J. Microbiol. Biotechnol. (2008), **18**(3), 410–416

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Cloning, Expression, and Characterization of Protease-resistant Xylanase from *Streptomyces fradiae* var. k11

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Received: July 4, 2007 / Accepted: September 6, 2007

The gene *SfXyn10*, which encodes a protease-resistant xylanase, was isolated using colony PCR screening from a genomic library of a feather-degrading bacterial strain *Streptomyces fradiae* var. k11. The full-length gene consists of 1,437 bp and encodes 479 amino acids, which includes 41 residues of a putative signal peptide at its N terminus. The amino acid sequence shares the highest similarity (80%) to the endo-1,4-β-xylanase from *Streptomyces coelicolor* A3, which belongs to the glycoside hydrolase family 10. The gene fragment encoding the mature xylanase was expressed in *Escherichia coli* BL21 (DE3). The recombinant protein was purified to homogeneity by acetone precipitation and anion-exchange chromatography, and subsequently characterized. The optimal pH and temperature for the purified recombinant enzyme were 7.8 and 60°C, respectively. The enzyme showed stability over a pH range of 4.0–10.0. The kinetic values on oat spelt xylan and birchwood xylan substrates were also determined. The enzyme activity was enhanced by Fe^{2+} and strongly inhibited by Hg^{2+} and SDS. The enzyme also showed resistance to neutral and alkaline proteases. Therefore, these characteristics suggest that SfXyn10 could be an important candidate for protease-resistant mechanistic research and has potential applications in the food industry, cotton scouring, and improving animal nutrition.

Keywords: Xylanase, *Streptomyces fradiae*, protease resistance

Xylan is one of the main components of hemicelluloses in plant cell-walls, and is the second most abundant polysaccharide in nature (after cellulose), accounting for approximately one-third of all renewable organic carbons on earth [23]. Endo-1,4-β-xylanases (E.C. 3.2.1.8) are glycosidases that catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan into short xylooligosaccharides [1]. Xylanases have widespread potential for biotechnological applications in the food, textile, animal-feed, waste-treatment, and paper industries. Although frequently used alone, xylanases are more commonly used in conjunction with other enzymes, such as cellulases, proteases, oxidases, and isomerases [4].

Based on the primary structure of their catalytic domains, glycosidases have been grouped into families (refer to the carbohydrate-active enzyme CAZY server at http://www.cazy.org/fam/acc_GH.html), where xylanases are normally confined to glycoside hydrolase families (GH) 10 and 11 [4]. Most of the xylanases in family 10 are large and modular in structure and usually consist of a catalytic domain, carbohydrate-binding module, and/or other functional domains joined by linker sequences [20].

Many xylanase genes from various microorganisms have already been cloned, and the encoded enzymes isolated and characterized. Several *Streptomyces* species, which are very active in the biochemical decomposition of a lignocellulosic biomass, have been reported to produce considerable amounts of xylanases: for example, *S. olivaceoviridis* E-68 [10–12], *S. lividans* [17, 26, 30], *S. thermoviolaceus* OPC-520 [28, 29], and *S. hallstedii* JM8 [24].

Furthermore, protease-resistant xylanases have potentially diverse applications in many industrial fields, yet few such xylanases have been reported. Accordingly, this study describes a xylanase gene, *SfXyn10*, from a feather-degrading strain *S. fradiae* var. k11. The gene was expressed in *E. coli*, and the purified enzyme, SfXyn10, characterized. SfXyn10 showed a very strong resistance to neutral and alkaline proteases, giving it potential application in bread making, cotton scouring, and improving animal nutrition.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Medium

S. fradiae var. k11 with a high feather-degrading capability was maintained in our laboratory and grown as described previously [14]. The *E. coli* JM109 strain was used as the host for the DNA manipulation, plasmid pUC19 (Takara, Dalian, China) and a pGEM-T Easy vector (Promega, Madison, U.S.A.) were used for the plasmid preparations and gene cloning, and the *E. coli* BL21 (DE3) strain with a pET-22b(+) vector (Novagen, San Diego, U.S.A.) was used for the xylanase expression. All the transformed *E. coli* were grown aerobically in a Luria-Bertani (LB) medium or on LB agar plates at 37°C and supplemented with ampicillin (100 μg/ml) to select the transformants.

