

Improving hydrophobicity of N-terminal increased molecular stability of mannanase Man1312

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Abstract— Protein terminals have important roles in molecular structural stability and in some occasions they have regulatory roles in catalytic reaction. To expand our understanding on the influences of distal residues mutation, we explored the molecular stability and kinetics of mannanase Man1312 mutants. For Man1312, the N-terminal loop was more disordered and changeable; therefore the mutations on N-terminal should have significant effects on enzymic properties. The experiment was investigated by spectrophotometer, circular dichroism and differential scanning calorimetry assays. As a result, positive mutations were found from sites of T2 and Q9 and double-site mutant ManY9G2 showed increased catalytic activity by 7.7% higher than Man1312. Meanwhile, ManY9G2 had significantly increased thermostability with promoted T_{opt} by 6°C and elongated $t_{1/2}$ by 7 min, which was resulted from the optimized intermolecular forces and a newly-built hydrogen bond in ManY9G2. In our experiment, the specific residues were mutated from hydrophilic to hydrophobic and the improved hydrophobicity on N-terminal had a positive impact on the properties of mannanase Man1312.

Highlights

We construct N-terminal multi-site mutants of mannanase Man1312.

We determine the enzyme activity and thermal dynamics of mannanase and its mutants.

Best mutations increase the hydrophobicity of N-terminal.

N-terminal mutation significantly improves the thermo-stability and slightly increases activity of mannanase.

Keywords— Mannanase, N-terminal, multi-site mutation, structural stability, activity.

I. INTRODUCTION

Hemicelluloses are a series of polysaccharides consisting of linear chain and branches in the plants cell walls which are associated to the cellulose and lignin forming lignocelluloses biomass (1). Mannan is the most abundant of polysaccharide present in softwood hemicelluloses and targeted by mannanase to degrade into oligosaccharides. β -Mannanases catalyze random hydrolysis of beta-1,4-mannosidic bonds by releasing a single β -D-mannose unit from the nonreducing end of manno-oligo saccharides (2).

In the past few decades, various mannanases were from isolated from plants (3), marine mollusk (4), and a body of bacteria and fungi (5, 6), but in general, production of β -mannanase by microorganisms is more promising because of its low cost, high production rate and readily controlled conditions (7).

Mannanases play important roles in fundamental biological processes and also have potential applications in various industries. They are widely used by industries including food processing, feed, oil mining, paper making, pharmaceutical, and second generation biofuel (8, 9) as well as manufacture of oligosaccharide (7). Meanwhile, mannanases could be used in prebiotic preparation which is expected to improve the growth performance of animal.

Mannanase Man1312 screened from *Bacillus subtilis* (*B. subtilis*) B23 has the mechanism of Glycoside Hydrolase Family 26 (GH26). Although the members of GH26 family display great diversity in their sequence and function, all of them have a canonical α/β hydrolase fold, which consists of eight β -sheets flanked by α -helices. This core architecture is the structural foundation as a stable scaffold to accommodate the catalytic residues and tolerate mutations at some degree without losing its molecular stability or catalytic machinery. The loop which connects α -helices and β -strand is important to molecular structural stability and activity as well. Pleiss team studied the sequence and structure of epoxide hydrolases and proposed that NC-loop might interact with the substrate by defining the substrate-binding pocket and regulating the accessibility of active site (10, 11). The residue Tyr on the NC-loop of epoxide hydrolases is involved in substrate binding, stabilization of the transition state, and possibly protonation of the epoxide oxygen (12, 13). Moreover, loops participate in the formation of the binding sites for metallic cations (14) and ligands (15), mediates protein-protein interactions (16), and modulates protein

stability (17). All evidences suggest that exploring the functional and evolutionary role of the loop may be important for understanding the adaptation and evolution of homologous enzymes.

