tiangen and New England Biolabs (Hitchin, UK), respectively. The low molecular weight calibration kit was from GE Healthcare (Pittsburgh, PA).
The substrate birchwood xylan was purchased from Sigma-Aldrich (St. Louis, MO). Minimal dextrose medium or minimal methanol medium for transformant selection, and buffered minimal glycerol-complex medium and buffered methanol complex medium for *P. pastoris* growth and induction, respectively, were prepared according to the manual of the *Pichia* Expression Kit (Invitrogen). All other chemicals were of analytical grade.

**Identification of key residues.** Multiple sequence alignment (MSA) of XynAS9 and six GH10 xylanases with different temperature optima (from 73°C to 90°C, see Fig. 1) were performed by using the ClustalW. The residues that might be related to their thermophilic properties were identified. The three-dimensional structures of XynAS9 and its five mutants (V81P, G82E, V81P/G82E, D185P/S186E and V81P/G82E/D185P/S186E) were modeled using Discovery studio 2.5.5 software (Accelrys, San Diego, CA) and the crystal structures of chain A (3CUF) (21) and chain E (3NDY) (22) as the templates.

**Molecular dynamic simulation (MDS).** The modeled structures of wild-type XynAS9 and its mutants were optimized using NAMD 2.7 and Gromacs v4.4.5 to investigate the possible mechanism of thermal stability increase. NAMD is a unique strategy for fast parallel MDS, using the CHARMM27 force field (23–26). The box size was 63.17 × 95.22 × 111.34 Å, which was large enough to equilibrate the protein within it. TIP3P water was used as the aqueous solution (27). Na⁺ was used as
counterion to neutralize the negative charges of proteins and the system was subjected to a steepest descent energy minimization with 10,000 steps. Each system was equilibrated in a NPT ensemble at 1 atm pressure. The structure of XynAS9 was optimized and simulated at 20 ns. The average conformation of the wild-type protein in the simulation was used as the template to construct variations with Discovery Studio 2.5.5. The wild-type and mutant enzymes were simulated for 20 ns with NAMD at 300 K or 400 K, respectively. For all simulations, the time step was defined as low as 2 fs, and cutoffs were 12 Angstroms (23). The data were calculated at 5 ns timescale from 15 ns to 20 ns. The analysis for root mean square deviation (RMSD) and root mean square fluctuation (RMSF) were performed using standard NAMD tools, and the hydrogen bonds and salt bridges were analyzed by VMD (http://www.ks.uiuc.edu/Research/vmd/). Analysis with Gromacs followed the same protocol of NAMD.

Generation, expression and purification of wild-type XynAS9 and its mutants.

By combining the results from MSA and MDS, the key residues related to XynAS9 thermostability were determined. Site-directed mutagenesis to replace specific residue(s) identified above was performed by overlap extension PCR (28). Mutations were introduced into the oligonucleotide primers (Table S1), and the overlapping ends of the fragments annealed to each other. Final products were purified using the gel-extraction kit (Tiangen), ligated into the pEASY-T3 vector, and sequenced. The gene fragments encoding the wild-type XynAS9 and mutant enzymes were inserted
into the expression vector pPIC9 at the EcoRI and NotI sites, respectively. The recombinant plasmids were linearized with BglII and then individually transformed into *P. pastoris* GS115 competent cell by electroporation. Recombinant expression was performed as described in the manual of the *Pichia* expression kit (Invitrogen).

To purify recombinant XynAS9 and its five mutants, the supernatants (~1 l) of the induced cultures were collected by centrifugation at 4°C. The clear supernatants were concentrated to 300 ml using the hollow fiber, followed by further concentration to 40 ml using vivaflow 50 ultrafiltration membranes with a 5 kDa molecular weight cut-off (Vivascience, Hannover, Germany). The xylanases in the supernatants were each precipitated by the addition of 80 ml acetone. The precipitates were collected by centrifugation for 10 min at 12,000 × g, 4°C, and resuspended in 20 mM Tris-HCl (pH 8.0). Each suspension was loaded onto the HiTrap™ Desalting column (GE Healthcare, Uppsala, Sweden) for desalination, and then applied to the HiTrap Q Sepharose XL 5 ml FPLC column (GE Healthcare). Proteins were eluted using a linear NaCl gradient (0–0.6 M) in the same buffer. Fractions showing xylanase activities were pooled, concentrated, and stored at 4°C.