DNA Manipulation

All the standard recombinant DNA techniques, including plasmid extraction, restriction endonuclease digestion, and DNA ligation, were performed as described by Sambrook *et al.* [25]. The *S. fradiae* var. k11 chromosomal DNA was isolated according to Yang *et al.* [34], and the DNA transformation performed by electroporation. The DNA purification kit, restriction endonucleases, T4 DNA ligase, and *LA Taq* DNA polymerase were all purchased from Takara Co. (Dalian, China).

Construction of Genomic Library and Gene Cloning

The xylanase gene (*SfXyn10*) fragment from *S. fradiae* var. k11 was cloned using two degenerate primers that were designed based on the amino acid sequences of family-10 xylanases from *Streptomyces* (http://www.cazy.org/fam/GH10.html). The forward and reverse primers were as follows: Sxyn10F (5'-TGGGACGTSGTSAAC-GAG-3') and Sxyn10R (5'-GGATGTCSAGYTCSGTGA-3') (where S and Y indicate C/G and T/C, respectively). The *S. fradiae* var. k11 chromosomal DNA was isolated and used as the template, and the PCR performed using *LA Taq* DNA polymerase and the following conditions: initial denaturation at 94°C for 2 min, followed by 32 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR product was then cloned into a pGEM-T Easy vector and sequenced.

A genomic library was constructed using the method of Yang *et al.* [34] with slight modifications. The chromosomal DNA of *S. fradiae* var. k11 was digested with Sau3AI and electrophoresed on a 0.8% agarose gel. The 5–10 kb fragments were purified from the gel, ligated into the BamHI site of pUC19, and then transformed into *E. coli* JM109 to construct the genomic DNA library. A colony PCR method [8] was used for the target gene isolation using two specific synthetic primers: SF (5'-GCCCGGCGCGACTCCAACC-TG-3') and SR (5'-CCGAGGGCCGCGAAGTTCTGG-3'). The amplification was carried out under the following conditions: 94°C for 8 min, followed by 32 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 5 min. After obtaining a clone carrying the candidate fragment, the recombinant plasmid was isolated and sequenced.

Gene Analysis

The sequence assembly was performed using programs from Vector NTI Suite 7.0 software, and the nucleotide sequence analyzed using the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The presence of a signal peptide in the deduced amino acid sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The DNA and protein sequence alignments were carried out using the BlastN and BlastP programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/).

Expression of SfXyn10 in *E. coli*

For the expression of SfXyn10 in *E. coli*, the coding sequence of a structural region without a predicted signal peptide was amplified by a PCR using the plasmid of the positive clone as the template. Two primers, SfxF (5'-TAGGATCCGGCCGAGACCAC-GCTCGGCG-3') containing a BamHI site (underlined) and SfxR (5'-TATAAGCTTTCAGGCGCGGGTCCACCGCTGGTT-3') containing a HindIII site (underlined), were designed to facilitate inframe cloning into a pET-22b(+) expression vector. The PCR was performed for 32 cycles, consisting of 94°C for 30 s, 62°C for 30 s, and 72°C for 1.5 min. The PCR product was then gel-purified, digested with BamHI and HindIII, and cloned into the corresponding sites of pET-22b(+). The recombinant plasmid, *pETSfXyn10*, was transformed into *E. coli* BL21 (DE3) competent cells, and the xylanase-producing recombinants identified using the Congo-red method [33].

A positive transformant harboring *pETSfXyn10* was picked from a single colony and grown overnight at 37°C in an LB medium with ampicillin (100 μg/ml). The culture was then inoculated into a fresh LB medium (1:100 dilution) containing ampicillin and grown aerobically at 37°C to an A_{600} of 0.6–0.8. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of either 0.6 or 0.8 mM, the cultures grown for an additional 4 h at 37°C or 6 h at 30°C, and the expression analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Sambrook *et al.* [25].

Purification of Recombinant SfXyn10

The recombinant cells were removed by centrifugation, and the supernatant concentrated using a Hollow Fiber Membrane Module (Tianjin Motian Membrane Eng. & Tech. Co., Ltd., Tianjin, China). Two volumes of ice-cold acetone were then added to the solution, and the precipitate collected by centrifugation. Thereafter, the pellet was dissolved in a minimal amount of buffer A (20 mM Tris-HCl, pH 8.0), the undissolved material removed, and the clear supernatant loaded onto a HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. The elution was performed with a linear gradient of NaCl from 0 to 1.0 M in buffer A at a flow rate of 3 ml/min. The fractions exhibiting xylanase activity were pooled, concentrated, and stored at 4°C for characterization. The protein concentration was assayed using the Bradford method [2].

