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Full Length Research Paper

Enhancement of the thermo-alkali-stability of xylanase II from *Aspergillus usamii* E001 by site-directed mutagenesis

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Replacing several serine and threonine residues on the Ser/Thr surface of xylanase II (Xyn II) from *Aspergillus usamii* E001 with arginines effectively increased the thermostability of the enzyme. The substitution of Ser and Thr residues on the Ser/Thr surface of the enzyme with four (ST4) or five arginines (ST5) led to an increase in optimal temperature of the enzymes by 2 and 5°C for the ST4 and ST5, respectively. The modified enzymes ST4 and ST5 showed 75 and 87% of maximal activities after incubated for 15 min at 55 ℃ compared to only 31% activity for wild-type enzyme. After incubated for 1 h at 55°C, ST4 and ST5 showed 61 and 77% of maximal activity compared to only 27% activity for wild-type enzyme. In addition, these mutations shifted the pH-dependence activity profile to the alkaline region by 1.0 pH units. Kinetic parameters of the four-arginine-substitution enzyme were maintained at the level of the wild-type enzyme with the K_m and V_{max} values of 5.39±0.20 mg ml^{-1} and 1410±67 U mg^{-1} protein, respectively. The five-arginine-substitution enzyme showed only slight alteration in K_m and V_{max} with K_m of 7.68±1.3 mg ml^{-1} and V_{max} of 1161±75 U mg^{-1} protein, indicating lower substrate affinity and catalytic rate. The study demonstrate that properly introduced arginine residues on the Ser/Thr surface of xylanase might be very effective in the improvement of enzyme thermostability and alkalistability.

Key words: *Aspergillus usamii*, thermostability, xylanase, site-directed mutagenesis, pH-dependence activity.

INTRODUCTION

Xylan is one of the major constituents of plant cell walls. As the most abundant hemicellulose, it accounts for more than 30% of the dry weight of terrestrial plants. Xylan thus belongs to the main food source of farm animals and also represents a major component of the raw material for many industrial processes ranging from baking to paper production (Beg et al., 2001; Polizeli et al., 2005). It consists of a backbone of β-1,4-linked xylopyranose residues (Subramaniyan, 2012), usually with branches of α-1,3-linked L-arabinose and α-1,2-linked D-glucopyranose (Souza et al., 2012; Sunna and Antranikian, 1996). Several enzymes such as endo-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase are involved in the hydrolysis of xylan polymers. Among them, the most important one is the β-1,4-xylanase (EC 3.2.1.8), which cleaves internal glycosidic bonds at random or specific positions of the xylan backbone and thus hydrolyzes xylan into xylooligosaccharide and xylose (Biely, 1985).

On the basis of sequence similarities and hydrophobic cluster analysis, endo-β-1,4-xylanases have been mainly grouped in families 10 and 11 of the glycosyl hydrolases (Collins et al., 2005; Jeya et al., 2009; Sibtain et al., 2009;

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Wu et al., 2011). Family 10 xylanases exhibit eightfold α/β barrel structures and have molecular masses>30,000, whereas family 11 xylanases have an all β-strand sandwich fold structure resembling a partly closed “right hand” and a lower molecular mass of ~20,000 (Hakulinen et al., 2003; Zhou et al., 2009). Although xylanases have been widely used, their application in the paper, pulp and feed industries is limited somewhat by certain enzymatic characteristic such as poor resistance to high temperature and narrow optimal pH range (Subramaniyan and Prema, 2002). Therefore, it is essential to improve the properties of xylanases for broader applications.

The 3D structures of several thermophilic and mesophilic xylanases including *Trichoderma longibrachiatum* (Uzuner et al., 2010), *Aspergillus niger* 400264 xylanase XYNB (Xie et al., 2011), and *Aspergillus kawachii* (Fushinobu et al., 1998) have been studied. This provides valuable information on correlation between 3D structures and stability of xylanases.

