J. Microbiol. Biotechnol. (2001), **11**(3), 534–538

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Asparagine Residue at Position 71 is Responsible for Alkali-Tolerance of the Xylanase from *Bacillus pumilus* A-30

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Received: January 30, 2001
Accepted: May 21, 2001

Abstract The *xynA* gene encoding an alkali-tolerant endo-1,4-β-xylanase (XYN) was cloned from the alkalophilic *Bacillus pumilus* A-30. The nucleotide sequence of a 974-bp DNA fragment containing the *xynA* was determined. An ORF of 684 nucleotides that encoded a protein of 228 amino acids was detected. Asparagine-71 of XYN from *B. pumilus* A-30 showed to be highly conservative in alkaline xylanases of family G/11, upon comparing the amino acid sequences of 17 family G/11 xylanases. Site-directed mutation of N71D of the *xyn*A gene resulted in a decrease of 12.4% in the specific activity and a significant decline in the enzyme activity in the alkaline pH range.

Key words: Alkali-tolerant, xylanase, gene cloning, site-directed mutagenesis

There has been an increasing interest in applying xylanases in the pulp and paper industry to reduce the quantities of hazardous chlorine chemicals used in the bleaching process. The beneficial effects of xylanase pretreatment on kraft pulp bleachability and Cl_2-saving effect were reported by Viikari [27] and Paice [16]. The xylan polymers that need to be removed in the pulp and paper processing 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-xylanase (XYN) (EC 3.2.1.8). Since the pulp bleaching process is carried out in alkaline pH, alkali-tolerant xylanases are suitable for the application in pulp and paper industry [15].

Xylanases have been classified into two families on the basis of sequence similarity [2]. Wong *et al.* [28] have divided xylanases into two categories; (1) the low molecular mass alkaline xylanases, and (2) the high molecular mass acid xylanases. These two groups roughly correspond to family G/11 and F/10, respectively, of the glycosyl hydrolases [5]. The family G/11 is composed of highly specific low molecular weight endoxylanases from eukaryotic and bacterial species, in which the sequence identity varied from 40 to 90% [21].

The alkaline xylanases are generally active in a wide pH range, from pH 5 to 9, whereas the acidic xylanases are active in a narrow pH range, from pH 3 to 6 [24]. Torronen *et al.* suggested that the pH profile of different xylanases was most likely to be dependent on the neighborhood residues of the acid/base catalyst [22]. Torronen and Rouvinen found that residue D33 in XYN I corresponding to N44 in XYN II, had an important role in determining the pH profile of the xylanase activity of the two major xylanases [23]. Krengel and Dijkstra speculated that the D37 residue at the corresponding position might also be involved in determining the low optimal pH level of xylanase that was produced by *Aspergillus niger* [10]. However, no site-directed mutagenesis data of aspartate (Asp) and asparagine (Asn) residues was presented regarding their effects on alkali-tolerance. More evidence is needed to reveal the mechanism of the alkali-tolerance of xylanases.

In our laboratory, a strain of alkalophilic bacteria named A-30, which produced extracellular alkali-tolerant xylanases, was isolated from alkaline soil and identified as a *Bacillus pumilus* strain [8]. In this research, the *xynA* gene encoding an alkali-tolerant xylanase from *B. pumilus* A-30 was cloned, expressed, and sequenced. The deduced amino acid sequence of the *xynA* gene was compared with that of other XYNs in family G/11, in order to determine a conserved amino acid residue for further study. The site-directed mutagenesis was carried out at Asn-71 of XYN of *B. pumilus* A-30 to analyze the role of the conserved Asn residue in the catalytic ability and its effect on the pH profile of the enzyme activity.

The primers for PCR were synthesized using a Gene Assembler (Shanghai Genecore Biotechnologies, China). PCR reaction was performed using a DNA Thermal Cycler

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480 (Perkin Elmer Co., U.S.A.) for 30 cycles at 94°C and 52°C, respectively, for 1 min, and at 72°C for 2 min. *Pfu* DNA polymerase and dNTP were derived from Promega Chemical Co., U.S.A. The recombinant DNA techniques used in this work were described by Sambrook [19]. The isolation of DNA fragments was performed by using an Agarose Gel DNA Extraction Kit (Boehringer Mannheim Co., U.S.A.).

