Studies on the key amino acid residues responsible for the alkali-tolerance of the xylanase by site-directed or random mutagenesis

Xiangmei Liu, Yinbo Qu[*], Fan You, Ying Liu

State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, PR China

Abstract

Asparagine (Asn)-71 of the xylanase (XYN) from *Bacillus pumilus* A-30 was found highly conserved in alkaline xylanases of family G/11. The mutated gene fragments containing different substitutions of Asn-71 was obtained by site-directed mutagenesis to study its role in the alkali-tolerant mechanism of xylanase. The xylanase activity was completely lost if Asn-71 residue was replaced by alkaline arginine (Arg) or lysine (Lys) residues, but obviously depressed with a shift in the pH optimum of the enzyme from 6.7 to 6.3 if substituted by serine (Ser) or aspartate (Asp) residues. No mutant with a shift of the pH optimum to a more basic value was found. Furthermore, N71D lost its activity in the alkaline pH range completely, while N71S did not lose as much as that of N71D. Except for Asn-71, the random mutagenesis to other residues of the xylanase was also studied. The alkali-tolerant mechanism of the xylanase was analyzed by their charged character, ionized state, and the hydrogen bond network of the residues surrounding the two catalytic residues on the basis of homology modeling of the mutated xylanases.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Alkali-tolerance; Xylanase; Site-directed mutagenesis; Asparagine-71; Homology modeling

1. Introduction

Xylan is the major hemicullulosic component of graminaceous plant, as well as hardwood. The xylan polymer can be hydrolyzed to xylo-oligosaccharides of various chain lengths by using several xylanolytic enzymes, of which the most important one is known as the endo-1,4-β-xylanases (EC 3.2.1.8). Xylanases are drawing increased attention because of their usefulness in facilitating the bleaching of krafe pulp, and in the textile industries [1,2]. Since all these industrial processes are carried out at neutral or alkaline condition, the alkali-tolerant xylanases are preferred for these applications.

On the basis of amino acid sequence homology and hydrophobic cluster analysis, xylanases can be divided into two major families of glycosyl hydrolases: families G/11 and F/10 [3,4]. The family G/11 is composed of highly specific low molecular weight endoxylanases, which share sequence identity varying from 40 to 90% [5]. All members of this family studied to date have strikingly similar 3D structures and active-site geometries [6]. However, the pH optima of family G/11 xylanases vary widely from acidic values as low as 2 to alkaline values as high as 11 [7]. Despite the two completely conserved and catalytically

* Corresponding author. Tel.: +86-531-8565234;
fax: +86-531-8565234.
E-mail address: quyinbo@sdu.edu.cn (Y. Qu).

essential glutamic acid residues (Glu-120 and Glu-209), which act as a nucleophile and an acid/base catalyst, respectively, in the double displacement reaction mechanism, residues at position Asn-71 of *Bacillus pumilus* A-30 XYN was found highly conserved [6,8]. In this family, all basic xylanases had an asparagine (Asn) while all acidic xylanases had an aspartate (Asp) at this position. The exchanges of the two residues by site-directed mutagenesis could dramatically change the pH profiles of the xylanase activity [7,9,10]. Torronen et al. had suggested that the pH profile of different xylanases was most likely dependent on the neighborhood residues of the acid/base catalyst [11]. This was proved by the xylanase from *Bacillus circulans* (BCX) that the pH optimum of BCX could be shifted from -1.1 to $+0.6$ pH units by mutating neighboring residues within the active-site [12]. However, there is no more data to explain the role of Asn/Asp residues on the pH-dependence of the xylanase catalysis in family G/11. Therefore, more evidence is needed in order to reveal the mechanism of the alkali-tolerance of the basic xylanases of this family.