The concentrations of the purified proteins were determined using the Bio-Rad Protein Assay Kit (Boston, MA). To remove N-glycosylation, purified enzymes were treated with 250 U of endo-β-N-acetylglucosaminidase H (Endo H) for 2 h at 37°C according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA). The deglycosylated and untreated enzymes were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (29).
Xylanase activity assay. The enzymatic activity of xylanase was determined by measuring the release of reducing sugar from birchwood xylan with the method of 3,5-dinitrosalicylic acid (DNS) (30). The standard reaction consisting of 100 μl of appropriately diluted enzyme and 900 μl of McIlvaine buffer (200 mM Na₂HPO₄, 100 mM citric acid, pH 6.5) containing 1% (w/v) xylan substrate was incubated at 70°C for 10 min, and then stopped with 1.5 ml DNS reagent. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute. Each reaction and its controls were run in triplicate.

Determination of kinetic parameters of XynAS9 and its mutants. The kinetic parameters, $K_m$, $k_{cat}$ and $V_{max}$, were determined in McIlvaine buffer (pH 6.5) containing 0.5–10.0 mg/ml birchwood xylan for 5 min. The kinetic values were determined by fitting the Lineweaver-Burk plot.

Measurement of optimal pH for activity and stability. The pH optimum of each enzyme was determined by measuring the xylanase activity at 70°C for 10 min in the pH range from 3.0 to 12.0. The pH stability of each enzyme was determined by measuring the residual activities under optimal conditions (pH 6.0 or 6.5, 73–90°C, 10 min) after pre-incubation at 37°C, pH 3.0–12.0 for 1 h. The buffers used were McIlvaine buffer (pH 2.0–8.0) as mentioned above and 50 mM glycine-NaOH (pH 8.0–12.0).
Optimal temperature and thermal stability studies. The optimal temperatures of XynAS9 and its five mutants were determined in the range of 30–95°C at optimal pH (6.0 or 6.5) with 10-min incubation time. Thermal stability was determined by measuring the half-life of enzyme inactivation ($T_{1/2}$) at 65–80°C and optimum pH. All purified enzymes were diluted to 100 μg/ml in McIlvaine buffer (pH 6.5) and incubated for different durations. The residual enzyme activities were measured under standard conditions.

To determine their thermal tolerance ($T_{50}$; the temperature at which 50% of activity is lost), wild-type XynAS9 and mutant enzymes were diluted to 50 μg/ml in McIlvaine buffer (pH 6.5) and then heated for 30 min at each temperature points within the range of 50–90°C at intervals of 5 degrees. After heating, the enzymes were immediately placed on ice for 10 min, the residual xylanase activity was then measured using the assay described above.

Circular dichroism (CD) for protein structure analysis. CD measurements of wild-type XynAS9 and its mutants were performed on a Chirascan spectropolarimeter (Applied Photophysics, Surrey, UK) (31) at 25°C. The concentrations of the proteins were diluted to 200 μg/ml (4.2 μM) in 10 mM PBS buffer. Spectra were continuously recorded from 190 nm to 250 nm using a 1 mm cell and a bandwidth of 1 nm over 3 scans at a scan rate of 120 nm/min. The results were analyzed by CDNN processing software.
Differential scanning calorimetry (DSC). DSC was performed on a Nano-DSC (TA Instruments, New Castle, DE) at a heating rate of 1°C/min and a scanning rate of 1°C/min. The proteins, 350 µg of each sample, were dissolved in 1 ml of 10 mM PBS buffer (pH 7.4). Before measurements, the samples were degassed in an evacuated chamber for 15 min at an atmospheric pressure of 386–640 mHg and then immediately loaded into the calorimeter cell, a 0.3-ml sample cell against a 0.3-ml reference cell. The same amount of buffer was measured as the baseline, which was subtracted from each measured scan. A constant pressure of 3 atm was maintained during all DSC experiments to prevent possible degassing of the solution on heating. The melting temperature, $T_m$, obtained from the DSC experiments corresponded to the maximum of the transition peak, and the test was repeated at least twice at temperatures between 25°C and 90°C.

