Structural characterization and molecular dynamics simulations studies of family-10 xylanase from *Bacillus firmus* K-1 complex with xylopentaose: a novel feature of the xylanase catalytic mechanism

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ABSTRACT

Xylanases hydrolyze the β-1,4-linked xylose backbone of xylans, the major hemicellulosic components of plant cell walls. They are especially useful in paper industry because they decrease the demand for chlorine-based chemicals in the wood pulp delignification process. In this study, 3D structure of Xyn10A, a family 10 xylanase from *Bacillus firmus* K-1 was generated by homology modeling using X-ray structure of xylanase from *Bacillus* sp. strain NG-27 as a template. The root mean square deviation (RMSD) of backbone atoms between the X-ray and homology modeled structures was 0.8 Å. Binding of xylopentaose (X_5) to the Xyn10A was investigated using molecular dynamics simulations via comparison the total energy, RMSD of Cα atom and root mean square fluctuation (RMSF) of free and complex forms of Xyn10A. In the reactive Xyn10A-X_5 conformation, in which the two catalytic sites, E149 and E255 are precisely positioned for the catalytic reaction, the -1 sugar moiety of the X_5 adopted a $^1C_4$ chair conformation for the Xyn10A. According to the RMSF, 13 amino acid residues in active site of complex form showed more flexibility than those of free form. The result suggested that they may implicate in binding to X_5 at subsites -3 to +2 of substrate-binding site through hydrogen bonding and stacking interactions. Furthermore, the RMSF of X_5 revealed that the substrate binding site of Xyn10A was more specific to three middle xylose moieties than two terminal xylose moieties. The role of sugar moiety interaction with Xyn10A in catalytic mechanism is discussed.

Keywords: Bacillus firmus, 3D structure, family 10 xylanase, homology modeling, molecular dynamics simulations

INTRODUCTION

Xylanases (EC 3.2.1.8) are glycoside hydrolases that catalyze the random hydrolysis of internal β-1,4 bonds of xylan backbones, a major component of plant hemicellulose (Wong *et al*., 1988). On the basis of amino acid similarities, generally xylanases have been classified into family 10 (formerly F) and family 11 (formerly G) glycosyl hydrolases (Henrissat, 1991) and a few of xylanases belong to families

5, 8, and 43 (http://afmb.cnrs-mrs.fr/CAZY/). The 3D structure of family 10 possessed an $(\alpha/\beta)_8$ fold whereas that of family 11 have predominantly β-sheets with one α-helix (Henrissat and Davies, 1997). In paper and pulp industry, xylanases were used to reduce the chlorine consumption in the bleaching process of kraft pulp and therefore to lower environmental pollution by organic halogens (Viikari *et al*., 1994). The pulp being produced by treating the wood batches at high temperature and basic pH, the subsequent enzymatic procedure need protein showing a high thermostability and optimally active in broad pH range.

The alkali-thermostable cellulase-free xylanase, Xyn10A from alkaliphilic *Bacillus firmus* K-1 (formerly known as *Bacillus sp.* strain K-1) (Ratanakhanokchai *et al*., 1999), belonging to family 10 glycosyl hydrolase was reported the amino acid sequence by Chang *et al.* (Chang *et al*., 2004). It shows optimally active at board pH range and high temperature, which is attractive to apply in pulp-prebleaching process of paper and pulp industry. Until now, 3D structures of several family 10 xylanases have been solved. However, 3D structure of only two xylanases produced from an alkalophilic organism have been determined. The objective of this study is to build the 3D structure of Xyn10A from *B. firmus* K-1 by homology modeling. The interaction of Xyn10A and its substrate (X_5), was then investigated for understanding the substrate-binding site and catalysis mechanism of the xylanase by molecular dynamics simulations.

METHODOLOGY

1. Homology modeling of Xyn10A

The 3D structure of Xyn10A was generated using the homology module of Insight II, which are well-known program (Insight II, 2001) based on X-ray structure of template which showed the highest identity percentage. The sequences of Xyn10A and template were aligned by homology module to identify the blocks, which are likely to contain structurally conserved region. All atomic coordinates of the residues in those blocks were transferred from template to build the modeled structure of Xyn10A. However, for the mismatch residues, only the atomic coordinate of C_α were transferred from template while the residual atomic coordinate were generated by using library. The homology-modeled structure was then energy-minimized by discover module of Insight II. The minimization was performed with a 5 Å-water layer in dimensions of 120 x 120 x 120 Å water box. First, the steepest descent algorithm was used for 1000 steps to remove close van der waals contacts, followed by the more efficient conjugate gradient algorithm until a tolerance of 0.01 kcal/mol/Å in the gradient is reached. After the minimization, the layer water was removed. The final structure was then evaluated by Procheck (Laskowski *et al*., 1993) and Verified 3D programs (Eisenberg *et al*., 1997).