Enzyme Assay

The xylanase activity was determined by measuring the release of reducing sugar from oat spelt xylan using a 3,5-dinitrosalicylic acid (DNS) reagent, as described by Miller [16]. The reaction contained 0.1 ml of the appropriately diluted enzyme and 0.9 ml of a McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid, pH 7.8) containing 1% (w/v) of oat spelt xylan (Sigma). After incubation at 60°C for 5 min, the reaction was stopped with 1.5 ml of the DNS reagent. The reaction was then boiled for 5 min, and the absorption

at 540 nm measured when the mixture reached room temperature. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per minute under the assay conditions.

Effect of pH and Temperature on Enzyme Activity

The optimal pH for the purified recombinant enzyme was determined in buffers ranging from pH 4.5–10.0 at 50°C, and the pH stability estimated by incubating the enzyme in buffers ranging from pH 2.0–12.0. The buffers used for the pH analysis were glycine-HCl buffer (0.1 M), pH 2.0 to 3.6; acetic acid/sodium acetate buffer (0.1 M), pH 3.6 to 5.0; McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid), pH 5.0 to 8.0; Tris-HCl buffer (0.1 M), pH 8.0 to 9.0; and glycine-NaOH buffer (0.1 M), pH 9.0 to 12.0. The optimal temperature for the purified enzyme was then determined at temperatures ranging from 20°C to 80°C in the optimal pH buffer. The thermostability of the purified recombinant enzyme was monitored by pre-incubating the enzyme in the absence of a substrate at 50, 55, 60, or 70°C, and then withdrawing aliquots at specific intervals to measure the residual enzyme activity under standard assay conditions.

Kinetic Analysis

The K_m, V_{max}, and k_{cat} values for the recombinant enzyme were determined by assaying the enzyme in a McIlvaine buffer (pH 7.8) at 50°C with 0.5–10 mg/ml oat spelt xylan and birchwood xylan as the substrates. The data were plotted according to the Lineweaver-Burk method.

Effect of Various Reagents on Enzyme Activity

The effect of different metal ions and chemical reagents on the recombinant enzyme activity was assessed under standard enzyme assay conditions in reactions containing 1 mM of $FeSO_4$, $FeCl_3$, LiCl, $CoCl_2$, $NiSO_4$, $MnSO_4$, $CrCl_3$, NaCl, KCl, $CaCl_2$, $CuSO_4$, $MgSO_4$, $ZnSO_4$, PbAc, $HgCl_2$, EDTA, or SDS.

Effect of Proteases on Enzyme Stability

To examine its resistance to different proteases, the purified recombinant enzyme (100 μg/ml) was incubated with 10 μg/ml trypsin (from bovine, pH 7.6, 25°C, 14,700 units/mg; Sigma), 250 μg/ml α-chymotrypsin (type II from bovine, pH 7.8, 25°C, ≥40 units/mg; Sigma), 20 μg/ml collagenase (type IV from *Clostridium histolyticum*, pH 7.4, 37°C, 527 units/mg; Sigma), 500 μg/ml subtilisin A (type VIII from *Bacillus licheniformis*, pH 7.5, 37°C, 10 units/mg; Sigma), 330 μg/ml proteinase K (type VIII from *B. licheniformis*, pH 7.5, 37°C, 30 units/mg; Amresco, Solon, U.S.A.), 10 mg/ml proleather (from *B. subtilis*, pH 10.0, 37°C, 10 units/mg; Amano Enzyme Inc., Nagoya, Japan), and 1 mg/ml alkaline protease (from *B. pumilus* SMJ-P, pH 10.0, 37°C, 1,000 units/mg), respectively. After a 1 h or 2 h incubation under the conditions specified above for each protease, the residual activity was measured under standard assay conditions. For the control sample, the recombinant enzyme was incubated under the same conditions in the absence of protease.

Nucleotide Sequence Accession Number

The nucleotide sequence for the xylanase gene (*SfXyn10*) from *S. fradiae* var. k11 was deposited in the GenBank database under Accession No. EF429086.