Substrate docking analysis. AutoDock 4.0 (http://autodock.scripps.edu/) was used as an appropriate docking tool to carry out the docking simulations of substrate xylohexaose and XynAS9 or mutants V81P/G82E and V81P/G82E/D185P/S186E. The output from AutoDock and all modeling studies as well as images were generated with PyMOL version 0.99 (http://pymol.en.uptodown.com/). Hydrophobic and hydrogen bonding interactions of docked molecules were compared using LigPlot program (32).
RESULTS

Identification of key residues. Based on the MSA results (Fig. 1), a regular pattern was identified from the consensus sequences. Compared with mesophilic counterparts, thermophilic xylanases with a temperature optimum of 80°C or higher have proline at sites 81 and 185 and glutamic acid at sites 82 and 186 with a higher frequency, corresponding to valine and aspartic acid and glycine and serine in XynAS9, respectively. By replacing the corresponding residues in XynAS9 with proline and glutamic acid, the modeled structure mutant V81P/G82E/D185P/S186E (Fig. 2) indicated that all mutation sites are at the end of a long secondary structure element, i.e. the mutations V81P and G82E located on the internal $\beta$-strand 2 of $\alpha/\beta_8$ domain and D185P and S186E on the surface region of the barrel at the N-terminus of fourth $\alpha$-helix. MDS analysis with NAMD showed that these mutations decreased the conformational flexibility of XynAS9 at temperature 400 K with low RMSD values (Fig. S1), suggesting that mutant V81P/G82E/D185P/S186E may have a more stable structure. Thus the crucial role of proline and glutamic acid substitutions in thermostability was supported by MDS results.

Generation, expression and purification of wild-type XynAS9 and its mutants.

The wild-type XynAS9 and its mutants were generated and expressed in Pichia and purified to electrophoretic homogeneity as described above. SDS-PAGE revealed that all recombinant enzymes had an apparent molecular weight of ~45–55 kDa, which were higher than their theoretical molecular weights (46 kDa). After treatment with
Endo H, all enzymes showed a single band corresponding to the theoretical mass (Fig. S2).

**Determination of kinetic parameters of XynAS9 and its mutants.** The specific activities and kinetic values of wild-type XynAS9 and its mutants were determined with birchwood xylan as the substrate (Table 1). Compared with the high specific activity of XynAS9 (630.3 U/mg), all mutants showed decreased specific activities, ranging from 78.5 to 206.9 U/mg. Moreover, these residue substitutions had profound impacts on the kinetics of all mutants. Except for the varying $K_m$ values from 0.60 to 4.02 mg/ml, all mutants were found with decreased $k_{cat}$, $k_{cat}/K_m$, and $V_{max}$ values. The results indicated that both residue substitutions V81P/G82E and far from it (D185P/S186E) decreased the catalytic efficiency of these mutant enzymes. Although the $K_m$ value of G82E for birchwood xylan decreased, which means an increased affinity to substrate, its low catalytic efficiency (low specific activity and $k_{cat}/K_m$ value) suggests that it might be inefficient in product releases.