2. Molecular dynamics simulations

Molecular dynamics simulations were achieved using the GROMACS package version 3.3.1 (Lindahl *et al*., 2001). Structure of the X_5 was built by biopolymer module of Insight II. The X-ray structure of *Pseudomonas fluorescens* xylanase in complex with X_5 (Lo *et al*., 2000) was used as reference for positioning

the X_5 in the active cleft of the modeled structure of Xyn10A. The Xyn10A free form and Xyn10A-X_5 complex form has been performed by using GROMOS96 force field. A cutoff distance of 9 Å was applied for Lennard-Jones interactions. To maintain the system at a constant temperature of 310 °K, a Berendsen thermostat was applied using a coupling time of 0.1 ps. Pressure was held at 1 bar, with a coupling time of 1.0 ps. The time step was set as 2 fs. The simulation cell was a cubic box with a minimum distance of 1 nm between protein and box walls, so that the protein would not interact directly with its own periodic image, given the cut off. The protein was solvated with water in a box containing simple point charge (SPC) water molecules. All systems were minimized using the steepest descent method for 1,000 steps. A simulation was initiated by dynamic equilibration of solvent molecules at 310 °K for 1 ns, followed by equilibration for 10 ns of simulation. All trajectories were kept to analyze every 10 ps. All graphic pictures in this study were drawn by Discovery Studio 2.0.1 program.

RESULTS AND DISCUSSION

1. Homology modeling of Xyn10A

1.1 3D structure generation

The 3D structure of Xyn10A was generated by homology module of Insight II based on the X-ray structure of family 10 xylanase from *Bacillus* sp. NG-27 (2F8Q chain A) (Manikandan *et al*., 2006), showing the highest identity percentage (77.9%). Although, amino acid sequence of Xyn10A contains 396 residues, only 350 residues were generated because 46 residues at N-terminal (M1 to N46) did not contain template structure. The successfully homology-modeled structure of Xyn10A is shown in Figure 1.
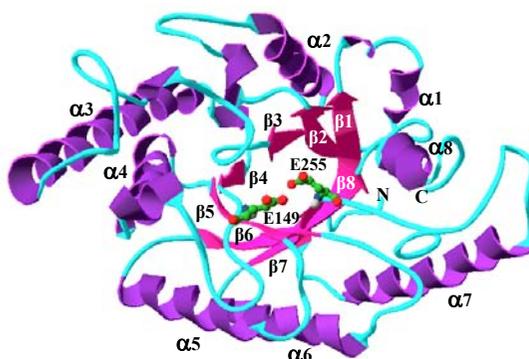
Figure 1 The homology-modeled structure of Xyn10A. The secondary structure
comprised of 8 α-helices (α) and 8 β-sheets (β) are shown. Two catalytic
sites are presented in ball-and-stick. The N- and C-terminals are indicated
as N and C, respectively.

The backbone RMSD between the 3D structures of Xyn10A and template was 0.8 Å. The small value of RMSD could be interpreted to mean that the two structures share common homology (Lee and Briggs, 2004). The structure of Xyn10A folds to form a standard $(\alpha/\beta)_8$ barrel fold typical for member of family 10 xylanases. On the basis of sequence alignment with the other family 10 xylanases, E149 at the C-terminus of β4 and E255 in the middle of β7 are identified as catalytic residues, that function as acid/base and nuclephile, respectively (Chang, *et al.*, 2004).

1.2 Protein structure validation

According to the Ramachandran plots of Xyn10A computed with the Procheck program and template (2F8Q chain A) showed that the spot distributions for the homology-modeled structure of Xyn10A were similar to the X-ray structure of template (Figure 2a) corresponding to the percentage of the residues in "most favored regions (the darkest area)" are 90.1% for template and 84.7% for homology-modeled Xyn10A structure (see detail in Figure 2b). The 3D-1D scores averaged over all residues checked by Verified 3D program were 0.49 for the Xyn10A and 0.47 for the template. The score value, for a well folded protein, should be above 0.1 (Eisenberg *et al.*, 1997). The results of structural validation revealed that the homology-modeled structure of Xyn10A is reasonable and acceptable.

