

A xylanase with high pH stability from *Streptomyces* sp. S27 and its carbohydrate-binding module with/without linker-region-truncated versions

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Received: 28 July 2008 / Revised: 27 November 2008 / Accepted: 5 December 2008 / Published online: 24 December 2008
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Abstract A xylanase gene, *xynAS27*, was isolated from a genomic library of *Streptomyces* sp. S27. The full-length gene consists of 1,434 bp and encodes 477 amino acids, including a putative signal peptide of 41 residues at its N-terminus. The mature xylanase comprises two functional domains, a family 10 glycoside hydrolase, and a family 13 carbohydrate-binding module (CBM), which were joined by a short Gly/Pro-rich linker region. The intact, the CBM-truncated and the CBM-linker-truncated versions of the mature proteins were expressed in *Escherichia coli* BL21 (DE3), purified to electrophoretic homogeneity and subsequently characterized. XynAS27 showed high pH stability over the pH range 2.2–12.0. XynAS27 may be a compelling tool for the food industry because it generates xylobiose (85% w/w) as the main product of xylan hydrolysis. The truncated versions showed less pH and thermal stability, and less affinity and hydrolytic activity to insoluble substrate than the intact one. These results indicate that the CBM of XynAS27 plays a key role in the hydrolysis of insoluble substrate, and the CBM and linker region are also important for the enzyme stability, and the linker region contributes more.

Keywords *Streptomyces* sp. S27 · Xylanase · Carbohydrate-binding module · Linker · Stability

Introduction

Xylan, one of the main components of hemicelluloses in plant cell walls, is the second most abundant renewable polysaccharide in nature (after cellulose; Prade 1996). Xylanases (EC 3.2.1.8) can hydrolyze β -1,4-glycosidic linkages in the xylan backbone into short xylooligosaccharides (Biely 1985). In recent years, xylanases have attracted considerable research interest due to their widespread potential applications in the food, animal feed, textile, waste-treatment, paper, and biofuel industries.

On the basis of primary structure comparisons of the catalytic domains, glycosidases have been grouped into 112 families of related sequences (refer to the carbohydrate-active enzyme CAZy server at http://www.cazy.org/fam/acc_GH.html). Xylanases are normally reported as being confined to glycoside hydrolase (GH) families 10 and 11 (Collins et al. 2005). Most xylanases in GH10 have multi-domain structures with a catalytic domain, one or more carbohydrate-binding modules (CBMs), and/or other functional domains joined by linker sequences (Ohmiya et al. 1997). CBMs are also divided into families based on amino acid sequence similarity. Currently, there are 52 defined CBM families (http://www.cazy.org/fam/acc_CBM.html).

In general, CBMs are appended to glycoside hydrolases that degrade insoluble polysaccharides (Boraston et al. 2004). Proteolytic excision or genetic truncation of CBMs from the catalytic modules results in a significant decrease in the binding ability or catalytic activity of the enzymes on insoluble substrates. For example, the family 9 CBM of *Clostridium stercoarium* xylanase (Xyn10B) (Ali et al. 2001), and family 22 CBM of *Clostridium thermocellum* xylanase (Xyn10C) (Ali et al. 2005b) showed high affinity to insoluble xylan. It is also reported that CBMs could enhance the stability of enzymes (Sunna et al. 2000; Ali

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et al. 2005a). However, Dias et al. (2004) reported that the thermal stability of the xylanase 10B from *C. thermocellum* was dependent on the linker region connecting CBM22 to the N-terminus of catalytic module instead of CBM. In this study, we cloned a GH10 xylanase (XynAS27) from *Streptomyces* sp. S27, which contained a catalytic domain, a CBM13 and a linker region between them. Then, we expressed and characterized the intact and the CBM with/without linker-region-truncated versions of XynAS27. The properties of these proteins were compared and analyzed. The results indicated that both the CBM and linker region had significant effect on the stability of XynAS27, and the linker region contributed more.

Materials and methods

Bacterial strains, plasmids and reagents

Streptomyces sp. S27, which exhibited xylanolytic activity, was isolated from the soil of Flaming Mountain in the Turpan Basin of Xinjiang, China, and we already cloned a GH11 xylanase from *Streptomyces* sp. S27 (Li et al. 2008b). *Escherichia coli* JM109 and BL21 (DE3) cells were grown aerobically in Luria–Bertani (LB) broth medium or agar plate supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin at 37°C for recombinant plasmid amplification and gene expression, respectively. The plasmid pUC19 (TaKaRa, Otsu, Japan) and the pGEM-T Easy vector (Promega, Madison, WI, USA) were used for plasmid preparations and gene cloning. The pET-22b(+) vector (Novagen, San Diego, CA, USA) was used for gene expression. The DNA purification kit, restriction endonucleases, T4 DNA ligase and *LA Taq* DNA polymerase with GC Buffer were purchased from TaKaRa. Oat spelt xylan and birchwood xylan were purchased from Sigma (St. Louis, MO, USA). The standard xylooligosaccharides, xylose, xylobiose, and xylotriose, were purchased from Wako Pure Chemical (Osaka, Japan); xylo-tetraose and xylopentano-se were purchased from Megazyme (Bray, Ireland). All other chemicals were of analytical grade.