**pH optima and stability studies.** The pH-activity profiles of wild-type XynAS9 and its mutants were essentially quite similar and showed two activity peaks at pH 6.0 or 6.5 (maximum) and pH 8.0 or 9.0 (Fig. 3A), respectively. The enzymes remained >55% of maximal activities at pH 5.0–9.0, and mutants V81P/G82E and V81P/G82E/D185P/S186E remained active even at pH 10.0 (>20% activity). All enzymes were stable at pH 4.0–11.0, and some enzymes retained active after 1-h
incubation under extremely acidic (pH 3.0; mutants G82E, V81P/G82E, and V81P/G82E/D185P/S186E) and alkaline (pH 12.0; wild-type XynAS9 and mutants V81P and D185P/S186E) conditions (Fig. 3B).

Temperature optima and thermostability studies. Five mutants showed improved activity at high temperatures (Fig. 3C). Among them, mutants V81P, G82E and D185P/S186E had a temperature optimum of 75°C, 80°C, and 80°C, respectively, 2–7°C higher than that of wild-type XynAS9 (73°C). Mutants V81P/G82E and V81P/G82E/D185P/S186E showed the greatest shift of the temperature optimum from ~73°C to 90°C. All these mutants showed 50–100% of the maximal activity at 85°C, much higher than that of wild-type XynAS9 (20% of the maximal activity). The temperature optimum increase (7°C) of the mutant G82E was more significant than that of V81P (2°C), and the sum of their increases (~9°C) was less than the gain in the double mutant V81P/G82E (~17°C). The four-fold mutant V81P/G82E/D185P/S186E showed the same increase in the apparent temperature optimum (~17°C). The results indicated that mutations at the positions identified by MSA had cumulative effect on enzyme activity at elevated temperatures.

Thermostabilities of wild-type XynAS9 and its mutants were assessed by their half-lives ($T_{1/2}$) (Table 2). Mutants V81P and D185P/S186E had a marginal increased $T_{1/2}$ value (1–2 folds). Mutant G82E was much more thermostable, increasing the $T_{1/2}$ by up to 3 folds at 65°C. And mutants V81P/G82E and V81P/G82E/D185P/S186E made the greatest improvement on the thermostability, increasing the $T_{1/2}$ by up to 16
folds at 65°C, 10 folds at 70°C, and 4 folds at 75°C and 80°C. The strongest effect on thermostability was therefore observed for the double mutant V81P/G82E, exactly in the same way as the highest increase in temperature optimum.

Thermal tolerance ($T_{50}$) of wild-type XynAS9 and its mutants was determined at temperature region from 50°C to 90°C (Fig. 3D). The $T_{50}$ value of XynAS9 was determined to be 62.0°C, and mutants V81P, G82E, V81P/G82E, D185P/S186E and V81P/G82E/D185P/S186E showed an increased value of 1.7°C, 6.2°C, 6.9°C, 2.3°C and 11.2°C, respectively. It was found that single mutation at position 82 with glutamic acid played a key role in the improvement of thermal tolerance and had additive effect when combined with V81P. The four-fold mutant V81P/G82E/D185P/S186E was most striking in thermal tolerance improvement.

**CD measurements.** CD analysis was conducted to check whether the improved thermal properties of mutants were caused by the secondary structure changes of proteins. As shown in Fig. 4A, the far-UV CD spectrum of XynAS9 exhibited pronounced maximum and minimum at 195 nm and 222 nm, respectively, which are characteristics of $\beta$-sheet and $\alpha$-helix structures in aqueous solution, respectively (33). Thus, XynAS9 is an autonomous structural protein that contains both $\alpha$-helix and $\beta$-sheet secondary structures as predicted from homology modeling. Residue substitutions didn’t change the secondary structure of mutants G82E and D185P/S186E, which had similar far-UV CD spectrum to XynAS9. In contrast, mutants V81P, V81P/G82E and V81P/G82E/D185P/S186E yielded different far-UV
CD spectra with deep minima near 200 nm, which is typical of irregular structure (33). The results may explain the significant contribution of residue substitution at site 81 with proline to improved thermal properties. Moreover, TmxB from *T. maritima* (1VBU) (14) and XynAS9 had different residues at positions 81 and 82 (Fig. 1), but their local structures in these positions are similar (modeled XynAS9 with 3CUF and 3NDY as the templates). The result indicates that the improved thermostability is still based on minor structural changes.