The chromosomal DNA of *B. pumilus* A-30 was isolated and used as the PCR template. The following two primers were designed for cloning the *xyn*A gene from the strain A-30: primer 1: 5'-GCTGCTGCAGAGGAGAGG-AATGACGAATGA-3' (*Pst*I), primer 2: 5'-GCAGGATCC-GACATGGTTCGTGTGCTGAAT-3' (*Bam*HI). The amplified fragment was cloned into plasmid pUC19 after being digested with restriction enzymes, *Pst*I and *Bam*HI. After transformation of the competent *E. coli* JM109 cells with the recombinant plasmids, the recombinants were screened on MacConkey agar plates supplemented with 50–100 μg/ml of Ampicillin. The white colonies, which indicated the clones carrying plasmids pUC19 with foreign inserts, were picked out and inoculated 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 M of NaCl for 20 min, the colonies producing clear halos, which indicated the putative clones containing hybrid plasmids with the inserts coding for xylanase, were picked out for further analysis.

Xylanase activity was determined by measuring the reducing sugar formed from oat spelts xylan (Sigma Chemical Co., U.S.A.) with the dinitrosalicylic acid reagent [14]. One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min. The concentration of protein was determined by the method of Lowry [13].

The DNA sequence was analyzed by using an automated sequencing system (Shanghai Genecore Biotechnologies, China). Amino acid sequences were compared by using the Clustal W (1.81) multiple sequence alignment program. Homologous amino acids were defined as either identical amino acids or conserved substitutions.

A 1.0 kb fragment, which was consistent with the deduced length (974 bp) between the two primers, was amplified by PCR. After transformation, a lot of recombinants with clear halos on the selected plates were screened. The recombinant plasmid, pUC19-2, derived from one of the positive clones, was isolated, identified, and sequenced (Fig. 1). The sequenced result of this 1 kb fragment revealed that the *xyn*A coding for the XYN activity was located in this fragment. An ORF of 684 nucleotides that encoded a protein of 228 amino acids was detected. This result is consistent with that reported by Fukusaki *et al.* [1] with an exception to some different nucleotides outside the ORF [12].

Only 4 members of the family G/11 including the XYN from *B. pumilus* were reported in 1991 [2]. Seventeen new members were added to this family in 1993 [6]. Currently,

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1    CTGCAGAGGAGAGGAATGACGAATGAATTTGAGAAAATTAAGACTGTTGTTTGTGATGTGTATTGGACTGACGCTTATACTGACGGCT
       Pst I                M  N  L  R  K  L  R  L  L  F  V  M  C  I  G  L  T  L  I  L  T  A
89   GTACCAGCCCATGCGAGAACCATTACGAATAATGAAATGGGTAACCATAGCGGGTACGATTATGAATTATGGAAGGATTATGGAAACACC
       V  P  A  H  A  R  T  I  T  N  N  E  M  G  N  H  S  G  Y  D  Y  E  L  W  K  D  Y  G  N  T
179  TCGATGACACTCAATAACGGCGGGGCATTTAGTGCAGGCTGGAACAATATCGGAAATGCTTTATTTAGAAAAGGGAAAAAGTTTGATTCC
       S  M  T  L  N  N  G  G  A  F  S  A  G  W  N  N  I  G  N  A  L  F  R  K  G  K  K  F  D  S
269  ACTAGAACTCACCATCAGCTTGGCAACATCTCCATCAATTACAACGCAAGTTTTAACCCAGGCGGGAATTCCTATCTATGTGTCTATGGC
       T  R  T  H  H  Q  L  G  N  I  S  I  N  Y  N  A  S  F  N  P  G  G  N  S  Y  L  C  V  Y  G
359  TGGACACAATCTCCATTAGCAGAATACTACATTGTTGATTCATGGGGCACATATCGTCCAACAGGAGCGTATAAAGGATCATTTTATGCT
       W  T  Q  S  P  L  A  E  Y  Y  I  V  D  S  W  G  T  Y  R  P  T  G  A  Y  K  G  S  F  Y  A
449  GATGGAGGCACATATGACATTTATGAAACAACCCGTGTCAATCAGCCTTCCATTATCGGGATCGCAACCTTCAAGCAATATTGGAGTGTA
       D  G  G  T  Y  D  I  Y  E  T  T  R  V  N  Q  P  S  I  I  G  I  A  T  F  K  Q  Y  W  S  V
539  CGTCAAACGAAACGTACAAGCGGAACGGTCTCCGTCAGTGCGCATTTTAGAAAATGGGAAAGCTTAGGGATGCCAATGGGGAAAATGTAT
       R  Q  T  K  R  T  S  G  T  V  S  V  S  A  H  F  R  K  W  E  S  L  G  M  P  M  G  K  M  Y
629  GAAACGGCATTTACTGTAGAAGGCTACCAAAGCAGCGGAAGTGCAAATGTGATGACCAATCAGCTGTTTATTGGCAACTAAAAAAGTCAA
       E  T  A  F  T  V  E  G  Y  Q  S  S  G  S  A  N  V  M  T  N  Q  L  F  I  G  N  ***
719  AGAAAAGAGCCGGGAGCAAAACTCCTGGCTTTTTCTATCATAATTTTTCAACTTCGACTCTGCCGGAAAAGAACGTCGCACCGCCGCCCA
809  TATCTGCCAAGCGATCAGGTGTGAGGCCATTCACCAAATGTTTTTTGCCTTTTTGGTCTGCCCATAATCCCTGGCTGACAACAACGCCAG
899  ATAACACATTTTGTCCGACTGACACGATCAGCTCGCATTCTCCTCGATCATTCAGCACACGAACCATGTCGGATCC 3′   974
                                                                                  BamH I
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Fig. 1. Nucleotide and deduced amino acid sequences of the *xynA* gene from *B. pumilus* A-30.
The primers are underlined and the position for site-directed mutagenesis is blocked.