B	2F8Q chain A	Xyn10A
Residues in most favored regions	284 (90.1%)	264 (84.7%)
Residues in additional allowed regions	31 (9.9%)	46 (14.7%)
Residues in generously allowed regions	0	2 (0.6%)
Residues in disallowed regions	0	0
Number of non-Gly and non-Pro residues	315 (100%)	312 (100%)
Number of end residues	2	2
Number of Gly residues	19	22
Number of Pro residues	18	14
Total number of residues	354	350

Figure 2 Ramachandran plots (A) and the results of structure validation calculated by procheck program (B) of the X-ray structure of *Bacillus* sp. NG-27 xylanase (2F8Q chain A) (left) and the homology-modeled structure of Xyn10A (right).

2. Molecular dynamics simulations

In order to study the interaction of the enzyme-substrate complex, free and complex forms of Xyn10A were subjected to MD simulations. Total energy of the system and RMSD of Cα atom from free and complex forms of Xyn10A and RMSD of Cα atom of X_5 were shown in Figure 3. During the MD simulations, the total energy level of complex form was notably higher (more negative value) than that of free form (Figure 3a). These results revealed that the structure of Xyn10A-X_5 complex was more stable than that of free form. The RMSD values of both forms of Xyn10A slightly increase before 5 ns (Figure 3b). After 5 ns, the RMSD values of the free form almost constant while complex form varied continuously corresponding to the RMSD values of X_5 (Figure 3c). Because of the interaction of Xyn10A and X_5, RMSD was consecutively changed unlike the free form.