In order to understand how the conserved Asp or Asn residues can modulate the pH profile of a xylanase, Asn-71 residue of the XYN from *B. pumilus* A-30 was investigated in our laboratory. In this paper, we described the effect of different substitutions of the Asn-71 residue on the enzyme activities by site-directed mutagenesis. Random mutagenesis to the whole DNA sequence of the xylanase was also carried out by error-prone PCR and mutator strain XL1-Red. Furthermore, the pH profiles of the enzyme activity of different mutants were studied and the mechanism of alkali-tolerance of the xylanase from *B. pumilus* A-30 was analyzed by the 3D structures of homology modeling.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

B. pumilus A-30 was used as the source of chromosomal DNA [8]. *E. coli* JM109 was used as the recipient strains for recombinant plasmids. Plasmid pUC19 was used as the cloning vector. Both *B. pumilus* and *E. coli* JM109 cells were grown in LB medium.

Ampicillin of a final concentration of 50–100 μg/ml in the medium was used for the selection of the transformants.

2.2. PCR reaction

The primers for PCR were synthesized using a Gene Assembler (Shanghai Genecore Biotechnologies, China). PCR reaction was performed using DNA Thermal Cycler 480 (Perkin-Elmer Co., USA) for 30 cycles with 94 and 52 °C for 1 min, respectively, and 72 °C for 2 min. *Pfu* DNA polymerase, *Taq* DNA polymerase and dNTP were purchased from Promega Chemical Co., USA.

2.3. DNA manipulations

The methods for isolation and purification of the chromosomal DNA and the plasmid DNA and the methods for restricted digestion, ligation, transformation of DNA were described by Sambrook et al. [13]. The isolation of DNA fragments was performed using Agarose Gel DNA Extraction Kit (Boehringer Mannheim Co., USA). The recombinant plasmids were transformed into competent *E. coli* JM109 using calcium chloride-heat shock.

2.4. Mutagenesis and cloning

The synthetic *xynA* gene fragment encoding the xylanase from *B. pumilus* A-30 was created by PCR using oligonucleotide primers: (1) 5′-GC TGCTGCAGA GGA GAG GAA TGA CGA ATG A-3′ (*Pst*I), and (2) 5′-G CAGGATCCG ACA TGG TTC GTG TGC TGA AT-3′ (*Bam*HI), and then cloned into the pUC19 plasmid under the control of an inducible *lac* promoter, as described by Liu et al. [8]. Primers used to mutate the Asn-71 codon were designed as follows: (3) 5′-G AAC AAT ATC GGA **NNN** GCT TTA TTT AG-3′, and (4) 5′-CT AAA TAA AGC **NNN** TCC GAT ATT GTT C-3′. Mutant gene fragments containing possible 20 kinds of different amino acids substitutions of Asn-71 residue were created by recombinant PCR using the earlier four primers. To create the gene encoding XYN-N71D, site-directed mutagenesis was also performed by recombinant PCR using another two oligonucleotide primers: (5) 5′-G AAC AAT ATC GGA **G**AT GCT TTA TTT AG-3′,

and (6) 5′-CT AAA TAA AGC AT**C** TCC GAT ATT GTT C-3′. Error-prone PCR was performed by the method described by Dieffenbach and Dveksler [14] using primers 1 and 2. Different mutant fragments were cloned into plasmid pUC19 after being digested with restriction enzymes, *Pst*I and *Bam*HI. The recombinants were screened on MacConkey agar plates supplemented with 50–100 ug/ml of ampicillin and then assayed for xylanase activity. XL1-Red competent cells of Stratagene Chemical Co. was also used as mutator strain in random mutagenesis.

2.5. Assay of xylanase activity

The xylanase activity on the plate was detected by inoculating the recombinants onto the selective LB agar plates containing 0.2% of oat spelts xylan. After staining with 0.1% of Congo red for 30 min and flooding with 1.0 mol/l of NaCl for 20 min, the colonies producing clear halos revealed putative clones with xylanase activity [8]. The size of the halos could indicate the xylanase activities of the recombinants. Xylanase activity was determined by measuring the increase in reducing sugar formation from oat spelts xylan (Sigma Chemical Co., USA) with the dinitrosalicylic acid reagent [15]. One unit xylanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min. Protein was determined by the method of Lowry for the calculation of the specific enzyme activity [16].

2.6. Nucleotide sequence determination and homology modeling

DNA sequences were analyzed using an automated sequencing system (Shanghai Genecore Biotechnologies, China). The figures of the 3D structure of the wild type and mutant xylanases were constructed by an automated structure modeling facility supplied by SWISS-MODEL (http://www.expasy.ch/swissmod/ SWISS MODEL.html). The interatomic distances were detected by using Swiss-PdbViewer, which is tightly linked to SWISS-MODEL.