We previously reported the characteristic of xylanase II from *A. usamii* E001 strain isolated from China (Wu et al., 2005). This was not stable at temperature above 50°C which prevents it to be used in industries that requires working conditions at high temperature. Previous studies by other people have shown that the stability of xylanases can be largely improved by either the introduction of disulphide bridges, single residue substitutions or chimeric alterations in the vicinity of the N-terminal region or the C-terminal region of the protein (Eva et al., 2010; Georis et al., 2000; Su et al., 2011; Wang and Xia, 2008; Yang et al., 2007). Several studies revealed that surface arginines have a role in protein stability (Vogt et al., 1997). Three-dimensional structure analyses of xylanases revealed that the flat face of Ser/Thr surface is conserved among family 11 xylanases (Rutchadaporn et al., 2006). Previous study has shown that mutation of amino acid residues on the Ser/Thr surface neither causes conformational change in the protein nor a change in enzyme catalysis. In addition, some of these mutations have been shown to increase thermostability of the xylanase from *T. Reesei* (Turunen et al., 2002).

In this work, arginine substitutions were introduced on the protein surface of *A. usamii* E001 xylanase II and a better thermostability of the xylanase was observed. To our knowledge, this is the first report on the improvement of thermostability of xylanase from *A. usamii*.

MATERIALS AND METHODS

Strains, plasmids and culture media

Escherichia coli DH5α and *Pichia pastoris* GS115 were from Invitrogen (USA). Buffered glycerol-complex medium (BMGY), buffered methanol-complex medium (BMMY), and minimal dextrose medium (MD) were prepared as described in the instruction manual of the Invitrogen *Pichia* expression kit (USA).

The plasmid pMD19-T-*xyn II* containing Xyn II gene (*xyn II*) from *A. usamii* E001 was prepared by our laboratory. Plasmid pPIC9k used as expression vector, was purchased from Invitrogen (USA).

Reagents

DNA Gel Extraction Kit, Taq polymerase, restriction enzymes and protein marker were purchased from TaKaRa Biotechnology (Dalian, China) Co. Ltd. Birchwood xylans, D-xylose and bovine serum albumin were purchased from Sigma Chemical Co., USA. Culture media were obtained from Invitrogen and Shanghai Sangon Co., Ltd. All other chemicals were of analytical grade.

DNA manipulations and analyses

Standard molecular cloning techniques were performed as descrybed by Sambrook (Sambrook et al., 1989). PCRs were done in a thermal cycler (GeneAmp PCR system 2400; PE Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were sequenced by Invitrogen (Shanghai, China) Co., Ltd.

Generation of Xyn II mutants

In planning the mutations, Swiss-Pdb Viewer (http://www.expasy.ch/spdbv/) and Insight II Molecular modeling software package version 2000 (Accelrys Inc., USA) were used as tools to examine the xylanase structure and to ensure that the mutations are not likely to cause conformational change.

Construction of yeast expression plasmids

Plasmid containing the xyn II gene

The plasmid pMD19-T-*xyn II* vector was used as a template to amplify Xyn II cDNA by PCR with the forward primer JN and reverse primer JZ (Table 1). The amplified fragment was gel-purified and digested with *Eco*RI and *Not*I, and then inserted into the *Eco*RI/*Not*I site of pPIC9K resulting in the recombinant plasmid which was confirmed by restriction analysis and nucleotide sequence analysis.

Mutant plasmids

Site-directed mutagenesis was performed using the overlap extension PCR methods as described previously (Ho et al., 1989), in which the mutations were introduced into the oligonucleotide primers (Table 1). DNA sequencing was again performed to verify that there were no other changes other than at the described codons.

Transformation of *P. pastoris*

The resulting plasmids were linearized with the restriction enzyme *Sac*I, and then transformed in *P. pastoris* GS115 by electroporation (7,500V/cm, 25 IF, 400X; Bio-RadGene Pulser, USA). The positive His^+ were selected on MD plate, and then confirmed by colony PCR. For xylanase production in shake-flask cultures, a single-copy transformant of each construct was selected according to Brunel et al (Brunel et al., 2004).

Expression and purification of recombinant protein

Single colonies of the transformants were initially inoculated into a flask containing 25 ml BMGY medium. After 24 h at 30°C and 250 rpm, the cultures were centrifuged at 3000 rpm for 5 min and resuspended by 50 ml BMMY medium to induce expression. The cells were allowed to grow for 96 h at 30°C, and methanol was added every 24 h to a final concentration of 0.5% (v/v) for inducing expression of the target protein. After 96 h growth, cultures were centrifuged at 10,000 rpm for 10 min and the supernatant was used as enzyme source. The crude xylanase was precipitated with

Table 1. Nucleotide sequences of used primers.