In our previous work, the active sites of mannanase Man1312 was detected and site-directed mutagenesis were performed on the active sites. H129, E159, H190, E191, W196, F197, W198, and W199 are the residues relevant to mannanase Man1312 activity and substrate binding; Moreover, mutants on H129, H190, and W198 had increased activity by 3.5-, 2.2-, and 3.8-fold, respectively. In this work, the disordered residues on distal loops were taken into accounts because they are relative to the molecular structural stability and in some occasions they have regulatory roles in catalytic reaction. In order to explore the relationship of the residues in distal loop and molecular stability and activity, the mutations on N-terminal loop were determined. Our work could provide insight into the integration of structure and function and had led to the design and construction of more stable and active mannanase.

II. MATERIALS AND METHODS

2.1 Computer-aided prediction for the structure of mannanase Man1312

The secondary structure, 3D structure modeling, Van der Waals' force (VDW) and hydrogen bond compute were performed by the Swiss Model server (<http://swissmodel.expasy.org/>) (18, 19, 20) using the template 2qha.1.A (the sequence identity 76.19). Structural modeling quality was evaluated with Protein Structure Validation Software (21) (PSVS, http://psvs-1_4-dev.nesg.org/). The visualization and analysis of structures were performed by PyMOL (22) and Swiss-PDB Viewer software.

2.2 Cloning, mutagenesis, expression and purification of enzymes

The decoded gene of mannanase Man1312 was transferred in *E. coli* TOP10 cells and can be cloned by the primers P1 (5'-ATGCCTACTAAGT-3') and P2 (5'-TGATTCAGCTATCTGT G-3'). Deletion mutations were constructed with the QuikChange Mutagenesis protocol (23) by pairs of reverse primers on the recombinant plasmid plasmid pBS-T-*man23* which carried the decoded gene of mannanase Man1312. Conditions of mutagenic PCR were 94°C for 4 min; 20 cycles of 90°C for 1 min, 55°C for 1 min and 72°C for 5 min; and finally extension at 72°C for 20 min. Successful introduction of deletion mutation was confirmed by DNA sequencing.

The PCR products were digested with *Bam*H I and *Eco*R I and linked with vector pHY-p43 and then transformed into *Bacillus subtilis* (*B. subtilis*) WB600. The details about expression and purification were described as previously (24).

2.3 Enzyme assay

Protein concentration was measured using the Bradford assay (25). The Mannanase activity assay was improved from the method of monitoring the release of reducing sugar (26, 27). One unit of enzyme activity was defined as the amount of enzyme liberating 1 μ mol mannose per minute at 50°C and pH 6.8. The activity formula was as follows:

$$\text{Mannanase activity (U/ml)} = 5.56C_e V_{de} / V_{je} V_s t$$

5.56	the mole value of 1.0 mg mannose, μ mol
C_e	amount of mannose produced from hydrolysis, mg
V_{de}	metered volume of enzyme solution, ml
V_{je}	volume of enzyme solution added into the reaction mixture, ml
V_s	volume of substrate solution, ml
t	time, min

$$\text{Specific activity of mannanase (units per milligram)} = 5.56C_e V_{de}^2 / C_p V_{je} V_{st}$$

C_p	amount of total proteins, mg
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2.4 Kinetic parameters of enzymes

T_{opt} was determined at the temperature range of 30~90°C under pH 5.8. T_{50} was determined at the temperature range of 30~90°C for 20 min and $t_{1/2}$ was determined at the optimum temperature for 1~40 min. The buffer was 50 mmol/l phosphate buffer.

V_{max} and K_m were acquired by fitting enzymatic activities as a function of substrate concentrations to the Michaelis-Menten equation using non-linear regression of the software GraphPad Prism 5.0. The parameter k_{cat} was obtained by using the equation $k_{cat} = V_{max} / [E]$, where [E] was the molar concentration of the enzymes.

2.5 Thermal dynamics of enzymes

The secondary structure of mannanase Man1312 and its mutants were monitored by Circular dichroism (CD) spectropolarimeter (JASCO J810, Japan) from 190 to 260 nm with the parameters as protein concentration 0.1 mg/ml, 0.1 cm path-length cuvette at 50 nm/min scan speed, response time 2 s, bandwidth 2 nm, and pitch 0.1 nm. For thermodynamic analysis, the spectra were recorded at 220 nm with the following parameters: protein concentration 0.2 mg/ml, and temperature interval 0.5°C/min from 40~90°C. T_m was determined through the change in the CD value.