RESULTS

Gene Cloning and Sequence Analysis

A 352 bp PCR product was amplified using the degenerate primers Sxyn10F and Sxyn10R, cloned into a pGEM-T Easy vector, and sequenced. Based on the partially identified sequence, SF and SR primers were synthesized and used for a PCR screening of the *S. fradiae* var. k11 genomic library. Approximately 5,000 recombinants were screened, and only one clone, pUCS-4007, was found to contain the gene *SfXyn10*. The insert of the plasmid DNA from pUCS-4007 was about 5 kb. After sequencing, one complete open reading frame (ORF) consisting of 1,437 bp was found. The overall G+C content of the ORF was 70.7%. The ORF initiated at the translation ATG start codon, and encoded a 478-residue polypeptide and TGA stop codon with a calculated molecular mass of 51 kDa. A typical signal peptide sequence and cleavage site between Ala41 and Ala42 were located at the N terminus. The mature polypeptide consisted of 437 residues with a calculated mass of 47 kDa. A potential Shine-Dalgarno ribosome-binding site sequence, 5'AAAGG3', was found 8 bp upstream of the ATG start codon.

The deduced amino acid sequence of the ORF was aligned with available protein sequences from the GenBank and SWISSPROT databases, as well as previous literature, and showed an extensive homology to an endo-1,4-β-xylanase belonging to the GH10 family. In addition, the sequence with a signal peptide shared a high sequence homology with xylanase A from *S. coelicolor* A3 (80.3% identity, 383/477 residues, GenBank Accession No. CAD55241), xylanase A from *S. lividans* (80.1% identity, 382/477 residues, AAC26525), xylanase I from *S. thermoviolaceus* (81.0% identity, 362/447 residues, BAD02382), and xylanase A precursor from *S. thermocyaneoviolaceus* (80.8% identity, 361/447 residues, AAF04600). The mature protein without a signal peptide shared the highest identity with the endo-1,4-β-xylanase from *S. olivaceoviridis* (85.3%, 372/436 residues, 1XYF_A). Thus, based on the sequence alignment, it was hypothesized that the mature SfXyn10 contained two functional domains, including a catalytic domain of GH10 from Met88 to Val337, substrate-binding domain from Gly355 to Arg477, and linker region. Two putative catalytic residues, Glu169 and Glu277, were also found in these conserved regions [7, 18].

Enzyme Expression and Purification

When the transformant harboring *pETSfXyn10* was cultivated in an LB/Amp medium, the cell lysate and medium supernatant showed a xylanase activity of 21.0 U/ml and 5.3 U/ml, respectively, after 0.6 mM IPTG induction at 37°C for 4 h. Meanwhile, the uninduced transformant and transformant harboring the empty pET-22b(+) vector showed no activity. The recombinant enzyme was then visualized by SDS-PAGE and Coomassie staining, and the molecular

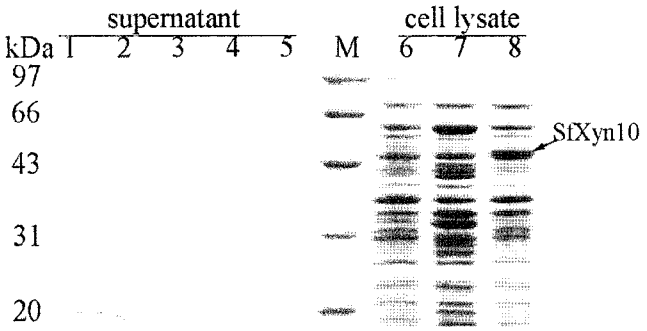

Fig. 1. SDS-PAGE analysis of recombinant SfXyn10 expressed in supernatant and cell lysate of *E. coli* BL21 (DE3) cells.
Lanes: M, standard protein molecular mass markers; 1, 6, BL21 with empty pET-22b(+) vector induced with IPTG; 2, 7, transformant harboring *pETSfXyn10* uninduced with IPTG; 3, 8, transformant induced with 0.6 mM IPTG at 37°C for 4 h; 4, transformant induced with 0.8 mM IPTG at 37°C for 4 h; 5, transformant induced with 0.6 mM IPTG at 30°C for 6 h.

mass was about 47 kDa (Fig. 1). Two other induction conditions were also analyzed to determine the optimal conditions that yielded the highest xylanase activity. According to the optimization analysis, the enzyme activity in the cell lysate and medium supernatant was 23.5 U/ml and 4.5 U/ml, respectively, after 0.6 mM IPTG induction at 30°C for 6 h; and 18.9 U/ml and 5.0 U/ml, respectively, after 0.8 mM IPTG induction at 37°C for 4 h. Thus, to purify the xylanase from the medium supernatant, induction with 0.6 mM IPTG at 37°C for 4 h was selected for further production of the recombinant xylanase.