Gene cloning

The xylanase gene (*xynAS27*) fragment from *Streptomyces* sp. S27 was amplified by polymerase chain reaction (PCR) with a set of degenerate primers (Sxyn10F, 5'-TGGG ACGTSGTSAACGAG-3'; Sxyn10R, 5'-GGATGTCSA GYTCSGTGA-3'; S and Y indicate C/G and T/C, respectively; Li et al. 2008c). Genomic DNA of strain S27 was isolated and used as the template. PCR was performed using *LA Taq* DNA polymerase with GC Buffer. The amplified product was purified and ligated with pGEM-T Easy vector for sequencing.

A genomic library was constructed as described (Li et al. 2008c). A colony PCR method (Bottoli et al. 1999) was used for the target gene isolation with two specific synthetic primers: SF (5'-CACCGCAACGACTGGATCGAG-3') and SR (5'-ACTCCTGCAGCGTGGTGCGG-3'). The clone carrying the candidate fragment was obtained, and the recombinant plasmid was isolated and sequenced.

Construction of expression plasmids

For the construction and expression of the mature peptide (without the predicted signal peptide) of XynAS27, the following primers were used: S27F (5'-TACCATGGCC GAGAGCACGCTCGGCGCC-3') containing an *NcoI* site (italicized) and S27R1 (5'-TATAAGCTTTCAGGCGC GGGTCCAGCGCTGGTTG-3') containing a *HindIII* site (italicized). The truncated derivatives, *xynAS27cd* (CBM-linker-truncated version) and *xynAS27cdl* (CBM-truncated version) were amplified using the primers S27F and S27R2 (5'-TATAAGCTTTCAGAGTGCCTCGAGGACGG CGGTG-3') or S27R3 (5'-TATAAGCTTTCAGCCGT CCCC GGCGCGGAG-3'), containing the *HindIII* sites (italicized). The PCR products were purified from an agarose gel, digested with *NcoI* and *HindIII*, and inserted into pET-22b(+) treated with the same restriction endonucleases. The recombinant plasmids, *p-xynAS27*, *p-xynAS27cd*, and *p-xynAS27cdl*, were transformed into *E. coli* BL21 (DE3) competent cells and confirmed by sequencing.

Expression and purification of the recombinant proteins

Cultivation and inductive expression of the recombinant proteins were conducted following the same procedure as described previously (Li et al. 2008a). For purification of the recombinant XynAS27, the supernatant of the culture was collected by centrifugation and concentrated using a Vivaflow 200 system (Vivascience, Hannover, Germany). The concentrated supernatant was loaded on a HiTrap Q Sepharose XL FPLC column (GE Healthcare, Uppsala, Sweden) previously equilibrated with buffer A (20 mM Tris–HCl, pH 8.5). Elution was performed with a linear gradient of NaCl from 0 to 1 M in buffer A for ten column volumes at a flow rate of 3 ml min⁻¹. Fractions exhibiting xylanolytic activity were combined, concentrated and loaded onto a Superdex 75 10/300 GL column (GE Healthcare) that was equilibrated with buffer B (50 mM phosphate buffer, pH 6.5). Proteins were eluted with one column volume of buffer B at a flow rate of 0.5 ml. The fractions containing XynAS27 were collected and concentrated.

XynAS27cd and XynAS27cdl were purified by HiTrap Q Sepharose XL FPLC column using the same method described above. The fractions with enzyme activity were concentrated and stored at 4°C before characterization.

The homogeneity of the purified enzyme was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 12% running gel (Laemmli 1970). The low-molecular weight calibration kit for SDS electrophoresis (GE Healthcare) was used as a standard. The proteins were visualized by staining with Coomassie brilliant blue R-250. The protein concentration was determined by the Bradford method (Bradford 1976) using bovine serum albumin as the standard.

Xylanase activity assay

Xylanase activity was determined by measuring the release of reducing sugar from soluble oat spelt xylan using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) with xylose as the standard. To prepare 1% (w/v) soluble xylan, 1 gram of xylan was suspended in 100 ml McIlvaine buffer (0.2 M Na₂HPO₄/0.1 M citric acid) at pH 6.5, and heated in a boiling water bath for 10 min. The supernatant was obtained as soluble xylan after centrifugation (10,000×g, 5 min). The assay was performed as described by Li et al. (2008a).

Enzymatic characterization

The optimal pH was determined with soluble oat spelt xylan in buffers at pH 2.2–12.0 at 55°C. The buffers used were McIlvaine buffer for pH 2.2 to 8.0, 0.1 M Tris–HCl for pH 8.0 to 9.0, and 0.1 M glycine–NaOH for pH 9.0 to 12.0. For pH stability, the enzyme was pre-incubated without substrate in buffers of different pH for 1 h at 37°C, and then the xylanolytic activity was measured under standard conditions.