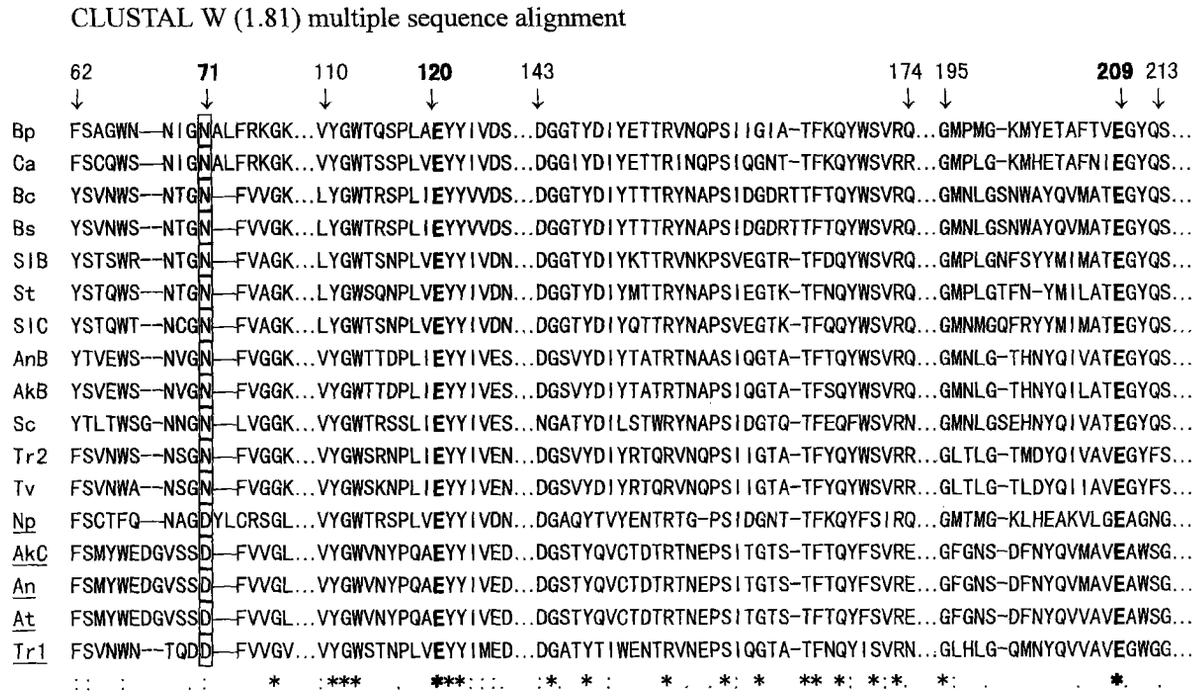

Fig. 2. Sequence alignment of representative family G/11 xylanases.
"*"=identical or conserved residues in all sequences in the alignment; ":"=conserved substitutions; "."=semi-conserved substitutions. The numbers of the amino acid refer to the XYN of *B. pumilus* A-30 (Fig. 1). The positions corresponding to Asn-71 are shown in black boxes. The genes for acidic xylanases are underlined. Bp: XYN from *B. pumilus* A-30 (this work); Ca: XYN from *C. acetobutylicum* [30]; AkC, AkB: XYNC and XYNB from *A. kawachii* [7, (GenBank) Accession: D38070]; An, AnB: XYN and XYNB from *A. niger* [(GenBank) Accession: A19535, D38071]; At: XYN from *A. tubingensis* [3]; Tr1, Tr2: XYNI and XYNII from *T. reesei* [25]; Bc: XYN from *B. circulans* [29]; Bs: XYN from *B. subtilis* [17]; SlB, SlC: XYNB and XYNC from *S. lividans* [20]; St: XYNII from *S. thermoviolaceus* [26]; Sc: XYN from *S. commune* [4]; Tv: XYN from *T. viride* [(GenBank) Accession: AJ012718]; Np: XYN from *N. patriciarum* [11].

the amino acid sequences of more than 20 xylanases belonging to the family G/11 have been reported. The amino acid sequences of 17 xylanases in family G/11 were compared. The result of the alignment of part of the amino acid sequences is shown in Fig. 2. It showed a divergence of sequence of the enzymes in family G/11. Most of the differences among the xylanases were found in the N-terminus.

It is well accepted that hydrolysis of xylan by xylanases occurs by a double displacement reaction that retains the configuration of the anomeric carbon. This mechanism involves two catalytic residues: One functions as a general acid or a general base, while the other acts as a nucleophile [18]. It could be seen in Fig. 2 