The RMSF was used to investigate the fluctuation of amino acid residues in enzyme structure as well as each atom in substrate molecule. The RMSF of Xyn10A and X_5 molecule, averaged at stable state (from 5-10 ns), as shown in Figure 4. The RMSF of most residues in complex form of Xyn10A showed a greater degree of fluctuation than free form (Figure 4a). Focused on amino acid residues in active site, the RMSF of 13 residues in the Xyn10A complex forms showed higher flexibility than those of free form. These residues, such as N49, K52, S55, W89, D153, Y193, K200, H226, W231-P232, W307, W315, and R319, possible implicated in substrate binding site of Xyn10A. The RMSF of X_5 found five flexible sections relating to five xylose moieties in X_5 molecule (Figure 4b). Three xylose moieties at middle site showed less flexible peaks than two peripheral xyloses units. These results suggest that three middle xylose moieties tightly bound to substrate binding site of Xyn10A. As two xylose moieties at terminal sites, the substrate binding site Xyn10A showed less specific, therefore Xyn10A should be able to accommodate sugar units which are decorated with the different kinds of substituents at these sites (Gruber, *et al.*, 1998).
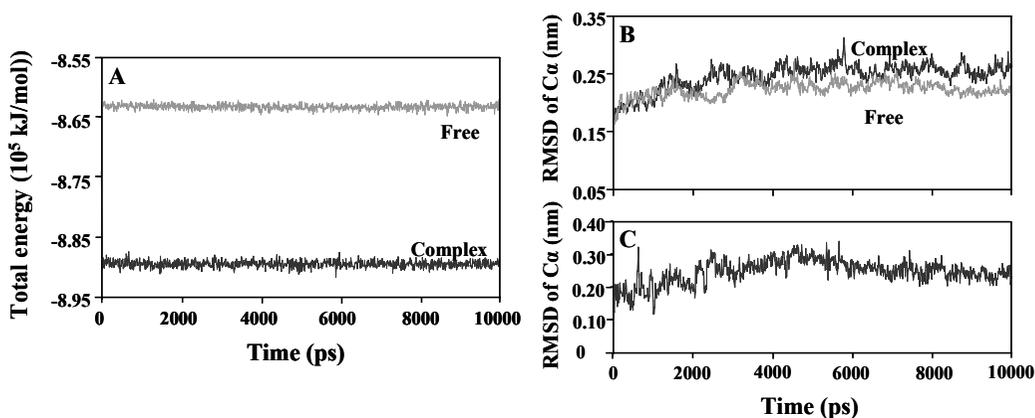
Figure 3 Time dependence during 10 ns of total energy (A) and RMSD of Cα atom (B) of Xyn10A free form and complex form and the RMSD of the X_5 (C).
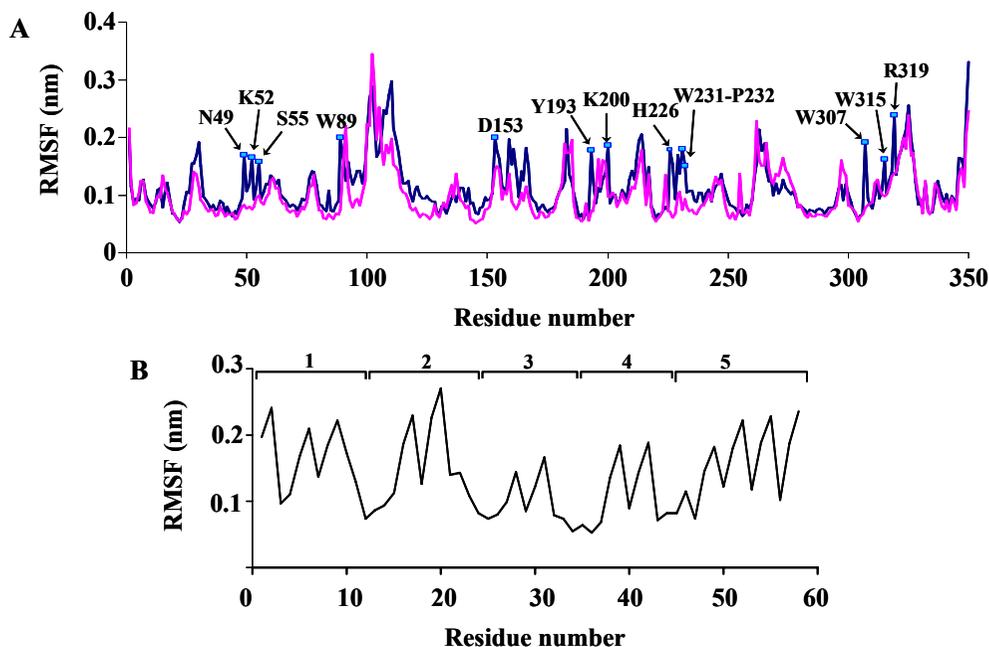
Figure 4 The RMSF profiles of the Xyn10A in free (pink) and complex (blue) forms (A) and the RMSF profiles of the X_5 (B) averaged from 5 to 10 ns of MD simulations.