3. Results and discussion

The *xynA* gene encoding an alkali-tolerant xylanase from *B. pumilus* A-30 was cloned, expressed and

sequenced. A high conservation of the Asn residue was found at position 71 of XYN of *B. pumilus* A-30 by comparing the deduced amino acid sequence with that of other alkaline xylanases in family G/11 [8]. Highly conserved amino acid residues located at specific positions in xylanase were expected to play an important role in the structure and function of the enzyme. So, Asn-71 of XYN was supposed to be involved in the alkali-tolerance of the xylanase from *B. pumilus* A-30.

In order to obtain the mutant *xynA* gene fragments containing 20 kinds of different amino acids substitutions of Asn-71, the recombinant PCR reactions were carried out by using three pairs of primer (primers 1 and 4, primers 2 and 3, primers 1 and 2, respectively). The mutant fragments were then cloned into plasmid pUC19 and more than 300 recombinants, which might included all 20 kinds of different amino acids substitutions of Asn-71, were selected and tested for their xylanase activities on the plate. More than 50% of the recombinants were found to completely lose their enzyme activities by the substitution of Asn-71. For the remaining active recombinants, the size of about 69% of the halos produced by the colonies in plate decreased evidently. Furthermore, an acidic shift of the pH optimum was detected in some active recombinants. No recombinant with a shift of the pH optimum to a more basic value was found. All of these suggested that Asn-71 had not only an important role in the catalytic ability but also an effect on the pH profile of the enzyme activity.

In order to realize the relationship between the enzyme activities and the substitutional residues for Asn-71, the mutant *xynA* gene fragments from some recombinants were verified by nucleotide sequence analysis. It was very interesting to find that two alkaline Arg or Lys residues was detected to replace Asn-71 residue among all of the four randomly selected inactive recombinants. While three hydroxyl Ser residues and two carboxylic Asp residues were detected to substitute Asn-71 residue among the five randomly selected recombinants with the pH optimum acidic shift. In addition, the xylanase activities were obviously depressed if substituted with Asp residue (Table 1).

Apart from the xylanase activity, the pH profiles of the xylanase of the N71S mutant and the N71D mutant were also compared with that of the wild type (Fig. 1). Both mutant xylanases caused a pronounced

Table 1
Characteristics of the wild type xylanase and the mutant xylanases of Asn-71 by site-directed mutagenesis

Xylanases	Amino acid residue at position 71	Charged character of the side-chain of amino acid residue at position 71	pH optimum	Specific enzymatic activity (IU/g)
Wild type XYN	Asn	None	6.7	258
XYN-N71S	Ser	None	6.3	206
XYN-N71D	Asp	Negative	6.3	31.9
XYN-N71K	Lys	Positive	ND	0
XYN-N71R	Arg	Positive	ND	0

N71S, N71D, N71K, N71R are the mutant xylanases caused by the substitutions of Asn-71 by serine, aspartate, lysine and arginine residues, respectively. ND: no detectable enzymatic activity.
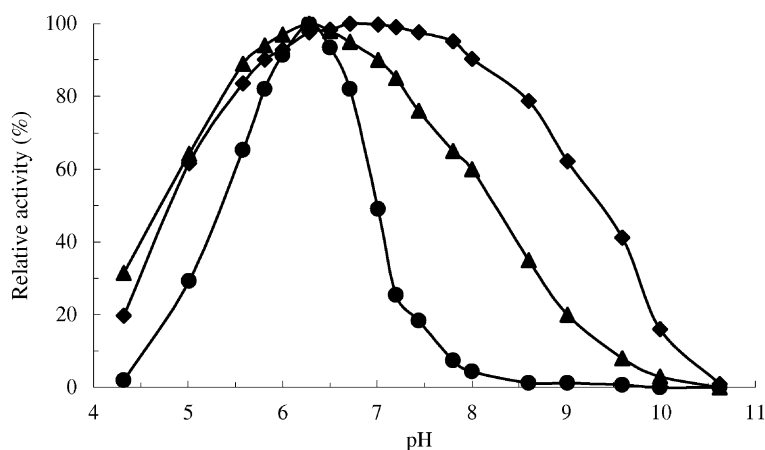