**DSC analysis.** DSC was performed to determine the $T_m$ values of wild-type XynAS9 and its mutants over the temperature range from 25°C to 90°C (Fig. 4B). The $T_m$ values of mutants V81P, G82E, V81P/G82E, D185P/S186E, and V81P/G82E/D185P/S186E were 69.1°C, 71.8°C, 75.1°C, 69.4°C and 75.2°C, respectively, which were 0.9–7.0°C higher than that of wild-type XynAS9 (68.2°C). It is in agreement with the results above that residue substitutions at sites 81 and 82 have most remarkable contribution to the thermal stability.

**Structural changes during MD simulation.** MDS at 400 K was used to interpret the effect of mutations on structural changes. During the course of simulation, the inner β-barrel stayed quite stable, but the loops and α-helix layer started to experience changes (Fig. S3). When mutated the residues at four-fold sites, several α-helices were unfolded and changed to long loops, especially around the positions 81/82. A long loop located after 81/82 (region 83–86 and 91–98) changed completely its
conformation. The local structure immediately before and after the positions 185/186 did not essentially change, but there were changes in other main chain regions nearby. Amphiphilic α-helices are important in the stabilization of the whole enzyme structure. The large changes from α-helices to long loops after the positions 81/82 could be relevant for the interpretation of the effects of mutations at positions 81 and 82. According to MDS, the eventual decrease of flexibility was observed in large areas of the protein with residue replacement (Fig. S1).

VMD analysis of hydrogen bonds and salt bridges. In the modeled structure of XynAS9, Val81 does not form any hydrogen bond with other residues, whereas there is a putative hydrogen bond between the main chain nitrogen of Gly82 and the main chain oxygen of Thr58. Within the distance of 6 Å, there are other four putative hydrogen bonds around Val81 and Gly82 (Fig. S4A), i.e. Asn83 to Met85 or Lys86, Thr80 to the carboxyl- or amino-group of Thr58, and five around Asp185 and Ser186 (Fig. S4C), i.e. Arg174 to Asp185 or Gln180 and Ser186 to Ala189 or Glu190. When replaced these residues with proline and glutamic acid, one or two additional putative hydrogen bonds are predicted to be formed between Glu82 and Asn83 (Figs. S4B and S5) and one additional bond between Glu186 to Tyr187 (Fig. S4D). Dihedral rotation of Ser186 does not make any hydrogen bonds, whereas rotated Glu186 can form several hydrogen bonds to the residues mentioned above, thus possibly strengthening the local structure. On the other hand, Glu186 is fully exposed to solution, whereas Glu82 appears to have favorable hydrophobic interactions in the hydrophobic part of
the side chain with Trp311 and strong polar environment for carboxyl group of Glu82, including ND2 of Asn83 and NE of Trp311 and side active site. Therefore, Glu82 fills very well the groove on the bottom of the active site is packed well against W311 and N83 (Fig. S5). The space filling with hydrophobic and polar interactions is likely to stabilize the local area around the cavity.

The protein structure changed considerably during the course of MDS, and new interactions were formed. The distances between Glu82 and Asp59, and Glu186 and Lys217 of mutant V81P/G82E/D185P/S186E, became short enough to form hydrogen bonds and even a salt bridge between Glu186 and Lys217. The same phenomenon was also observed in the distance change of Glu82 and Lys316. The putative salt bridge between Glu82 and Lys316 may stabilize the structures of the big loop from Trp311 to Trp319 and the β-strand in close proximity. Asp59 and Asp312 are separated from Glu82 at the distances of 3.2 and 4.9 Å, respectively; within this distance a force of mutual electrostatic repulsion might produce. VMD analysis indicates that unfolded protein may create new interactions between natively distant positions and thus in some degree stabilize the unfolded structure.