3. Prediction of possible amino acid residues in each subsite of Xyn10A

The electrostatic surface structure of Xyn10A in complex with X_5, averaged during stable state of MD simulations, was depicted in Figure 5a. In surface structure of Xyn10A mostly found negatively charge residues (red), aspartic acid and glutamic acid. The residues surrounding the two catalytic sites showed neutral charge. The X_5 bound to subsites -3 to +2 in substrate-binding site of Xyn10A by rotating the reducing end (anomeric carbon) to the right whereas the non-reducing end turned to the left in case of E149 and E255 locating at top and bottom, respectively. The hydrogen bonding (< 4 Å) and stacking interactions (≤ 5 Å) were calculated for prediction the possible amino acid residues binding to X_5 in each subsite (-3 to +2) of Xyn10A, as shown in Figure 5b and see detail in Table 1. Five aromatic residues formed stacking interactions with xylose rings at individual subsites such as W307 and W315 at subsite -1, W89 at subsite -2 and Y193 and W231 at subsite +2. Moreover, at subsite +2 found that WP sequence-structure interaction motif which was aromatic ring of W231 is sandwiched between P232 and the xylose ring. The motif also presents in the template structure (Manikandan *et al*., 2006). Hydrogen bonding interaction, occurring between hydroxyl groups of xylose moieties and side chains of amino acid residues, found in all subsites including H226 at subsite -1, K52 at subsite -2, S55 and N49 at subsite -3, R319 at subsite +1 and D153, Y193 and K200 at subsite +2. The geometry of xylose ring of X_5 at subsite -1 was likely to adopt a $^1C_4$ chair conformation for catalytic mechanism of Xyn10A similar to other family 10 xylanases.
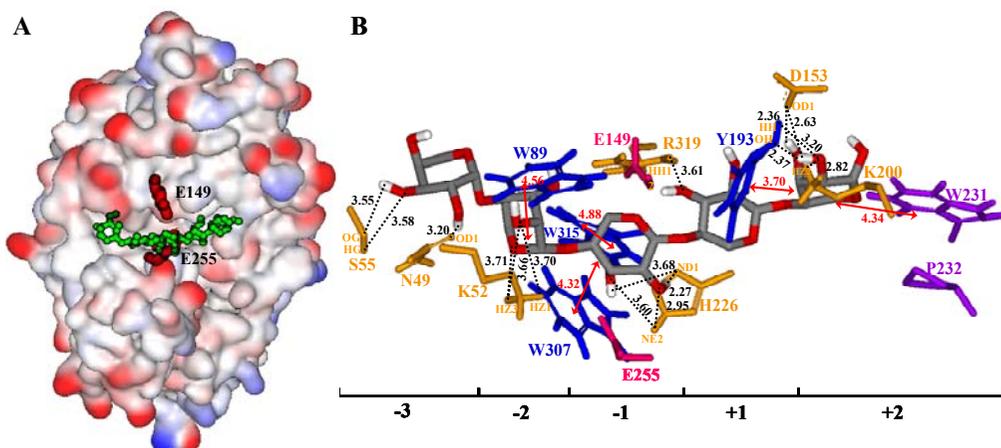
Figure 5 Electrostatic surface representation of the Xyn10A in complex with X_5 (a). The surface potentials of color property from red, negatively charged to blue, positively charged. Amino acid residues in each subsite (-3 to +2) formed hydrogen bonding (----) and stacking (↔) interactions (in Å) with X_5 are shown in orange and blue, respectively (b). Two catalytic sites locating between -1 and +1 are shown in pink. W231-P232 motif at subsite +2 is indicated in violet.

Table 1   Summary of possible stacking interactions and hydrogen bonds in each subsite of Xyn10A binding to X_5

Subsite	Amino acid residues formed bonds with xylose molecules in each subsite (distance in Å)				
	Stacking interaction		Hydrogen bond		
	Substrate ring	Side chain	Substrate atom[a]	Side chain	Atom
-3	Xyl 1	-[c]	OH3	S55	OG (3.55)
					HG (3.58)
			OH2	N49	OD1 (3.20)
-2	Xyl 2	W89 (4.56)	OH2	K52	NZ3 (3.66)
			O[b]		NZ3 (3.71)
			OH2		HZ1 (3.70)
-1	Xyl 3	W307 (4.88)	OH2	H226	NE2 (2.95)
		W315 (4.32)	H2		ND1 (2.27)
			OH3		NE2 (3.60)
			H3		ND1 (3.68)
+1	Xyl 4	-[c]	OH3	R319	HH2 (13.61)
+2	Xyl 5	Y193 (3.70)	H2	D153	OD1 (2.63)
		W231-P232 (4.34)	H3		OD1 (2.36)
			OH2	Y193	OH (3.20)
			H2		HH (2.37)
			OH2	K200	HZ2 (2.82)

[a] Labeled number at hydroxyl group and hydrogen atom indicated the position of carbon atom in each xylose ring
[b] Endocyclic oxygen
[c] No stacking interaction

CONCLUSION

The 3D structure of Xyn10A was achieved by homology modeling. Subsequently, the interaction of homology-modeled structure of Xyn10A in complex with X_5 was studied by using MD simulations. Result of comparison of total energy and RMSD of Cα atom between the structures of Xyn10A-X_5 complex and free form revealed that the complex form relatively equilibrated after 5 ns. Combination of the RMSF of residues in active site and the computation of hydrogen-bonding and stacking interactions showed that 13 amino acid residues played a pivotal role in binding to X_5 and occupied at subsites -3 to +2 in substrate-binding site of Xyn10A. The Xyn10A more tightly bound to three middle xylose moieties than two terminal xyloses since it preferred to bind xylan without branch chain at the middle site. At terminal sites, it might be specific to bind to decorated xylose. The adaptation of xylose units at subsie -1 in a $^{1}C_4$ chair conformation extremely involved in catalytic mechanism of Xyn10A.

ACKNOWLEDGMENTS

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