Fig. 1. Effects of pH on the xylanase activities of XYN-N71D (●), XYN-N71S (▲) and wild type XYN (◆).

shift in the pH optimum of the enzyme from 6.7 to 6.3. Although, the optimal pH of the two mutant xylanases did not show a great change, it was obvious that the N71D lost its activity in the alkaline pH range, and the effective pH range of N71D became much narrower. While the N71S did not lose its activity in the alkaline pH range as much as that of the N71D. The equivalent mutant (N35D) of the xylanase from *B. circulans* of family G/11 had 20% increase in activity and a much more significant acidic shift (−1.1 pH units) [7]. Though the differences between the two xylanases remained unclear, it was distinctly that both mutants with the substitution of Asp for Asn lost their activities in the alkaline pH range compared with their wild type xylanases. This is consistent with previous studies of family G/11 xylanases that xylanases with an aspartate residue instead of an asparagine residue at the corresponding position have a lower pH optimum [7,9].

Table 2
Characteristics of the mutant xylanases by random mutagenesis

Xylanases	Methods for mutagenesis	Substitutions of amino acid residues	pH optimum	Specific enzyme activity (IU/g)
Wild type XYN	None	None	6.7	258
XYN-F10L/T133S	Error-prone PCR	10:Phe(F)-Leu(L), 133:Thr(T)-Ser(S)	6.3	139
XYN-F225V	Error-prone PCR	225:Phe(F)-Val(V)	6.3	228
XYN-G199R	Mutator strain	199:Gly(G)-Arg(R)	6.3	142

It is well accepted that hydrolysis of xylan by xylanases occurs by a double displacement reaction. This mechanism involves two catalytic residues: one functions as a general acid or a general base, while the other acts as a nucleophile [17]. Some of the conserved amino acid residues in the neighborhood of the two catalytic residues were involved in a hydrogen bond network formed in the catalytic active center. The replacement of the uncharged Asn residue by the positively charged Lys or Arg residue made the xylanase activity completely lost, while by the negatively charged Asp residue largely decreased the xylanase activity and made the xylanase activity completely lost in the alkaline range. The replacement by the Ser residue with hydroxyl side-chain caused a shift in the pH optimum of the xylanase and a decrease in its activity by ∼20%. All of these suggested that Asn-71 might be involved in the catalytic active center and was critical for the alkaline tolerance of the xylanase.

In order to search other residues of the xylanase besides Asn-71, which are involved in alkaline tolerance of the xylanase, the random mutagenesis to the whole DNA sequence of the xylanase was also carried out by error-prone PCR and mutator strain XL1-Red. On the basis of screening more than 4000 recombinants, no mutant with a shift of the pH optimum to a more basic value was found, but some mutants with an acidic shift of the pH optimum were detected. Three of them were selected and studied (Table 2). The pH profiles of the three mutant xylanases were also compared with that of the wild type (data not shown). All three mutant xylanases had similar pH profiles as that of N71S, and they did not affect their activity in the alkaline pH range as much as that of N71D.

The overall structures of the xylanases in family G/11 are similar and have been described as a partially closed right hand. Up to date more than five refined crystal structure of xylanases have been published [9,11,18–20]. The theoretical 3D structure of the xylanase from *B. pumilus* A-30 was obtained from SWISS-MODEL, an automated protein modeling server running at the GlaxoSmithKline in Geneva, Switzerland. The *B. pumilus* A-30 xylanase had a characteristic fold (Fig. 2a) which was unique for family G/11 xylanases. The xylanase fold into a single ellipsoidal domain with the "fingers" at the bottom, the "palm" at the right hand side and the "thumb" at the top of the molecule as represented in Fig. 2. The