The optimal temperature was determined at pH 6.5 (McIlvaine buffer) from 20°C to 80°C. The thermostability was monitored by pre-incubating the enzyme without substrate in McIlvaine buffer (pH 6.5) for various periods at 65°C. The residual enzyme activity in each case was then assayed under standard assay conditions.

The effect of different metal ions and chemical reagents on the recombinant enzyme activity was assessed with soluble oat spelt xylan in 0.1 M phosphate buffer (pH 6.5) at 60°C. The reactions contained 1 mM or 10 mM of NaCl, KCl, CaCl₂, LiCl, CoCl₂, NiSO₄, CuSO₄, MgSO₄, FeCl₃, ZnSO₄, Pb(CH₃COO)₂, FeSO₄, AgNO₃, HgCl₂, SDS, EDTA, or β-mercaptoethanol.

The K_m , V_{max} , and k_{cat} values for the purified recombinant enzymes were analyzed by the Lineweaver–Burk method. The enzyme activity was assayed at 60°C in McIlvaine buffer (pH 6.5) containing 1–10 mg ml⁻¹ soluble oat spelt xylan or birchwood xylan as substrate.

The analysis of hydrolysis products degraded by the purified enzymes was carried out using high-performance anion-exchange chromatography (HPAEC) as described previously (Li et al. 2008b).

Insoluble substrate degradation and binding assays

Insoluble xylan was prepared from oat spelt xylan using the method of Irwin et al. (1994) with slight modifications. Xylan (2 g) was suspended in 40 ml deionized water, then the pH was adjusted to 10.0 with 1 M NaOH and the solution was stirred gently at room temperature for 1 h. The xylan suspension was centrifuged at 3,000×g for 5 min, resuspended in deionized water, and heated in a boiling water bath for 10 min with gentle stirring. The suspension was then centrifuged (3,000×g, 5 min) and washed twice with deionized water and finally with McIlvaine buffer (pH 6.5). The pellet was then suspended in 20 ml 95% ethanol and filtered through Whatman No. 1 filter paper. The pellet was dried and ground as finely as possible with a mortar and pestle.

The degradation of insoluble oat spelt xylan by the purified xylanases was tested under standard conditions using McIlvaine buffer (pH 6.5) containing 1% (w/v) insoluble xylan as the substrate. The activity of the purified enzymes on soluble oat spelt xylan was used as positive control. The affinity of the xylanases to insoluble xylan or microcrystalline cellulose (MC) was measured as follows: the purified enzyme (1 U) was mixed with 0.05 g insoluble oat spelt xylan or MC (Shanghai Hengxin Chemical, Shanghai, China) suspended in 500 μl McIlvaine buffer (pH 6.5). After 1 h of gentle shaking at 20°C, the preparation was centrifuged at 10,000×g for 5 min and the xylanase activity in the supernatant was determined. The loss of activity from the supernatant was assumed to be due to the loss of purified enzymes which bound to the pelleted insoluble xylan or MC.

Nucleotide sequence accession numbers

The nucleotide sequence for the *Streptomyces* sp. S27 xylanase gene (*xynAS27*) was deposited in the GenBank database under accession number EU660498.

Results

Cloning and sequence analysis of the xylanase

A 352-bp DNA fragment of xylanase was amplified from *Streptomyces* sp. S27 by PCR using degenerate primers and sequenced. The primers S9F and S9R were synthesized based on the partially identified sequence, and used for PCR screening of the *Streptomyces* sp. S27 genomic library. Approximately 3,000 transformants were screened, and one clone (PS2920) contained the gene *xynAS27*. The insert of the plasmid DNA from PS2920 is approximately 6 kb, including a complete open reading frame (ORF) of 1,434 bp. The ORF has about 67.7% G+C content, starts

with an ATG, and terminates with a TGA codon. The ORF encodes a 477-residue polypeptide that includes a signal peptide of 41 residues.

The deduced amino acid sequence of the ORF was aligned with available protein sequences from the GenBank database. The highest identity was 87.8% with the xylanase A from *Streptomyces coelicolor* A3(2) (GenBank accession no. CAD55241) for which the genome was sequenced, followed by a xylanase A precursor from *Streptomyces lividans* (87.6%, AAC26525). On the basis of homology comparison in GenBank, the residues Ala42 to Leu341 of XynAS27 correspond to the catalytic domain of xylanases belonging to GH10. Two putative catalytic site residues, Glu169 and Glu277, are located in the catalytic domain (Moreau et al. 1994; Fujimoto et al. 2000). Downstream of the catalytic domain is a 124-residue sequence (Asn354 to Ala477) exhibiting a high degree of similarity to CBM13 which is made of three similar subdomains, α -, β - and γ -subdomain (Fujimoto et al. 2000; Fig. 1). Between the catalytic domain and CBM13 there is a short Gly/Pro-rich

region from Asn342 to Gly353, which potentially acts as a linker (Fig. 1).