that two glutamate residues were conserved in all the sequences. These two conserved glutamate residues had been proved to be the catalytically active residues in XYNs of family G/11 [24]. The active sites of XYN of *B. pumilus* were proven to be Glu-120 and Glu-209 by Ko *et al.* [9].

Apart from the active sites, a high conservation of the Asp or Asn residue was found at position 71 of XYN of *B. pumilus* (Fig. 2). It was interesting to see that all acidic xylanases of family G/11 had an aspartate residue while all basic xylanases consisted of an asparagine residue at this position. Highly conserved amino acid residues located at specific positions in XYN are expected to play an important role in the structure and function of the enzyme. As a result, Asn-71 of XYN of *B. pumilus* was targeted for the site-directed mutagenesis to investigate whether the residue is a key amino acid responsible for the alkali-tolerance of the enzyme.

Primers used to mutate the Asn-71 codon were designed as follows (the substituted nucleotides are underlined): primer 3: 5'-GAACAATATCGGAGATGCTTTATTTAG-3', primer 4: 5'-CTAAATAAAGCATCTCCGATATTGTTC-3'. Three PCR reactions were carried out to obtain the fragments harboring the mutant *xyn*A (N71D). First, fragments I (240 bp) and II (749 bp) were amplified by using the primers 1, 4 and 2, 3, respectively, and the isolated 1 kb fragments carrying the *xyn*A gene as the template. Then, fragment III (974bp) was amplified by using the primers 1 and 2 and the purified fragments I and II mixture as the template. After being digested with the restriction enzymes *Pst*I and *Bam*HI, fragment III was cloned into pUC19 to construct the recombinant plasmid pUC19-D2, which might have the insert coding for XYN-N71D.