unusual cord could also be seen in this figure (Fig. 2a). The two catalytic residues of the xylanase from *B. pumilus*, which were proven to be Glu-120 and Glu-209 by Ko et al. [21], were observed to locate on either side of the extended open cleft. The Asn-71
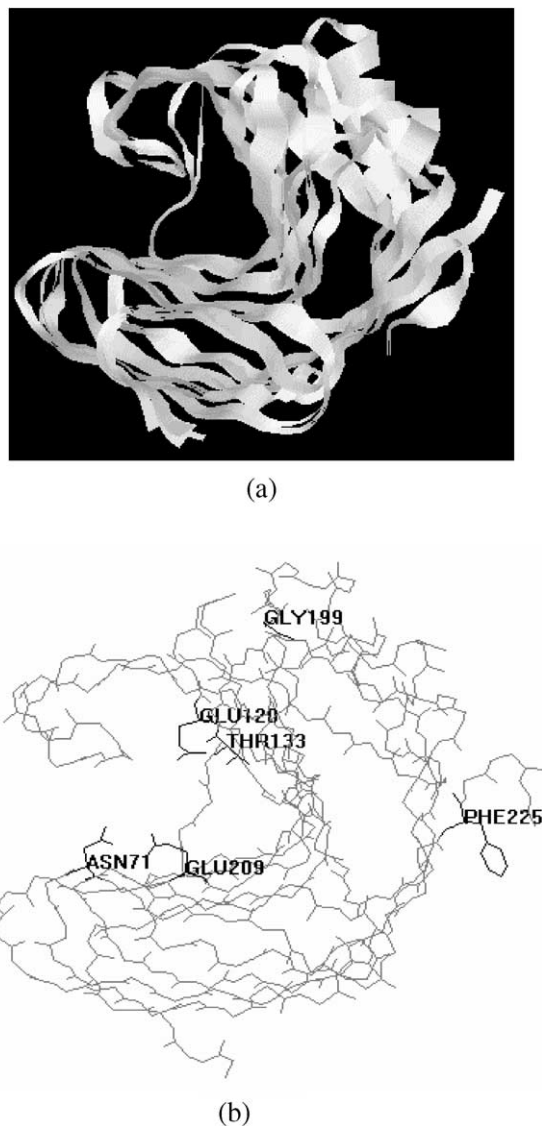

(a)

(b)