Differential scanning calorimetry (DSC) was performed on DSC200F3 Differential Scanning Calorimeter (NETZSCH, German) with protein concentration 1.0 mg/ml. Denaturing curves were recorded from 40 °C to 90°C at a rate of 1°C/min.

2.6 Statistical Analysis

Data were presented as the mean±standard error of the mean. Results were compared with the analysis of variance and Fisher's protected least-significant difference tests, with a significance of $P<0.05$.

III. RESULTS

3.1 A mutant library on N-terminal loop was developed to discover key positions relevant to enzyme properties

Mannanase Man1312 has a typical $(\beta/\alpha)_8$ barrel structure of GH26 according to its amino acid sequence similarities. The N-terminal loop of Man1312 includes the first 11 residues from H1 to Q11 (Fig. 1). Saturation mutagenesis was applied on each site of N-terminal loop to identify catalytic effects of the substitutions by other amino acid residues at the each position. The saturation mutant library contained 220 clones, but significant mutations were observed on site T2 and N9 (Fig. 2). Three mutants showed an enhanced thermo-stability activity but without activity loss (Table 1).

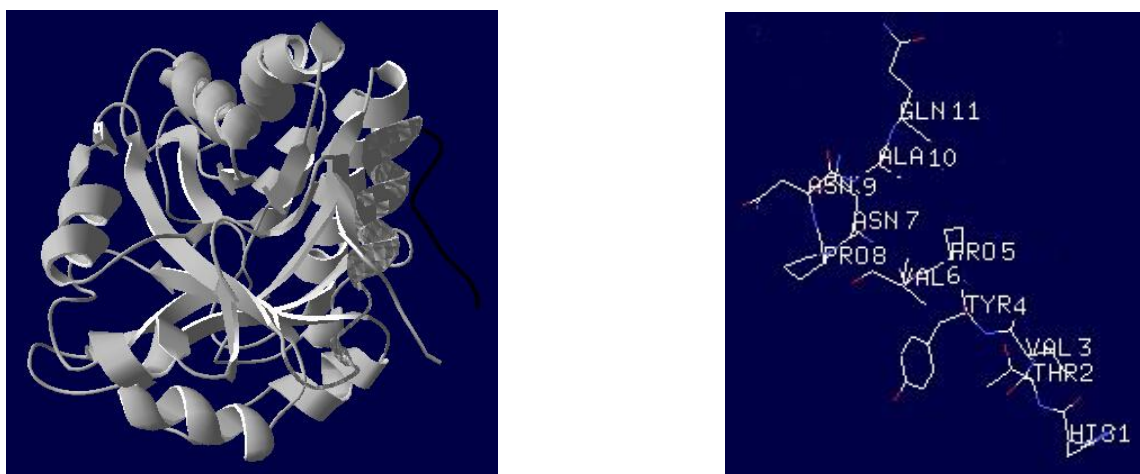


FIG. 1 THE TERTIARY STRUCTURE OF MANNANASE MAN1312

A structure of mannanase Man1312; b residues on N-terminal of mannanase Man1312. The black represented N-terminal region which is the research target in this paper and its sequence is H-T-V-Y-P-V-N-P-N-A-Q.

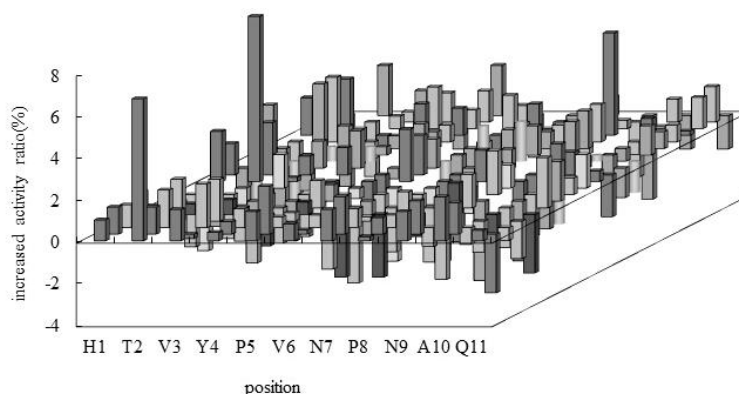


FIG. 2 THE ACTIVITY INCREASING RATIO IN THE SATURATION MUTANT LIBRARY

The saturation mutant library contained 220 clones, among which significant mutations were observed on site T2 and N9 with the substitution of alanine and glycine on T2 and tyrosine on N9.