Expression and purification of the recombinant proteins

The gene fragments of *xynAS27*, *xynAS27cd*, and *xynAS27cdl* obtained by PCR amplification with the genomic DNA of *Streptomyces* sp. S27 as a template contained 1,308, 900 and 936-bp encoding 436 (Ala42 to Ala477), 300 (Ala42 to Leu341), and 312 (Ala42 to Gly353) amino acid residues, respectively. After induction with IPTG, the xylanase activity in the medium supernatant was 9.8, 5.2 and 24.6 U ml⁻¹, respectively.

The recombinant xylanases in the culture supernatants were purified to electrophoretic homogeneity by ion exchange chromatography or gel filtration chromatography. The resulting specific activity of the purified XynAS27, XynAS27cd, and XynAS27cdl was 790.7, 1,278.8, and 1,250.7 U mg⁻¹, respectively. SDS-PAGE analysis revealed protein bands with sizes of about 47 kDa (XynAS27),

Fig. 1 The nucleotide and deduced amino acid sequence of *xynAS27* gene from *Streptomyces* sp. S27. The 41-amino acid putative signal peptide is *underlined*. The putative catalytic amino acids are *boxed*. The cleavage sites of *xynAS27cd* and *xynAS27cdl* gene fragment are showed by *arrow*, respectively. *Dotted line* indicates the putative location of linker region and carbohydrate-binding module (CBM), consisting of α , β and γ subdomains. The Gln-X-Trp repeats are *shaded in gray*. The translational stop codon is indicated by *asterisk*

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1  ATGGGCTCATGCCCTTCCCAGATCCGTTGTCGGCGGAAGCTCCGCGCTCTGTGCTGGCGCTGGTCCGGCGCTCCTCGGTGTGGTC
1  M G S H A L P R S V V R R K L R A L L L A L V A G V L G V V
91  GCCGCACTGGTCGCGCCACCGTCCGCTCAGGCCGCGAGAGACGCTCGGGCCGCGGGCCGAGAGCGGCCCTACTTCGGTCGCGC
31  A A L V A P P S A Q A A E S T L G A A A A Q S G R Y F G V A
181 ATCGCCGCGAACAGGCTGAGCGACTCGACGTACGCGACGATCGCGCCCGTGAGTTCAACTCGGTGACGGCCGAGAACGAGATGAAGATC
61  I A A N R L S D S T Y A T I A A R E F N S V T A E N E M K I
271 GACGCCACCCAGCCGAGCGGGCCAGTTCAACTTCACCGCCGCGACCGCTTACAACCTGGGCGGTGACAGAACGCAAGGAGGTGCGC
91  D A T Q P Q R G Q F N F T A A D R V Y N W A V Q N G K E V R
361 GGCCACACCTGGCTGGCACTCCAGCAGCCGCGTGGATCAGAACCTCAGCGCCAGCGCACTGGCTCAGGCGATGATCGACCACATC
121 G H T L A W H S Q Q P G W M Q N L S G S A L R Q A M I D H I
451 AACGGCTGATGAGCCACTACAAGGGAAGATCGCGCAGTGGGACGTCGTGAACGAGGCTTTCGCGCAGCGAGTTCGGGAGCCCGCGC
151 N G V M S H Y K G K I A Q W D V V N [ ] A F A D G S S G A R R
541 GACTCCAACCTGCAGCGCACCGGCAAGACTGGATCGAGGTGCGCTTCCGACCGCGCGCCGCGCCGACCCGTCGCCAACTCTGTCTAC
181 D S N L Q R T G N D W I E V A F R T A R A A D P S A K L C Y
631 AACGACTACAACGTCGAGAAGTGGCACTGGCCCAAGACCCAGGCCATGTACCGCATGGTGAAGGACTTCAAGCAGCGCGCGTCCGATC
211 N D Y N V E N W T W A K T Q A M Y R M V K D F K Q R G V P I
721 GACTGCGTCCGCTTCCAGTCCGACTTCAACAGCGCGACCCCTACAACAGCAACTTCCGACCCAGCTGTCAGGAGTTCGCGCCCTCGG
241 D C V G F Q S H F N S G S P Y N S N F R T T L Q E F A A L G
811 GTGACGTGGCCATCACCAGAACTCGACATCCAGGGAGCCTCGCCCTCGACCTACGCCCGGTGGTCAAGCACTGCCTGGCCGCTCCCGT
271 V D V A I T [ ] L D I Q G A S P S T Y A A V V N D C L A V S R
901 TGCCCTCGGTGTCACCGTCTGGGCGTGGCGACAGCGACTCGTGGCGCTCGGAGCACACGCCGCTGTGTCTACAACAACGGCAGCAAG
301 C L G V T V W G V R D S D S W R S E H T P L L F Y N N G S K
991 AAGCCCGGTACACCGCGTCTCGAGCACTCAACGGCGGCTCCACCACTCCGCCCGCGGGACGGAACACGATCAAGGGCGTCCGT
331 K P A Y T A V L D A L N G G S T T P P P G D G N T I K G V G
          ↑[ linker ]↑
1081 TCGGGCCGCTGCCTGGACGTGCCAACCGCAGCACCCGACCGGACCCAGCTCCATCTGTGGGACTGCCACAACGGCACCACAGCAG
361 S G R C L D V P N A S T T D G T Q L H L W D C H N G T N Q Q
          α-subdomain
1171 TGGACGTACCAATGCCGCGAACTCAGGCTACGGCAACAGTGCCTGGACGCCCGCGCACCGGTAACGGTGCCAAGGTCCAGATC
391 W T Y T N A G E L R V Y G N K C L D A A G T G N G A K V Q I
          β-subdomain
1261 TACAGCTGTGGGTGGGACAACAGAGTGGCGCTGAACCTCCAGCGATCCATCGTGGCGTCCAGTCCGGCTCTGCCTCGACGCC
421 Y S C W G G D N Q K W R L N S D G S I V G V Q S G L C L D A
          γ-subdomain
1351 GTCGGGGCCGACCGCAACGGCACCTGTATCCAGCTCTACTCTGTCGAACGGCAGCAACAGCGCTGGACCCCGCGCTGA
451 V G A G T A N G T L I Q L Y S C S N G S N Q R W T R A *