Primer name	Sequence (5′→3′)
JN	5′-CGGAATTCAGTGCCGGTATCAACTATG-3′[a]
JZ	5′-ATTTGCGGCCGCTTAAGAAGATATCGTGAC-3′[a]
T18R-F	5′-GTGACTTC**AGA**TACGACGAG-3′[b]
T18R-R	5′-CTCGTCGTA**TCT**GAAGTCACCA-3′[b]
S22R-F	5′-GATACGACGAG**AGA**ACCGGGAC-3′[b]
S22R-R	5′-CCCGGT**TCT**CTCGTCGTATC-3′[b]
S55R-F	5′-CACCTAC**AGA**GCCCAATAC-3′[b]
S55R-R	5′-ATTGGGC**TCT**GTAGGTG-3′[b]
S59R-F	5′-CCCAATAC**AGA**GCTTCTAGC-3′[b]
S59R-R	5′-CTAGAAGC**TCT**GTATTGGGCAG-3′[b]
S179R-R	5′-ATCGTGAC**TCT**GGCACTGCCAG-3′[b]

[a] The bold and underlined sequences show the restriction enzyme *Eco*R I site (JN) and *Not* I site (JZ). [b] The boldface nucleotides represented mutagenesis sites.

ammonium sulfate (60% saturation) followed by centrifugation at 10,000 rpm for 15 min at 4°C. The precipitated proteins were then resuspended in Na_2HPO_4-citric acid buffer (0.2 mol/L Na_2HPO_4, 0.1 mol/l citric acid; pH6.0). Desalting was performed on a Sephadex G-25 column. The fractions containing xylanase were applied onto a Sephadex G-100 column and eluted with the same buffer, and then fractions containing the enzyme were pooled and concentrated. The purified protein was used for biological assays.

Enzyme and protein assays

The reaction mixture consisted of 2.4 ml of a 0.5% (w/v) suspension of birchwood in deionized water and 0.1 ml of a suitably diluted enzyme solution in 50 mM Na_2HPO_4-citrate. After incubation at 50°C for 15 min, reducing sugars were determined by the dinitrosalicylic acid methods (Miller, 1959). The absorbance of the supernatant was measured at 540 nm. As standard, different concentrations of monomeric D-xylose, ranging from 0 to 4 mM prepared in the same conditions were used. One unit (U) of xylanase activity was defined as the amount of enzyme that liberated 1 µmol of xylose equivalents from xylan per minute. Protein was measured by the method of the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. The results were means of duplicate determination on triple independent measurements.

Enzyme properties

The optimal temperature of purified xylanase was determined after 15 min incubation in the presence of 0.5% (w/v) substrate at different temperatures ranging from 35 to 65°C. Relative activity was calculated as enzyme activity at indicated temperature divided by maximal activity at optimal temperature. For thermostability, the enzyme solutions were incubated at 55°C in the presence of substrate for various times. The residual xylanase activity was measured by 15 min incubation at each optimal temperature for Xyn II, ST4 and ST5, respectively. Residual relative activity was calculated as enzyme activity at indicated time divided by activity at time zero.

The pH profile of purified xylanase was evaluated by incubating the enzymes for 15 min in the presence of 50 mM appropriate buffers: Na_2HPO_4-citric acid buffer (pH 3.0 to 7.0), KH_2PO_4-NaOH buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0). The activity of each sample was quantified at its optimal temperature. Relative activity was calculated as enzyme activity at indicated pH divided by maximal activity at optimal pH.

Further study on the pH stability of each sample was carried out at its optimal temperature by pre-incubation of the enzyme solutions in the aforementioned buffer systems in the absence of substrate at 40°C for 1 h. The pH values of various reaction solutions were adjusted to the optimum pH. Then they were subjected to xylanase activity assay. Relative activity was calculated as enzyme activity at indicated pH divided by maximal activity.

For determining the reaction rate, different substrate (birchwood xylan) concentrations were used, ranging from 1.25 to 20.0 mg/ml. The reaction rate versus substrate concentration was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and K_m and V_{max} were determined from the Lineweaver-Burk plot.