TABLE 1
ENZYME ACTIVITY AND KINETICS PARAMETERS OF MANNANASE MAN1312 AND ITS MUTANTS T2A, T2G AND N9Y

	Mannanase amount (mg/ml)	Mannanase activity (U/mg)	T_{opt}	T_{50}	$t_{1/2}$
Man1312	8.5 ± 0.1	245 ± 0.5	60°C	65°C	25 min
ManT2A	8.65 ± 0.1	253 ± 0.5	62°C	66°C	26 min
ManT2G	8.6 ± 0.1	260 ± 0.5	62°C	66°C	26 min
ManN9Y	8.7 ± 0.1	254 ± 0.5	65°C	68°C	30 min

3.2 The double mutant was constructed to build top variants

From the single-site saturation mutations, the substitution of alanine and glycine on T2 and tyrosine on N9 showed significant improvement on enzyme properties. The first top substitution was N9Y, therefore tyrosine was the best and exclusive mutation on position 9. The second top substitution was T2G and the third was T2A, both of which mutated on position 2. Two double-site mutants were constructed, each of which combined the N9Y substitution with T2G and T2A respectively. The assessment of two double-site mutants illuminated the combination of N9Y and T2G was the best variants with 7.7% higher activity and 28% longer half-life (Table 2).

TABLE 2
ENZYME ACTIVITY AND KINETICS PARAMETERS OF MANNANASE MAN1312 AND ITS MUTANTS

	Mannanase amount (mg/ml)	Mannanase activity (U/mg)	K_m (mg/L)	K_{cat} (s^{-1})	T_{opt}	T_{50}	$t_{1/2}$ (min)
Man1312	8.5±0.1	245±0.5	9.5±0.5	8.2±0.2	60°C	65°C	25
ManY9A2	8.65±0.1	256±0.5	8.1±0.3	9.2±0.2	65°C	67°C	28
ManY9G2	8.7±0.2	264±0.5	7.2±0.2	10.4±0.2	66°C	69°C	32

As for double mutant ManY9G2, the residues were mutated from Thr of site 2 and Asn of site 9 to Gly and Tyr. Threonine and asparagine are both hydrophilic amino acids, but glycine and tyrosine are hydrophobic, therefore the double mutant improved hydrophobicity of N-terminal which could be a reason of optimized properties of ManY9G2.

3.3 The double-site mutant ManY9G2 exhibited more stable on thermal dynamics

According to the thermal denaturation spectra (Fig. 3a), the T_m values of double-site mutant ManY9G2 was 69°C, which was 5°C higher than that of Man1312. Taking into account that the mutant T_m value was basically consistent with $t_{1/2}$ value, it could be concluded that the enhanced enzyme thermo-stability might be caused by the increase of T_m . Meanwhile, from the ΔG^0 profiles between the native and denatured states, the slopes of ManY9G2 decreased more slowly than that of Man1312 (Fig. 3b). Correspondingly, the Man1312 unfold more quickly than ManY9G2. Because of the higher T_m and the slower decreasing ΔG^0 , ManY9G2 was more thermostable. To obtain further information on the thermodynamic stability, thermal unfolding experiments were carried out by DSC (Fig. 4). As the above results, T_m value of mutant ManY9G2 was ~5°C higher than Man1312.