Fig. 2. The representative view of the overall structure of the xylanase from *B. pumilus* A-30 (generated by SWISS-MODEL program). (a) The shape of a "right hand" model, with the "fingers" being at the bottom, the "palm" at he right hand side and the "thumb" at the top of the molecule; (b) the two catalytic residues (Glu-120 and Glu-209) and the residues studied in this research are shown.
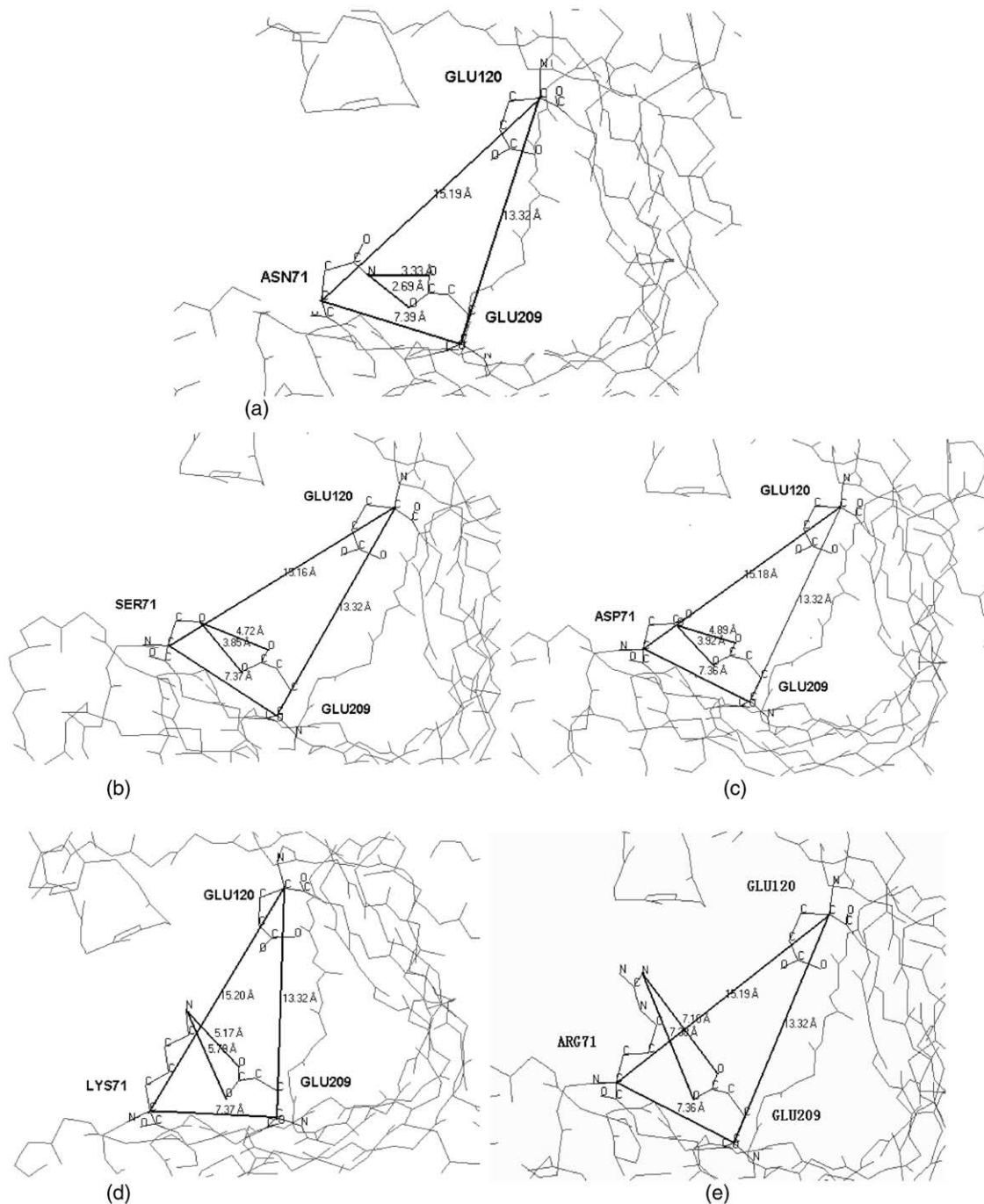
Fig. 3. Schematic illustration of the structure of xylanases showing the interatomic distances. The distances between the C^α of the two catalytic residues (Glu-120 and Glu-209) and the residues at position 71 are compared. The distance between the two carboxylic O of the acid/base catalyst residue (Glu-209) and Asn-71 N^δ, Ser-71 O^χ, Asp-71 O^δ, Lys-71-N^ϕ, Arg-71 N^γ, respectively, are shown. (a) Wild type XYN; (b) XYN-N71S; (c) XYN-N71D; (d) XYN-N71K; (e) XYN-N71R.

residue of the xylanase was observed to locate near the mouth of the cleft (Fig. 2b).

Single site mutagenesis on Asn-71 studied in this research did not change the overall structure of the enzymes, but some wispy structural differences were detected by measuring the distances between the atoms of the two catalytic residues (Glu-120 and Glu-209) and the mutant residue at position 71 of the xylanases (Fig. 3). Although, the data obtained based on the theoretical structure model, the following possibilities might be suggested [6,7,12]. (i) Single site mutagenesis at Asn-71 could not make much change to the structure of the xylanases. (ii) Different site-chains of residue at position 71 might have different effects on the hydrogen bond network to the acid/base catalyst residue (Glu-209). (iii) N71R and N71K did not show detectable activity probably because of the bulkiness of their side-chains, which hindered the binding of the substrates.

The substitution of Asn-71 residue with Ser residue just enhanced the polar of the amino acid, while the substitutions with Asp, Lys or Arg residues changed the charged character of the amino acid. So, both the charged character and ionized state of the residue at position 71 might influence the enzyme activities and the alkali-tolerance of the xylanase. On the other hand, the Asn side-chain can serve not only as a hydrogen acceptor, but also as a hydrogen donor. Therefore, the change of Asn-71 might not only disturb the hydrogen bond network surrounding the two active catalytic residues, but also influence the maintenance of the proper ionized state of the catalytic residues for their catalytic action.