The method of how to identify enzyme half-life was performed as described by Friend and Shahani (Friend and Shahani, 1982).

RESULTS

Expression of Xyn II in P. pastoris and its characterization

The xylanase activity of the recombinant Xyn II was quantitatively assayed by DNS method. The recombinant Xyn II showed the specific activity of 3139±61 U mg^{-1} protein (Table 2). The purified recombinant Xyn Ⅱ had maximal activity at pH4.2 and 50°C. When using birchwood xylan at concentrations ranging from 1.25 to 20.0 mg ml^{-1}, the enzymatic reaction was found to follow Michaelis-Menten kinetics with K_m and V_{max} values of 5.56 ± 0.2 mg ml^{-1} and 1347 ± 69 U mg^{-1} protein, respectively (Table 2).

One drawback of this enzyme is that it is not stable at 55°C and at high temperatures. It retained only 50% of maximal activity after incubation for 10 min at 55°C in standard sodium phosphate buffer at pH4.2 without substrate (Table 3).

Generation of mutant enzymes

In order to generate xylanase mutants with improved thermostability, the three-dimensional structure of *A.*

Table 2. Enzymatic properties of the recombinant Xyn II and the mutants.

Strain	Mutation	Specific activity (U mg^{-1} protein)	V_{max} (U mg^{-1})	K_m (mg ml^{-1})
Xyn II		3139±61	1347±69	5.56±0.2
ST4	T18R,S179R,S59R, S55R	3210±65	1410±67	5.39±0.2
ST5	T18R,S179R,S59R,S55R,S22R	2910±59	1161±75	7.68±1.3

The specific activity and kinetic parameters were determined at pH 4.2, 4.5, 4.8 and at 50, 52, 55°C for the recombinant Xyn II, the ST4 and the ST5 enzymes, respectively.

Table 3. The half-life (min) of the recombinant Xyn II and the mutant enzymes.

Strain	$t_{1/2}$ at 55°C min)	
	No substrate	With substrate
Xyn II	10±1	11±1
ST4	9±2	128±12
ST5	6±1	204±9

The half-lives were determined at pH 4.2, 4.5, 4.8 and at 50, 52, 55°C for the recombinant Xyn II, the ST4 and the ST5 enzymes, respectively.

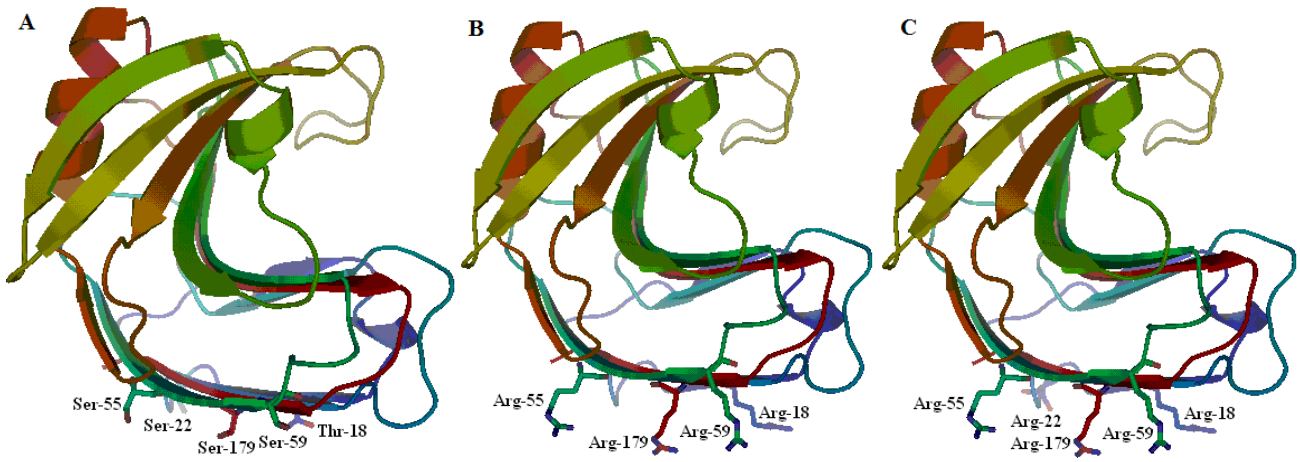

Figure 1. Predicted structures of Xyn II (A), ST4 (B) and ST5 (C).