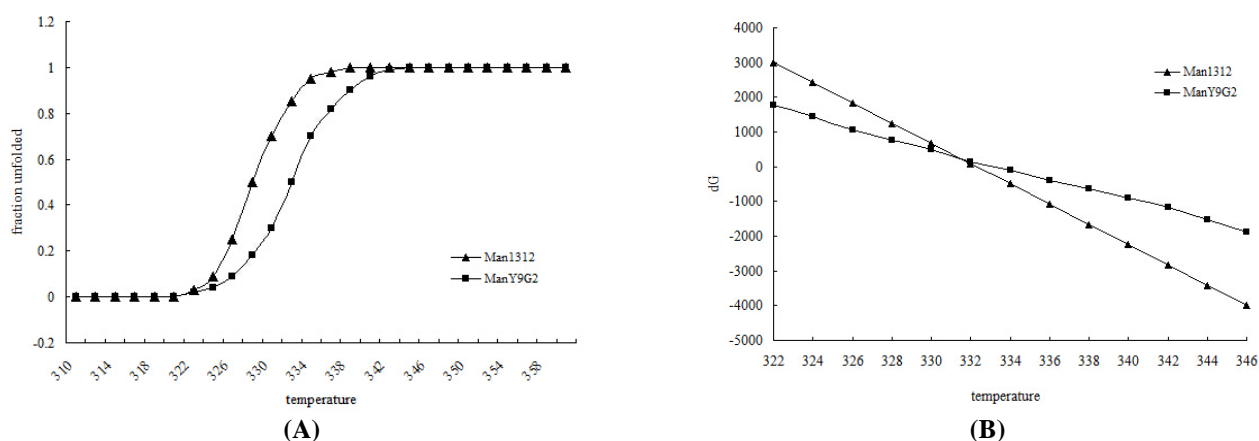


FIG. 3 FRACTION UNFOLDED (A) AND MOLAR GIBBS FREE ENERGY CHANGES (ΔG^0) (B) DURING THE THERMAL DENATURATION PROCESS OF MANNANASE

The CD data were measured at 220 nm from 310 to 360 K at a heating rate of 0.5 °C/min and were converted to an unfolded fraction through the equation ($f_u = (y - y_n)/(y_d - y_n)$), where y is the ellipticity observed at the given temperature and y_n and y_d are the characteristic ellipticities of the folded and unfolded protein. ΔG^0 (dG, J/mol) was calculated by the standard van't Hoff equation ($\Delta G^0 = -RT(f_u/(1 - f_u))$).

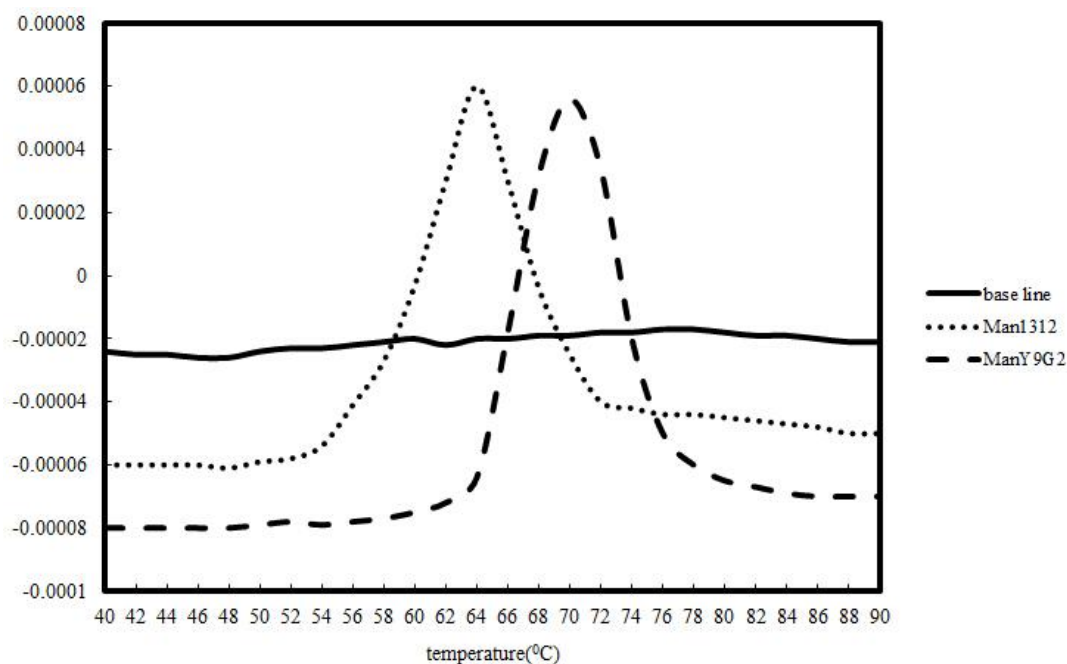


FIG. 4 CD SPECTRA PROFILES OF MAN1312 AND DOUBLE-SITE MUTANT MANY9G2
CD spectra were recorded from 190 to 260 nm. Protein concentration of 0.1 mg/ml was used for the analysis.