Docking of xylohexaose into XynAS9 and mutant V81P/G82E. Docking simulation with AutoDock 4.0 (Fig. S6) showed that xylohexaose was docked into the substrate binding pocket of XynAS9 by residues Asn83, Trp123, Glu166, Glu170, Asn210, His243, Glu271, Trp311 and Trp319. The schematic diagrams of protein-ligand interactions by Ligplot showed that the catalytic residue Glu271
formed a strong intermolecular hydrogen bond with xylohexaose-O25. Gly82 formed a hydrophobic interaction with xylohexaose. One potential hydrogen bond was formed between Asn83 and xylohexaose-O18. When xylohexaose was docked with the modeled structure of mutant V81P/G82E, the filling of the bottom of the catalytic canyon by Glu82 forced the substrate to occupy a higher position at this end of the canyon, therefore changing the interaction sites with the enzyme. The catalytic Glu271 may form one additional hydrogen bond with xylohexaose-O23, and Glu82 may form three potential hydrogen bonds, two with xylohexaose-O11 and one with xylohexaose-O12. Glu82 and Tyr317 may compete to form a hydrogen bond with xylohexaose-O11. Moreover, Asn83 in mutant V81P/G82E may form two hydrogen bonds with xylohexaose-O13/14 instead of one with xylohexaose-O18 in XynAS9.

The docking result of mutant V81P/G82E/D185P/S186E was the same as that of the mutant V81P/G82E (data not shown). This result suggests that residue substitution with glutamic acid at site 82 may form new hydrogen bonds, increase the stability of enzyme-substrate complex and consequently improve the enzyme activity at high temperatures.

DISCUSSION

XynAS9 from *Streptomyces* sp. S9 contains a (α/β)₈ barrel catalytic domain and has weak thermostability (20). Quite much knowledge exists about the stabilizing factors in (α/β)₈-barrel enzymes, including efficient packing of the hydrophobic core, the presence of prolines at the N-termini of α-helices, cavity filling and stabilization of
loops and N- and C-terminal regions (34, 35). In this study, we developed several highly thermostable mutants of XynAS9 by combining MSA, MDS and site-directed mutagenesis techniques. MSA of various GH10 xylanases with different temperature optima provides the most direct and readily available information related to enzyme properties. Computational design methods provide more precise guidance for enzyme engineering and make mutation more efficient. According to the results of MSA and MDS, four key residues probably involved in enzyme activity and stability at high temperatures were identified, and five mutants were then constructed. Compared with wild-type XynAS9, all five mutants V81P, G82E, V81P/G82E, D185P/S186E and V81P/G82E/D185P/S186E showed enhanced resistance to high temperatures, and the double- and four-fold mutants V81P/G82E and V81P/G82E/D185P/S186E showed the greatest shift of 17°C in temperature optima and best thermostability at high temperatures (i.e. 85°C).

Glutamic acid has an anionic carboxylate that easily forms hydrogen bonds and salt bridge and has helix-forming propensity, which contributes to thermostability (36, 37). For example, replacement of aspartic acid with glutamic acid made mesophilic Aspergillus awamori glucoamylase thermostable up to 70°C (37); glutamic acid substitution increased the $T_m$ of T4 lysozyme by 1.5°C by forming a salt bridge (38); the modified xylanase A of Streptomyces lividans with glutamic acid substitution showed longer half-life at 60°C than its wild-type enzyme (39). Here we also introduced glutamic acid residues into XynAS9, which may produce additional putative salt bridges, hydrogen bonds and hydrophobic interactions (Figs. S4 and S5).
The mutant glutamate side chain of G82E fills a groove on the bottom of active site canyon with some favorable side chain interactions like hydrogen bonds and therefore the improved packing and filling of the groove may play a central role in thermostability. The same space filling can be seen in *T. maritima* xylanase 10B (1VBU) and *Cellulomonas fimi* XYN10A (3CUF) by the side chain of glutamic acid (data shown in PDB database). Besides these molecular interactions, the mutation S186E at the end of helix might increase the length of helix, thus creating higher rigidity to the region. In both regions there are mainly acidic amino acids nearby the introduced glutamic acid (plus one lysine close to S186E), the roles of these ionic interactions remain unclear however. These interactions in combination contributed to the more stable conformation, and finally improve the enzyme robustness at high temperatures.