usamii Xyn II was determined by using insight II molecular modeling software. Several charged amino acids (threonine and serine) were found on the Ser/Thr surface of the protein (Figure 1A). From software analysis, arginine substitutions on the Ser/Thr surface of Xyn II from *A. usamii* are not likely to cause conformational change. We replaced Thr or Ser residues with arginines in order to improve thermostability of this enzyme, resulting in two mutant strains named ST4 and ST5. The ST4 strain contained T18R, S179R, S59R and S55R mutations (Figure 1B) while the ST5 mutant strain contained T18R, S179R, S59R, S55R and S22R mutations (Figure 1C).

Effect of mutations on the enzyme activity

The substitution of Ser and Thr residues on the Ser/Thr surface of the enzyme with four (ST4) or five arginines (ST5) led to an increase in optimal temperature of the enzymes by 2 and 5°C for the ST4 and ST5, respectively (Figure 2). The engineered arginine residues on the Ser/Thr surface clearly increased the thermostability of the enzyme in the presence of substrate. The modified enzymes ST4 and ST5 showed 75 and 87% of maximal activities after incubation for 15 min at 55°C compared to only 31% activity for Xyn II. After incubation for 1 h at 55°C, ST4 and ST5 showed 61 and 77% of maximal activity compared to only 27% activity for Xyn II (Figure 3). These substitutions had a clear effect on the pH-dependence activity profile. The pH optimum of the ST5 was shifted to the alkaline region by 1.0 pH units (Figure 4). Four arginines on the Ser/Thr surface also shifted the pH optimum upwards, but to a smaller extent than five

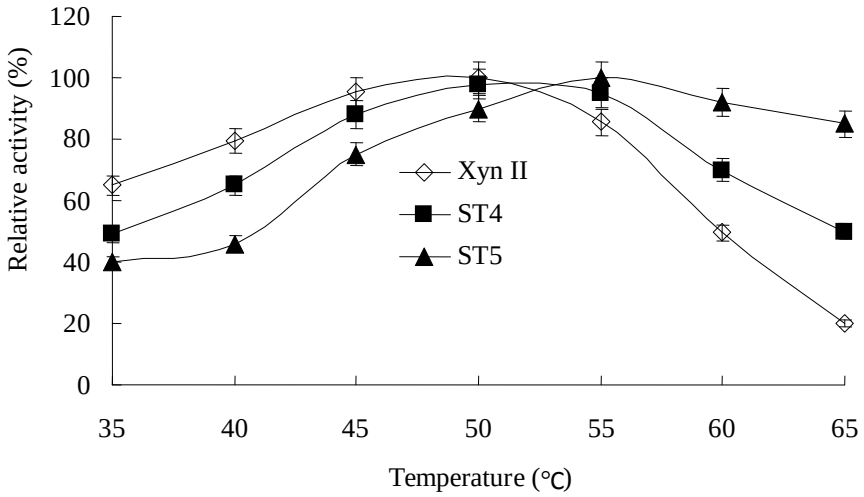

Figure 2. Effect of temperature on enzyme activity of mutants.

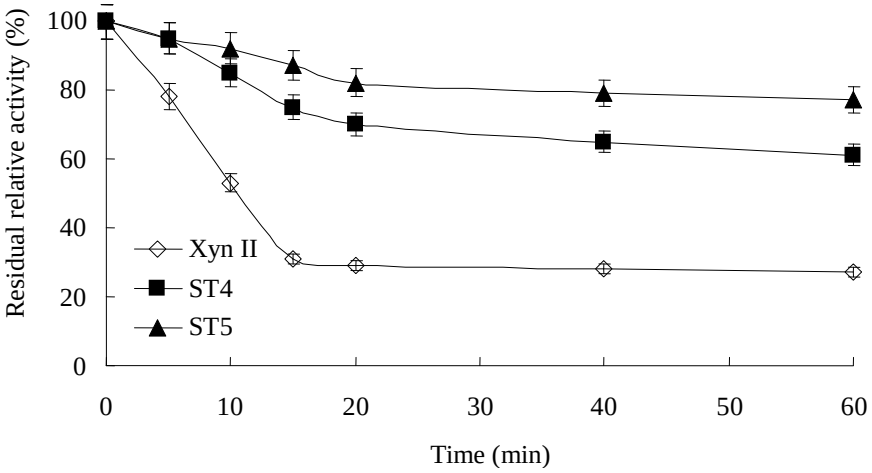

Figure 3. Effect of temperature on enzyme stability of mutants.