To compare the secondary structural between Man1312 and its mutant ManY9G2, the CD spectra were presented to evaluate secondary structural elements of helix, sheet, and coil which characterized negative CD spectrum bands at 222 and 210 nm, 215 nm, and 195 nm, respectively.

Both of CD profiles had two clear minima at 210 and 222 nm (Fig. 5), in responding to GH16 hydrolase having both α -helix and β -sheet features. The CD spectra of Man1312 and ManY9G2 exhibited similar minima at 222 nm, however the amplitude decreased on ManY9G2 comparing with Man1312. The irregular coil had a characterized negative band at \sim 195 nm, the spectra minima of ManY9G2 at 210 nm shifted toward higher wavelengths, indicating that the contents of helix and strand on ManY9G2 increased relatively. Thus, the results stated mutant ManY9G2 increased enzyme stability.

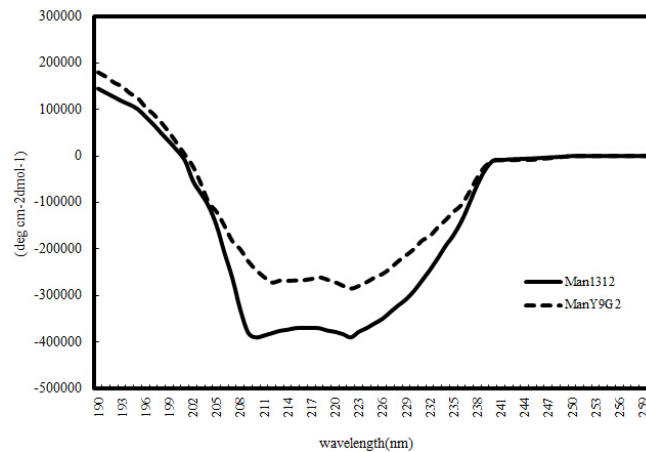
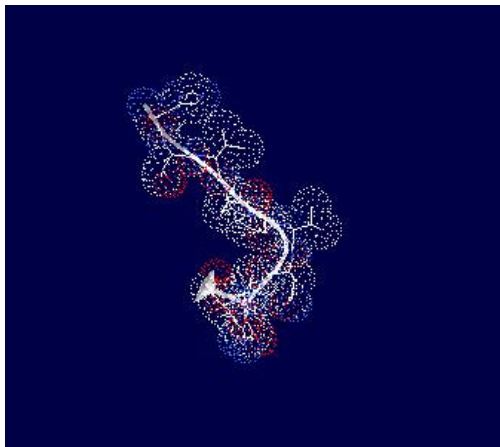


FIG. 5 THERMAL UNFOLDING OF MAN1312 AND DOUBLE-SITE MUTANT MANY9G2 MONITORED BY DSC SCANNING

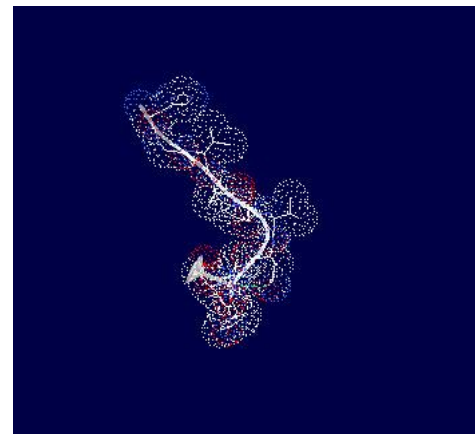
Protein concentration of 1.0 mg/ml was used for the analysis. Denaturing curves were recorded from 40°C to 90°C with a rate of 1°C/min.