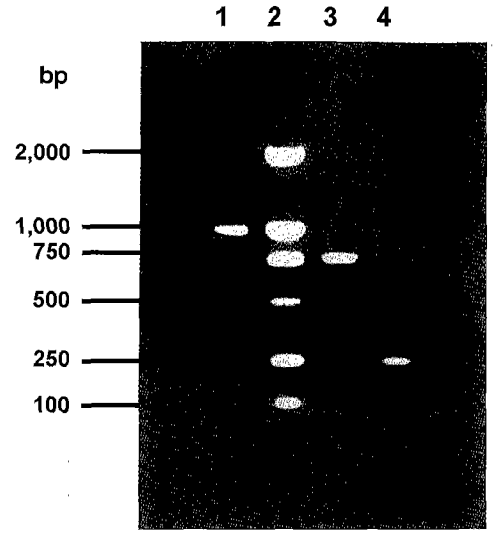

Fig. 3. Agarose gel electrophoresis showing three PCR-amplified fragments for XYN-N71D.
Line 1, fragment III (974 bp); Line 2, DNA Marker (TaKaRa); Line 3, fragment II (749 bp); Line 4, fragment I (240 bp).

Figure 3 shows the agarose gel electrophoresis of the three PCR amplified fragments for XYN-N71D. As illustrated in the figure, the three fragments were consistent with the deduced length (240 bp, 749 bp, 974 bp) between each two primers, respectively. The mutated site of the *xyn*A (N71D) was confirmed by a method of nucleotide sequencing of the gene. The data showed that Asn was substituted by Asp that resulted from the change in the sequence from AAT to GAT. The XYNs produced by *E. coli* JM109 harboring plasmids pUC19-D2 (N71D) and pUC19-2 (wild-type), respectively, were analyzed. The specific activity of the XYN-N71D decreased to 12.4% compared with that of the wild-type XYN (decreased from 258 to 31.9 units g^{-1} of the protein) that was measured at pH 6.5. The comparison of pH profiles of XYN-N71D and the wild-type XYN is shown in Fig. 4. The mutant xylanase (N71D) had a shift of the optimal pH level from 6.7 to 6.3. Although the optimal pH of XYN-N71D did not show a great change, it was obvious that the XYN-N71D lost its activity in the alkaline pH range, and the effective pH range of XYN-N71D became much narrower. According to the above results, which were observed by replacing Asn-71, it could be concluded that Asn-71 was responsible for the alkali-tolerance of the XYN. The Asn side-chain can serve not only as a hydrogen acceptor, but also as a hydrogen donor. Therefore, the change of N71D might not only disturb the hydrogen bond network surrounding the two active catalytic residues, but also influence the maintenance of the proper ionization state of the catalytic residues for their catalytic action as well. Further studies on the structure of the mutated and the wild-type XYNs are necessary in order to provide more conclusive evidence in regards to the

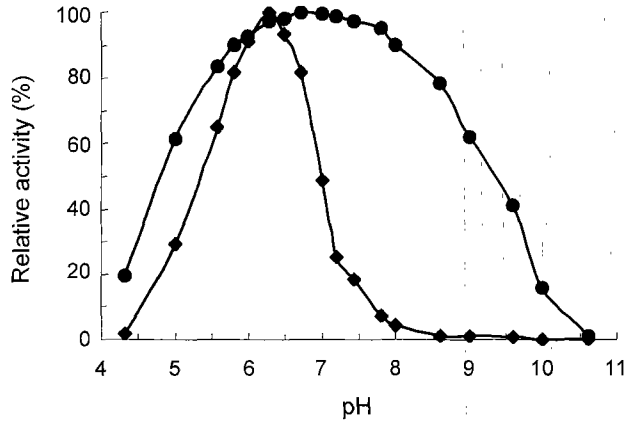

Fig. 4. Effects of pH on the xylanase activities of XYN-N71D and wild-type XYN.
—●—, wild-type XYN; —◆—, XYN-N71D.

impact of the residue at this position for the enzymes on their pH profiles. In addition, the K_m values of 19.6 and 6.15 mg/ml and the optimal temperatures of 40°C and 50°C for the mutated and the wild-type XYNs, respectively, were measured and compared. The substitution of the uncharged Asn residue to the negatively charged Asp residue changed the ionic and hydrogen bonds inside the protein molecule, which could have lead to the changes of K_m and optimal temperature.

In conclusion, Asn-71 of XYN from *B. pumilus* A-30 showed to be highly conservative in alkaline xylanases of family G/11. Site-directed mutation of N71D of the *xyn*A gene led to the decrease in specific activity of the xylanase, especially in an alkaline pH range.

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