The recombinant xylanase was then purified to electrophoretic homogeneity from the culture supernatant by acetone precipitation and anion-exchange chromatography (Table 1). As a result, the specific activity of the purified SfXyn10 was 304.2 U/mg after 6.7-fold purification, with a final activity yield of 12.7%. Additionally, the purified enzyme yielded a single band with a molecular mass of 47 kDa, as determined by SDS-PAGE (Fig. 2).

Effects of pH and Temperature on Enzyme Activity

The enzyme activity was optimal at pH 7.8 (at 50°C), whereas over 80% of the peak activity was achieved between pH 6.5 and 8.5 (Fig. 3A). The enzyme remained stable over a wide pH range and fully stable from pH 4.0 to 10.0 after incubation at 37°C for 1 h (Fig. 3B). The optimal temperature for the enzyme activity was 60°C at pH 7.8 (Fig. 3C). The

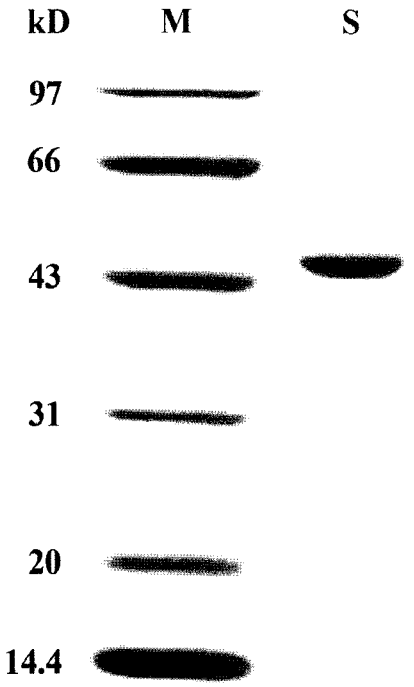

Fig. 2. SDS-PAGE analysis of purified recombinant SfXyn10.
M: standard protein molecular mass markers; S: purified SfXyn10.

enzyme remained quite stable at 50°C after incubation for 30 min at pH 7.8 and more than 40% of the activity was retained after treating the enzyme at 60°C for 5 min (Fig. 3D).

Kinetic Properties

The K_m, V_{max}, and k_{cat} values on oat spelt xylan were 1.09 mg/ml, 217.39 μmol/min·mg, and 170.83 s^{-1}, respectively. In addition, the K_m, V_{max}, and k_{cat} values using birchwood xylan as the substrate were 0.41 mg/ml, 131.58 μmol/min·mg, and 103.40 s^{-1}, respectively.

Effect of Various Reagents on Enzyme Activity

The xylanase activity in the presence of different metal ions or chemical reagents is shown in Table 2. The activity was enhanced 61.9% by Fe^{2+} and strongly inhibited by Hg^{2+} (100.0%) and SDS (94.4%). The addition of other reagents had little or no effect on the recombinant SfXyn10 activity.

Table 1. Summary of purification procedure for recombinant SfXyn10 expressed in *E. coli* BL21 (DE3).

Purification steps	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture supernatant	1,200	6,377.4	139.41	45.7	100
Hollow fiber membrane module	300	4,630.8	56.32	82.2	72.6
Acetone precipitation	26	4,122.6	25.12	164.1	64.6
Anion-exchange chromatography	5	812.3	2.67	304.2	12.7