3.4 Molecular analysis around mutated sites on N-terminal was performed by computer-aided software

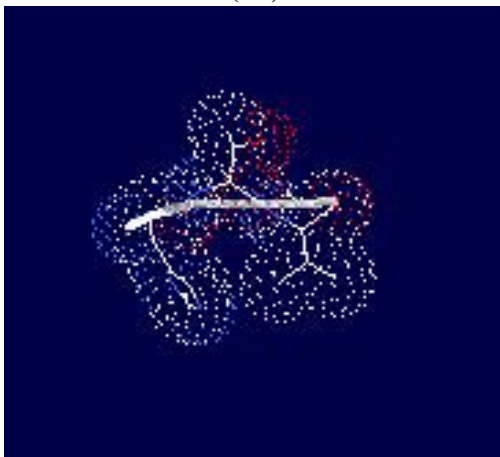
From the VDW prediction (Fig. 6), ManY9G2 formed stronger van der Waals force around position 9, but became weak around position 2. ManY9G2 showed more stable comparing to Man1312, indicating that substitution on position 9 had more important effects on thermal stability than position 2. On the other side, around position 9, one more hydrogen bond was built after being mutated from Asn to Tyr (Fig. 7), but there is no newly-built hydrogen bonds appear around position 2. It can be perceived that the changes around position 9 should be main reasons for thermos-stability of mannanase ManY9G2.



(6A)



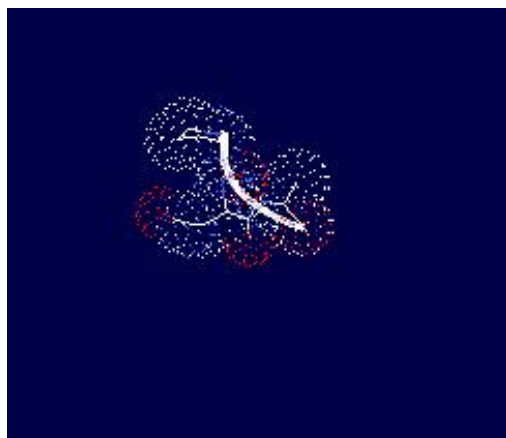
(6B)



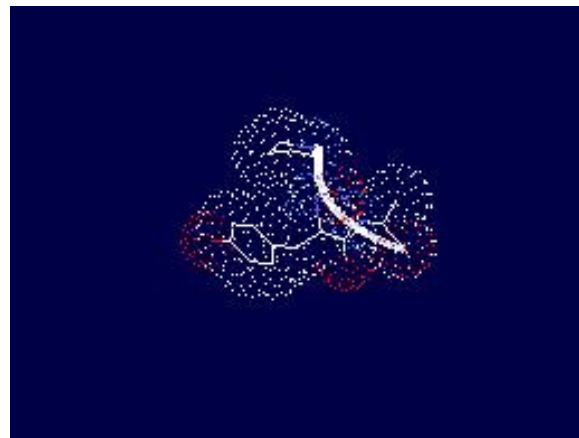
(6C)



(6D)



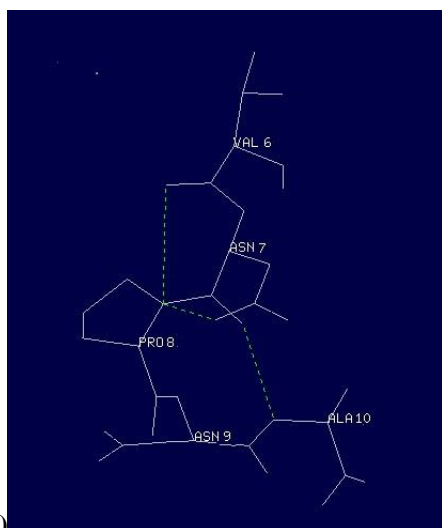
(6E)