Besides glutamic acid, proline is highly prevalent in thermophilic proteins because it has a side chain of distinctive cyclic structure that locks its backbone and leads to an exceptional conformational rigidity in the turns and loops. Introduction of proline residues at certain positions has been used successfully to improve the thermostabilities of many enzymes (40–45). In the present study, we introduced proline at sites 81 and 185, which are located at the second position of the β-turn and the N-cap of the α-helix (Fig. 2), respectively. Proline substitution at site 81 probably affected the local structure with lower RMSD values (as shown in Fig. S7). After position 81, there is a curvy loop (starting from 82) that is probably stabilized by the rigid proline and enables the enzyme to perform its catalytic function at higher
temperature. Thus, we can conclude that proline substitutions also are one of the key
factors responsible for the improved thermal properties of the mutated XynAS9.

Considering the increases in temperature optima and $T_m$ values of mutants G82E
and V81P (7°C and 3.6°C v.s. 2°C and 0.9°C), the effect of glutamic acid is clearly
more significant than that of proline. The combination of the two mutations in
adjacent positions with apparently differing stabilization mechanisms was additive in
the improvement of temperature optimum. Therefore, both improved rigidity of the
main chain and improved side chain interactions contributed to the final effect in the
combination mutant. These results showed that the enzyme had a strong weak point
on the bottom of the active site canyon that was largely repaired by only two
mutations.

It is known that substrate itself increases the thermostability of xylanases (35). In
the presence of substrate, hydrogen bonds were formed between xylohexaose and
catalytic residues and Glu82 of mutant G82E/V81P, respectively, as shown in the
corresponding glutamic acid of C. fimii xylanase (3CUJ) and Bacillus subtilis xylanase
(2B46) that forms two hydrogen bonds to the substrate (data shown in PDB
databases). These interactions may increase the robustness of the
xylanase-xylohexaose complex that benefits the adaptability and stability of XynAS9
over high temperatures. Experimental results that the increases of mutants G82E and
V81P in apparent temperature optima were greater than that in melting temperature
further confirms the role of substrate in the stabilization of enzymes at high
temperatures. These improved interactions between enzyme and substrate can
remarkably improve the tolerance of enzymes to high temperatures. Modified XynAS9 with double to four fold mutations showed significant improvement in enzyme thermostability and activity at alkaline conditions. These characteristics make them favorable for wide industrial application, especially in the bleaching of kraft pulp. However, the price of these stabilizing mutations could be a lowered catalytic activity. While the mutations improved both apparent temperature optimum and thermostability, there was a clear trend to decreased catalytic efficiency. It indicates how sensitive the balance between activity and stability can be and the increase of enzymatic performance at higher temperatures may require a compromise of the catalytic efficiency. This phenomenon can be interpreted by stability-function hypothesis that enzyme residues involved in function are not optimized for stability and vice versa (46). The consequence could then be that the increased rigidity leads to lowered activity, as it was observed in our molecular dynamic simulations that the mutations decreased structural flexibility.

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Protein Eng. 11:783–788.


Figure legends

**FIG 1** MSA of GH 10 xylanases with various temperature optima using the ClustalW program. TmxB, the xylanase from *Thermotoga maritima*; TpXyl10B, the xylanase 10b from *Thermotoga petrophila* RKU-1; XYL10C, the endo-β-1,4-xylanase from *Bispora* sp. MEY-1; XyIA, the β-1,4-endoxylanase from *Thermobifida alba*; Xyle, the endo-1,4-xylanase from *Penicillium canescens*; XynC, the xylanase from *Phanerochaete chrysosporium*; and XynAS9, the xylanase from *Streptomyces* sp. S9. The catalytic residues are indicated with *. The mutation sites are gray.