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33 kDa (XynAS27cd), and 34 kDa (XynAS27cdl), corresponding to the predicted sizes (Fig. 2).

Effect of pH and temperature on enzyme activity and stability

XynAS27 and XynAS27cd had optimal activity at pH 6.5 and 60°C, and XynAS27cdl showed optimal activity at pH 5.5 and 65°C (Fig. 3a and b). Both truncated versions changed the profiles of pH or temperature versus enzyme activity in some degrees. The pH stability assays showed that no significant loss of activity occurred to XynAS27 during 1-h pretreatment in pH ranging from 2.2 to 12.0. In the case of XynAS27cd and XynAS27cdl, the stability drastically decreased when the pretreatment pH was above 10.0 (Fig. 3c). XynAS27 had good thermal stability, retaining about 25% of the initial activity after preincubation without substrate for 1 h at 65°C. Under the same conditions, XynAS27cdl was almost inactive and XynAS27cd lost all activity after incubation at 65°C for only 5 min (Fig. 3d).

Effect of various metal ions and chemical reagents on enzyme activity

Addition of most reagents under test had little effect on the activity of the three purified xylanases. The activities of these enzymes were almost completely inhibited by Hg^{2+} and SDS, and strongly inhibited by Cu^{2+} . The metal ion, Ag^+ (1 mM), inhibited about 60% of the activity of XynAS27, and less than 20% of the activities of XynAS27cd and XynAS27cdl. In the presence of 10 mM Fe^{3+} , Zn^{2+} , Pb^{2+} , Co^{2+} , or Ni^{2+} , the activity of XynAS27 was partially inhibited, and the activities of both truncated versions were inhibited only a little by them. β -mercaptoethanol (10 mM)

enhanced the activities of XynAS27, XynAS27cd, and XynAS27cdl by about 1.5-, 1.7-, and 1.4-fold, respectively.

Kinetic analysis

The K_m , V_{max} , and k_{cat} values for XynAS27, XynAS27cd, and XynAS27cdl are shown in Table 1. The K_m and V_{max} values for XynAS27cd and XynAS27cdl were a little higher than those for XynAS27.

Analysis of hydrolysis products

The products generated by hydrolysis of soluble oat spelt xylan and birchwood xylan by purified XynAS27, XynAS27cd, and XynAS27cdl were analyzed by HPAEC. As shown in Fig. 4, xylose and xylobiose were the major hydrolysis products after degradation with XynAS27. For all of the three enzymes and both substrates, the composition and proportion of the hydrolysis products was quite similar. After 12 h of incubation, about 15% (w/w) of the total reaction products was xylose, and 85% was xylobiose.

Degradation of insoluble xylan and binding assays

The activity of three enzymes on the insoluble oat spelt xylan was tested under the standard conditions. Comparing with the soluble xylan, the activities of XynAS27, XynAS27cd, and XynAS27cdl on the insoluble xylan were lower, about 56%, 23% and 24% of the activities on soluble substrate, respectively. For the binding studies, CBM13 showed strong binding ability to insoluble xylan and MC. XynAS27 could bind nearly completely to insoluble xylan and MC; however, XynAS27cd and XynAS27cdl were unable to adhere to insoluble xylan, and only about 20% of both truncated versions could bind to MC.