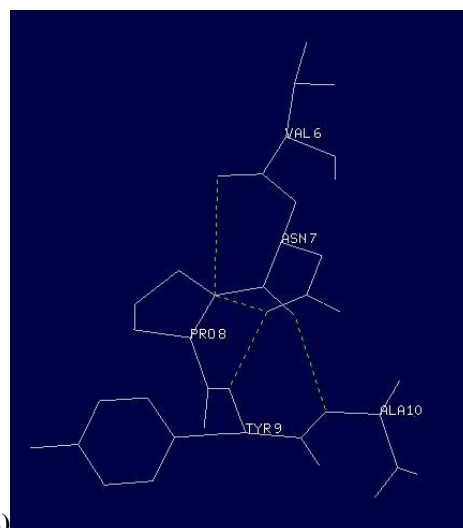
(6F)

FIG. 6 THE VDW ANALYSIS ON N-TERMINAL OF MAN1312 AND MANY9G2

A N-terminus of Man1312; b N-terminus of ManY9G2; c position 2 surrounding of Man1312; d position 2 surrounding of ManY9G2; e position 9 surrounding of Man1312; f position 9 surrounding of ManY9G2.



(7A)



(7B)

FIG. 7 HYDROGEN BONDS ANALYSIS AROUND POSITION 9 OF MAN1312 AND MANY9G2

Dash lines represented hydrogen bonds formed by inter-molecules. a hydrogen bonds around position 9 of Man1312 (before mutation); b hydrogen bonds around position 9 of ManY9G2 (after mutation). Before being mutated, three hydrogen bonds were formed around position 9 between Val6-Pro8, Asn7-Pro8, and Asn7-Ala10; after being mutated, besides three bonds formed before, one more hydrogen was newly built between Asn7- Tyr9.

IV. CONCLUSION

According to the hydrophobic cluster analysis, Mannanase Man1312 has been classified into GH26 family. The $(\alpha/\beta)_8$ barrel is one of GH26 hydrolase features and enzymes in this family display a broad variety of activity, including endo- β -1,4-mannanases, exo-acting β -mannanase (28), β -1,3:1,4-glucanase (29) and β -1,3-xylanase activities (30). For this reason, they are one of the most widely used groups of biocatalysts in industry.

In our previous work, the active sites were clarified and the expression system of mannanase Man1312 decoded gene was optimized. Furthermore, rational evolution strategy was performed on α/β fold of Man1312 to improve the molecular stability. The present study focused on the relationship of mutations on the N-terminal loop and molecular stability and activity basing on the structural analysis.

Our results suggest that the N-terminal loop could modulate both catalytic activity and stability of Man1312 and the mutations increased Man1312 thermostability and activity. The double mutant ManY9G2 increased catalytic activity by 7.7% higher than Man1312 and meanwhile, it increased T_{opt} by 6°C and $t_{1/2}$ elongated by 7 min in compare with Man1312.

From the spectra and modeling, ManY9G2 increased contents of the helix and strand and thus, led to a slow decreased ΔG^0 tendency in the thermal denaturation process, which indicated ManY9G2 had more mechanical stabilities and higher stiffness. Moreover, around the mutated positions 9, ManY9G2 formed stronger van der Waals force due to its greater electron cloud overlap and formed a new hydrogen bond between Tyr9 and Asn7, indicating that substitution on position 9 had more important effects on thermal stability.

Similar to our work, Zhai constructed a loop-replacement mutant (L6RM) and solution nuclear magnetic resonance data show that the L6RM results in significant chemical shift changes in the loop and surrounding regions. The interactions with the L6RM loop stabilize the enediolate intermediate toward the elimination reaction catalyzed by the LDM (31). Results from Pertusa suggested the N-terminal domain of TRPM8 had a critical contribution to thermal and chemical sensitivity. Single point mutations caused a comparable increase in the responses to cold and menthol (32). The five N-terminal mutations might confer structural stability, and hence, prevent the overall thermal unfolding of the mesophilic *Streptomyces olivaceovirdis* xylanase (33). Dumon researched on the 15 most thermostable xylanases, the results showed that the N-terminal region was more susceptible to thermal unfolding (34).

Our results demonstrate that the specific mutations on N-terminus can have a significant impact on chemical properties of mannanase Man1312. With the better understanding of sequence-structure-function relationship of the second structure in different α/β hydrolases, the more benefit for industrial applications would be achieved through engineering this considerable group of enzymes.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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