arginines (Figure 4). The pH-stability region in the presence of the substrate had also shifted to a more alkaline pH in ST4 and ST5 compared with the wild-type Xyn II (Figure 5).

The kinetic parameters of ST4 and ST5 were determined. The K_m and V_{max} values of the ST4 enzyme for birchwood xylan were 5.39 ± 0.2 mg ml^{-1} (n = 3), and 1410 ± 67 U mg^{-1} protein (n = 3), respectively, which were similar to those of the recombinant Xyn II. The ST5 enzyme showed slightly lower affinity for substrate with K_m of 7.68 ± 1.3 mg ml^{-1} (n=3) and a slight decrease in V_{max} with the value of 1161 ± 75 U mg^{-1} protein (n = 3) (Table 2).

The ST4 enzyme showed high specific activity similar to that of the recombinant Xyn II with the values of 3210 ± 65 U mg^{-1} and 3139 ± 61 U mg^{-1} protein (n = 3) for the ST4 and the recombinant Xyn II, respectively, when tested in the conditions of substrate concentration 5 mg ml^{-1} for 15 min at optimal temperature for each enzyme. The ST5 enzyme had a slightly lower specific activity than that of the recombinant Xyn II and the ST4 with the value of 2910 ± 59 U mg^{-1} protein (n = 3) (Table 2). However, this decrease of specific activity of the ST5 enzyme is likely to be insignificant when used in industrial applications.

DISCUSSION

In our study, we tested how a systematic increase in the amount of arginines on the protein surface affects the stability and functional properties of *A. usamii* Xyn II. The *A. usamii* Xyn II has low thermostability and is inactivated rapidly above 55°C in the presence of substrate. This result indicates that at 55°C in the presence of substrate, its conformation or structure might rapidly changed,

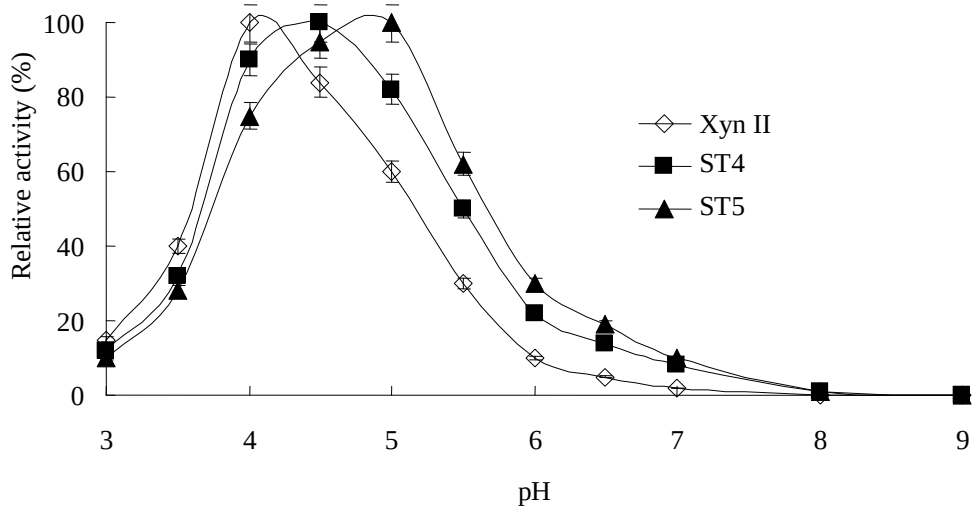

Figure 4. Effect of pH on enzyme activity of mutants.