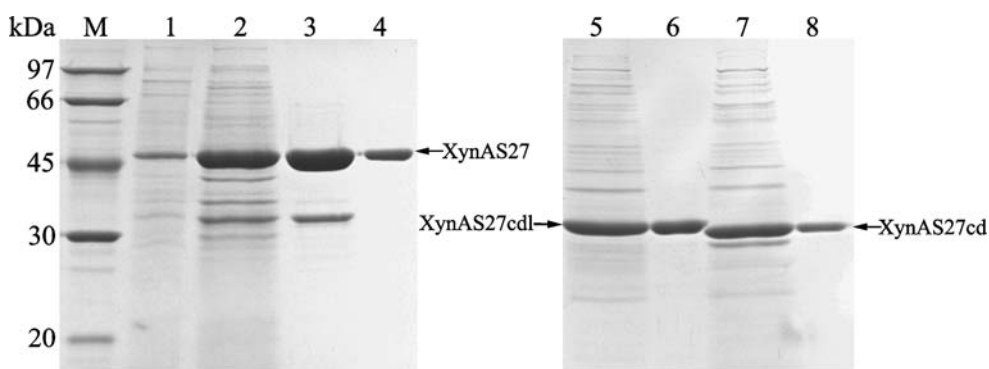


Fig. 2 SDS-PAGE analysis of the protein purification. Lane M, standard protein molecular weight markers; lanes 1, culture supernatant of the induced transformant harboring *p-xynAS27*; lanes 2, 5 and 7, concentrated culture supernatants containing XynAS27, XynAS27cdl and XynAS27cd, respectively, using Vivaflow 200; lane

3, combined fractions containing xylanolytic activity after ion exchange chromatography for XynAS27; lane 4, purified XynAS27 after gel filtration chromatography; lane 6 and 8, purified XynAS27cdl and XynAS27cd, respectively, after ion exchange chromatography

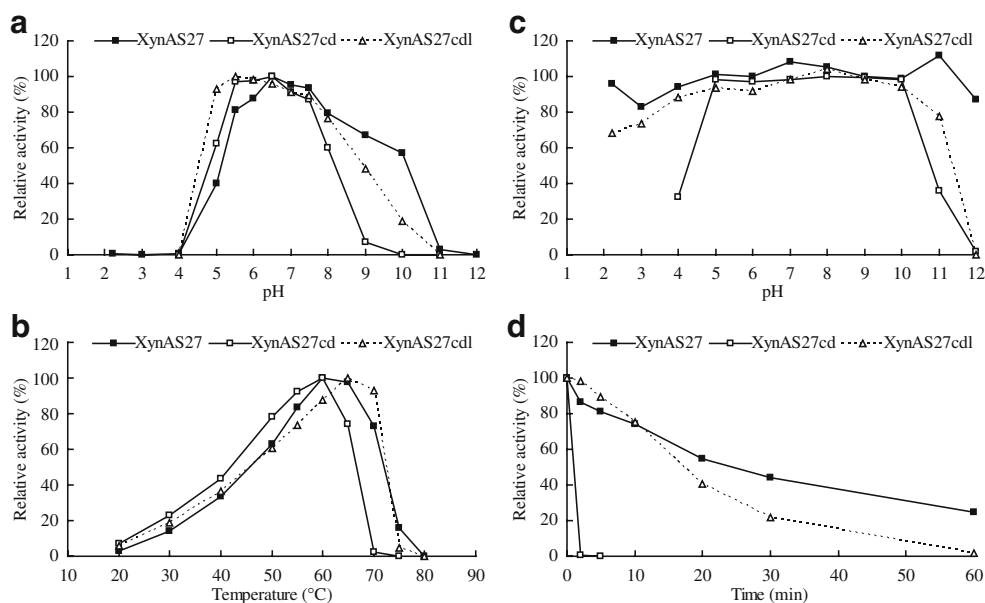


Fig. 3 Characterization of the purified XynAS27, XynAS27cd, and XynAS27cdl. **a** Effect of pH on xylanase activity. The assay was performed at 55°C in buffers ranging from pH 2.2 to 12.0. **b** Effect of temperature on xylanase activity. **c** pH stability of xylanase activity. After incubating the purified enzyme at 37°C for 1 h in buffers

ranging from pH 2.2 to 12.0, the activity was measured in McIlvaine buffer (pH 6.5) at 60°C. **d** Thermostability of the purified proteins. The enzyme was pre-incubated at 65°C in McIlvaine buffer (pH 6.5), and aliquots were removed at the specified time points for measurement of residual activity at 60°C

Discussion

Several *Streptomyces* species that are very active in the biochemical decomposition of lignocellulosic biomass produce considerable amounts of xylanases (Johnson et al. 1988; Shareck et al. 1991; Jiang et al. 2006). In this study, we cloned, expressed and characterized a xylanase (XynAS27) from *Streptomyces* sp. S27. XynAS27 showed very high pH stability and predominantly generated xylobiose as the hydrolysis products. In comparison with the carboxyl-terminal truncated versions, XynAS27 also displayed better affinity and higher hydrolytic activity to insoluble xylan, and exhibited better thermal and pH stability.