**FIG 2** The three-dimensional structure model of mutant V81P/G82E/D185P/S186E. Glu166 and Glu271 are putative catalytic residues.

**FIG 3** Enzymatic properties of wild-type XynAS9 and its mutants. (A) pH-dependent activity profiles. (B) pH stability. (C) Temperature-dependent activity profiles. (D) Enzyme inactivation at different temperatures. Symbols: wild-type XynAS9 (□) and mutants V81P (●), G82E (○), V81P/G82E (-), D185P/S186E (■), and V81P/G82E/D185P/S186E (▲).

**FIG 4** (A) Far-UV CD spectra of wild-type XynAS9 and its mutants in 10 mM PBS (pH 7.4). (B) Thermograms were measured using DSC. The calorimetric recordings for XynAS9 and its mutants were scanned at 1°C/min in 10 mM PBS (pH 7.4) with the protein concentration of 350 μg/ml, respectively. Symbols: wild-type XynAS9 (─)
and mutants G82E (—), V81P (◻◻◻◻◻◻), V81P/G82E (—), D185P/S186E (−−) and V81P/G82E/D185P/S186E (—).
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U/mg)</th>
<th>(K_m) (mg/ml)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (ml/s·mg)</th>
<th>(V_{max}) (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XynAS9</td>
<td>630.3 ± 12.5</td>
<td>1.03 ± 0.21</td>
<td>790.2 ± 14.3</td>
<td>760.3</td>
<td>950.8 ± 23.12</td>
</tr>
<tr>
<td>Mutant V81P</td>
<td>155.2 ± 2.1</td>
<td>3.19 ± 0.91</td>
<td>218.4 ± 3.8</td>
<td>68.2</td>
<td>268.4 ± 8.52</td>
</tr>
<tr>
<td>Mutant G82E</td>
<td>78.5 ± 1.4</td>
<td>0.60 ± 0.01</td>
<td>75.7 ± 3.1</td>
<td>121.4</td>
<td>90.3 ± 3.14</td>
</tr>
<tr>
<td>Mutant V81P/G82E</td>
<td>206.9 ± 9.6</td>
<td>1.05 ± 0.15</td>
<td>212.5 ± 7.4</td>
<td>202.5</td>
<td>255.6 ± 8.33</td>
</tr>
<tr>
<td>Mutant D185P/S186E</td>
<td>90.7 ± 3.4</td>
<td>4.02 ± 1.32</td>
<td>184.4 ± 6.4</td>
<td>45.8</td>
<td>221.9 ± 7.30</td>
</tr>
<tr>
<td>Mutant V81P/G82E/D185P/S186E</td>
<td>160.4 ± 5.3</td>
<td>2.21 ± 0.79</td>
<td>235.6 ± 6.8</td>
<td>106.5</td>
<td>283.5 ± 7.97</td>
</tr>
</tbody>
</table>
**TABLE 2** The half-lives ($T_{1/2}$) of wild-type XynAS9 and its mutants for thermal inactivation. $^a$

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$T_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65°C</td>
</tr>
<tr>
<td>XynAS9</td>
<td>16</td>
</tr>
<tr>
<td>Mutant V81P</td>
<td>27</td>
</tr>
<tr>
<td>Mutant G82E</td>
<td>45</td>
</tr>
<tr>
<td>Mutant V81P/G82E</td>
<td>255</td>
</tr>
<tr>
<td>Mutant D185P/S186E</td>
<td>35</td>
</tr>
<tr>
<td>Mutant V81P/G82E/D185P/S186E</td>
<td>228</td>
</tr>
</tbody>
</table>

$^a$ The enzyme activity was assayed at pH 6.5 and 70°C for 10 min.