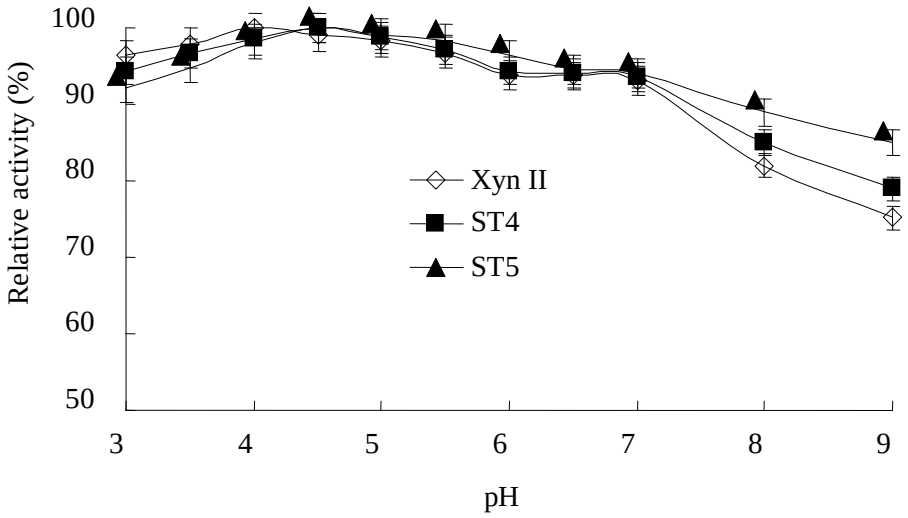

Figure 5. Effect of pH on enzyme stability of mutants.

resulting in the loss of enzyme activity. This effect might be similar to *T. reesei* xylanase II which was inactivated rapidly due to conformational change caused by high temperature (Janis et al., 2001).

The effect of the Ser/Thr surface arginines on thermostability was complex. These arginines decreased the thermostability in the absence of the substrate. However, in the presence of the substrate, the arginine mutations on the Ser/Thr surface considerably increased the enzyme activity at elevated temperature. The half-life in the presence of substrate was increased after the introduction of arginines. Thus, when the destabilizing effect of the mutations was neutralized by the substrate, a clear stabilizing effect of the Ser/Thr arginines was revealed.

Several studies indicate that there is a correlation between protein stability and the number of arginines on the protein surface. The comparison of mesophilic proteins and their thermophilic counterparts has revealed that thermophilic proteins have, on average, a higher arginine content on the protein surface. The higher stability may be due to (i) the arginine δ-guanido moiety has reduced chemical reactivity with other chemicals due to its high p*K*a and resonance stabilization, (ii) the arginine δ-guanido moiety containing positive charge provides surface area for charge to charge interactions thus providing opportunity to participate in multiple noncovalent interactions and (iii) the arginine residues has high p*K*a (approximately 12), thus it is able to maintain ion pairs of net positive charge at elevated temperature (p*K*a values drop as the temperature increases) (Kumar et al., 2000; Rutchadaporn et al., 2006: Vogt et al., 1997). The 3D homology modeling of *A.usamii* Xyn II and the structural

features are shown in Figure 1. The Ser/Thr surface is a major part of the outer surface of the double-layered β-sheet and replacements of serine and threonine residues on this Ser/Thr surface by arginine residues had no effect on the overall structure of the enzyme. Our study demonstrate that enhancing the thermostability of Xyn II from *A. usamii* E001 can be accomplished by introduction of multiple arginine residues on the Ser/Thr surface. Since most family 11 xylanases contained the Ser/Thr surface, it is likely that introduction of arginine residues on the surface could be a promising approach to improve thermostability of the enzymes without alteration of specific activity and kinetic parameters.

In addition, the introduction of arginines into the Ser/Thr surface resulted in a shift of the pH-dependence activity profile to alkaline pH by 1.0 pH units. A clear shift in the pH-stability region was observed when four or five arginines were introduced into the Ser/Thr surface. These results indicate that the Ser/Thr surface of family 11 xylanases has a role in determining the functional properties of the catalytic site. However, an increase in the number of arginines is not in itself sufficient to cause an acidic enzyme to function in highly alkaline conditions.

In conclusion, our results indicatethat replacing several serine and threonine residues on the Ser/Thr surface of Xyn II with arginines effectively increased the thermostability of the enzyme. The mutants generated in this study will be instrumental for further research on the relationship between the structure and function of xylanases.

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