XynAS27 showed some distinct enzymatic properties when compared with other highly identical xylanases, such as

xylanase A from *S. lividans*, XYNA from *S. olivaceoviridis* and SfXyn10 from *Streptomyces fradiae*. They shared the same optimal temperature of 60°C, but varied in the optimal pH: XynAS27 (pH 6.5), xylanase A (pH 6.0; Morosoli et al.

Table 1 Kinetic properties of the purified enzymes

	XynAS27	XynAS27cd	XynAS27cdl
Oat spelt xylan			
V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	1361.2±83.3	2119.0±258.5	1389.3±66.7
K_m (mg ml^{-1})	1.3±0.2	2.9±0.4	1.4±0.3
k_{cat} (s^{-1})	1066.2±65.3	1165.5±142.2	789.6±37.9
Birchwood xylan			
V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	771.2±44.6	1204.7±106.6	1074.3±43.1
K_m (mg ml^{-1})	0.9±0.2	1.2±0.1	1.1±0.2
k_{cat} (s^{-1})	604.1±34.2	662.6±58.6	610.6±24.5

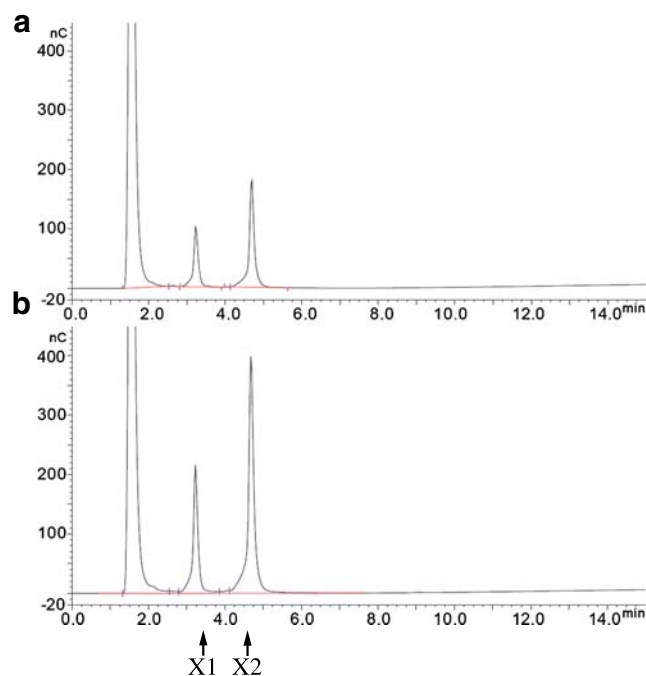


Fig. 4 HPAEC analyses of the hydrolysis products by XynAS27. **a** The hydrolysis products of oat spelt xylan **b** The hydrolysis products of birchwood xylan. The positions of standard oligosaccharides, xylose (X1) and xylobiose (X2), are shown by arrows

1986), XYFA (pH 5.6; Zhang et al. 2002), and SfXyn10 (pH 7.8; Li et al. 2008c). Furthermore, XynAS27 showed good thermal stability, retaining more than 85% of the initial activity after incubation at 60°C for 1 h (data not shown). Under the same conditions, xylanase A from *S. lividans* retained only 30% of the initial activity (Morosoli et al. 1986), and SfXyn10 from *S. fradiae* was almost inactive after incubation at 60°C for 30 min (Li et al. 2008c). XynAS27 also showed high pH stability over a wide pH range, maintaining over 80% of the activity after treatment in buffers ranging from pH 2.2 to 12.0 at 37°C for 1 h. But most xylanases, such as SfXyn10 from *S. fradiae* (Li et al. 2008c), STX-I from *Streptomyces thermoviolaceus* (Tsujiibo et al. 1992), Xys1 from *Streptomyces halstedii* (Ruiz-Arribas et al. 1995) and a xylanase from *B. halodurans* (Mamo et al. 2006), maintained stable only in the range of pH 4.0–10.0 or pH 5.0–9.0. XynAS9 from *Streptomyces* sp. S9 retained good stability over the much wider pH range of 4.0–12.0 (Li et al. 2008a), but failed under extreme acidic condition in comparison with XynAS27. Few GH10 xylanases from bacteria could retain stable in both the acid and alkaline pH range. With regard to the effects of various metal ions and chemical reagents, the activity of XynAS27 was completely inhibited by SDS, and was not affected by Fe²⁺ (data not shown). In contrast, SDS had little effect on the activity of XYNA from *S. olivaceoviridis* (Zhang et al. 2002), and Fe²⁺ could completely inhibit the activity of STX-I from *S. thermoviolaceus* (Tsujiibo et al. 1992).