In addition, the structures of homology modeling of the three mutant xylanases by random mutagenesis were also obtained. Only one residue (Thr-133) in mutant xylanase F10L/T133S was found to locate inside the cleft, while the other two residues (Phe-225, Gly-199) of the mutant xylanases (F225V and G199R) were out of the catalytic center (Fig. 2b). The substitution of polar threonine (Thr) residue with polar Ser residue in the catalytic center might disturb the internal hydrogen bond network of the cleft. While the mutations out of the active center by F225V and G199R might somewhat influence the dimensional structure of the cleft. Therefore, all three mutant xylanases changed their pH optima.

In conclusion, using site-directed mutagenesis, we were able to confirm that the substitutions of Asn-71 of the xylanase led to the decrease in specific activity of the xylanase, especially in an alkaline pH range. Though other sites of residue could also influence the pH optimum of the xylanase, they did not affect their activity in the alkaline pH range as much as that of Asn-71. In addition, all mutant xylanases studied in this paper changed their pH optima to a more acidic value. So, the Asn-71 of XYN from *B. pumilus* A-30 plays a critical role in the alkali-tolerance of the family G/11 xylanases. More kinetic data are needed in order to reveal the alkali-tolerant mechanism of the alkaline xylanase of family G/11.

References

[1] J. Buchert, M. Tenkanen, A. Kantelinen, L. Viikari, Biores. Technol. 50 (1994) 65.

[2] M.L. Nikupaavola, M. Ranua, A. Suurnakki, A. Kantelinen, Biores. Technol. 50 (1994) 73.

[3] N.R. Gilkes, B. Henrissat, D.G. Kilburn, R.C. Miller, A.J. Warren, Microbiol. Rev. 55 (1991) 303.

[4] B. Henrissat, Biochem. J. 280 (1991) 309.

[5] A. Torronen, C.P. Kubicek, B. Henrissat, FEBS Lett. 321 (1993) 135.

[6] A. Torronen, J. Rouvinen, J. Biotechnol. 57 (1997) 137.

[7] M.D. Joshi, G. Sidhu, I. Pot, G.D. Brayer, S.G. Withers, L.P. McIntosh, J. Mol. Biol. 299 (2000) 255.

[8] X.M. Liu, M. Qi, J.Q. Lin, Z.H. Wu, Y.B. Qu, J. Microbiol. Biotechnol. 11 (2001) 534.

[9] U. Krengel, B.W. Dijkstra, J. Mol. Biol. 263 (1996) 70.

[10] S. Fushinobu, K. Ito, M. Konno, T. Wakagi, H. Matsuzawa, Protein Eng. 11 (1998) 1121.

[11] A. Torronen, A. Harkki, J. Rouvinen, EMBO J. 13 (1994) 2493.

[12] M.D. Joshi, G. Sidhu, J.E. Nielsen, G.D. Brayer, S.G. Withers, L.P. McIntosh, Biochemistry 40 (2001) 10115.

[13] J. Sambrook, E.F. Fritsch, T. Mariatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989.

[14] C.W. Dieffenbach, G.S. Dveksler, PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1995.

[15] G.L. Miller, Anal. Chem. 31 (1959) 426.

[16] D.H. Lowry, J. Biol. Chem. 193 (1951) 265.

[17] M. Roberge, C. Dupont, R. Morosoli, F. Shareck, D. Kluepfel, Protein Eng. 10 (1997) 399.

[18] A. Arase, T. Yomo, I. Urabe, Y. Hata, Y. Katsube, H. Okada, FEBS Lett. 2 (1993) 123.

[19] W.W. Wakarchuk, R.L. Campbell, W.L. Sung, J. Davoodi, M. Yaguchi, Protein Sci. 3 (1994) 467.

[20] A. Torronen, J. Rouvinen, Biochemistry 34 (1995) 847.

[21] E.P. Ko, H. Akatsuka, H. Moriyama, A. Shinmyo, Y. Hata, Y. Katsube, I. Urabe, H. Okada, Biochem. J. 288 (1992) 117.