The major products of xylan hydrolysis by XynAS27 were xylose and xylobiose. Similar hydrolysis products have been reported from many xylanases (Tsujiibo et al. 1992; Ali et al. 1999; Li et al. 2008a). XynBS27 from *Streptomyces* sp. S27 (Li et al. 2008b), XynB from *Thermotoga maritima* (Jiang et al. 2004) and an endoxylanase from *Bacillus* sp. (Jeong et al. 1998) hydrolyzed xylan to produce about 75%, 66%, and 60% xylobiose, respectively. XynAS27 released about 85% of the total reaction products as xylobiose after incubation with xylan for 12 h. Xylobiose stimulates the growth of intestinal *Bifidobacteria*, which is beneficial for human health in that it inhibits proliferation of pathogenic bacteria and promotes absorption of nutrients (Moure et al. 2006). As a food ingredient, xylobiose is also used in anti-obesity diets because it is low in calories and does not produce dental caries (Vázquez et al. 2000). However, production of xylobiose is time-consuming and costly (Moure et al. 2006). Thus, the application of XynAS27 to produce xylobiose is attractive.

XynAS27 from *Streptomyces* sp. S27 contains a (β/α)₈-barrel of the GH10 catalytic domain, a family 13 CBM, and a Gly/Pro-rich linker region between them. The CBM of XynAS27 exhibited high sequence identity (92.6%) to that of the xylanase (FXYN) from *S. olivaceoviridis* E-86 (PDB ID: 1XYF). The crystal structure of FXYN containing the

CBM13 has been studied by Fujimoto et al. (2000; 2002) and CBM13 has been found to have a structure similar to ricin B-chain. CBM13 has three similar subdomains, as suggested from a triple-repeat sequence, which are assembled against one another around a pseudo-threefold axis (Fujimoto et al. 2000). The triple-repeated sequences have been referred to as the (Gln-X-Trp)₃-domain (Hazes 1996), and CBM13 in XynAS27 displays the typical triple-repeated sequences (Fig. 1). Gln probably plays an important role in substrate binding, and Trp may be involved in the formation of the hydrophobic core (Fujimoto et al. 2000).

To evaluate the role of CBM13 in XynAS27, the enzyme activity and binding ability of the CBM-truncated version (XynAS27cdl) were compared with those of XynAS27. The catalytic activity on insoluble xylan of XynAS27cdl was less than half of that by XynAS27. It suggests that CBM13 could enhance the activity of XynAS27 on insoluble xylan, presumably by increasing the frequency of encounters between the catalytic domain and insoluble substrate. The results from the binding assays support the role of CBM13 in increasing the interaction with insoluble substrates. XynAS27 could completely bind to insoluble xylan and MC, but the CBM-truncated version failed to bind to insoluble xylan and bound MC less efficiently than did XynAS27. The binding ability to insoluble substrates has been reported for many other CBMs (Ali et al. 2001; Mangala et al. 2003; Ali et al. 2005a).

Removal of CBM13 and linker region could affect the enzymatic properties of XynAS27, particularly the enzyme stability. XynAS27 displayed better pH stability than the two truncated versions, indicating that CBM13 and linker region of XynAS27 played an important role in pH stability. A similar phenomenon has been detected for Xys1L with a family 2 CBM from *S. halstedii* JM8 (Ruiz-Arribas et al. 1995, 1997). However, fusion of the *B. halodurans* xylanase and a family 6 CBM did not modify the pH activity and stability (Mangala et al. 2003). In some reports, the thermal stability of the fusion of CBM with catalytic domains was less than that of the original one (Kataeva et al. 2001; Mamo et al. 2007). In the other reports, fusion of CBM had no effect or resulted in a better thermal stability (Mangala et al. 2003; Ali et al. 2005a, b). However, some data suggested that the small linker between CBM and catalytic domain but CBM made a significant contribution to the overall stability of the xylanase (Dias et al. 2004). In this study, the thermal stability of XynAS27cdl was worse than that of XynAS27, but fairly better than that of XynAS27cd. This result indicated that the linker region of XynAS27 displayed an important effect on thermal stability, and CBM13 also contributed to the enzyme stability.

In conclusion, XynAS27 from *Streptomyces* sp. S27 displayed high pH stability from pH 2.2 to 12.0, and produced xylobiose as the predominant hydrolysis product.

These favorable properties make XynAS27 a promising candidate for various applications. Comparative study of enzymatic properties, hydrolytic activity, and binding assays indicate that CBM13 of XynAS27 is not only responsible for binding to insoluble substrates, but also contributes to the thermal and pH stability of the enzyme. Furthermore, the linker region connecting CBM and catalytic domain plays a more important role in the enzyme stability. Future studies will help us achieve a better understanding of the structure–function relationship of enzyme.

Acknowledgments This work was supported by National High Technology Research and Development Program of China (863 Program; No. 2007AA100601) and National Key Technology R&D Program of China (No. 2006BAD12B05-03).

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