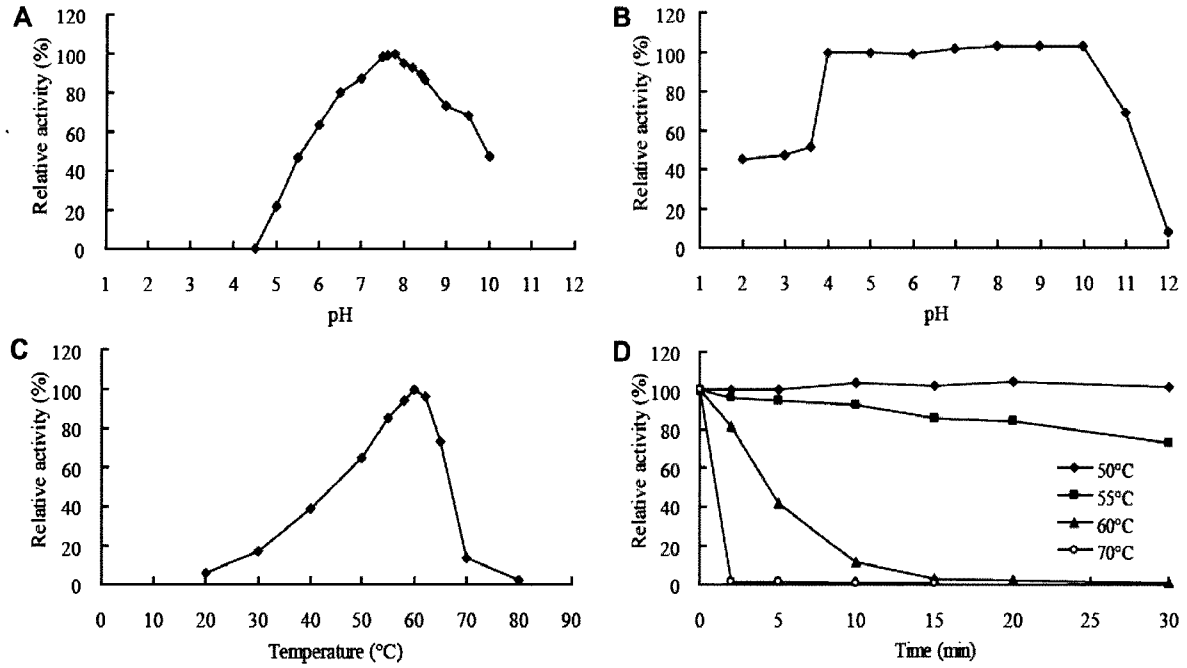

Fig. 3. Characterization of recombinant SfXyn10.
A. Effect of pH on activity. The assay was performed at 50°C in pH 4.5–10.0. **B**. pH stability of recombinant SfXyn10. After incubating the enzyme at 37°C for 1 h in buffers pH 2.0–12.0, the activity was measured in a McIlvaine buffer (pH 7.8) at 60°C. **C**. Effect of temperature on activity. The enzymatic activity was measured in a McIlvaine buffer (pH 7.8). **D**. Thermostability of recombinant SfXyn10. The enzyme was pre-incubated at 50, 55, 60, or 70°C in a McIlvaine buffer (pH 7.8), and aliquots removed at specific time points to measure the residual activity at 60°C.

Protease Resistance

The recombinant SfXyn10 exhibited a strong resistance to neutral and alkaline proteases. As shown in Fig. 4, the enzyme activity virtually unchanged after incubation for 2 h with commercial proteases, including trypsin, chymotrypsin, collagenase, subtilisin A, proteinase K, and proleather, plus retained over 60% of its activity following treatment with high concentrations of an alkaline protease from *B. pumilus*, which contained a variety of alkaline proteases as an industrial preparation.

Discussion

This report described the cloning of a GH10 xylanase secreted by *S. fradiae* var. k11, plus a genomic library was constructed and screened using a colony PCR method. Although a number of hydrolases, such as serine proteases from strains of *S. fradiae*, have already been reported [14, 27], *SfXyn10* is the first xylanase gene cloned from *S. fradiae*.

SfXyn10 showed some distinct enzymatic properties when compared with xylanases XYFA from *S. olivaceoviridis* and xylanase A from *S. lividans*, which exhibited the highest sequence homology to SfXyn10. Although these xylanases shared the same optimal temperature of 60°C, the optimal pH for SfXyn10, XYFA, and xylanase A was 7.8, 5.6, and 6.0, respectively [19, 35]. Furthermore, whereas xylanase I (STX-I) from *S. thermoviolaceus* was completely inhibited by Fe^{2+} [28], the activity of SfXyn10 was enhanced 61.9% by Fe^{2+}. SfXyn10 also remained fully stable after treatment with buffers ranging from pH 4.0 to 10.0 at 37°C for 1 h, which was a wider pH range when compared with the enzymes mentioned above.

Strain *S. fradiae* var. k11 effectively hydrolyzes feathers and human hair, and 6 genes encoding secreted proteases have already been cloned from this strain, with two having been identified by the current authors [13–15]. When *S.*

Table 2. Effect of metal ions and chemical reagents on recombinant SfXyn10 activity.

Reagent[a]	None	Fe^{2+}	Fe^{3+}	Li^{+}	Co^{2+}	Ni^{2+}	Mn^{2+}	Cr^{3+}	Hg^{2+}
Relativity activity (%)	100.0	161.9	100.3	101.9	109.6	102.0	110.7	102.7	0.0
Reagent[a]	Na^{+}	K^{+}	Ca^{2+}	Cu^{2+}	Mg^{2+}	Zn^{2+}	Pb^{2+}	EDTA	SDS
Relativity activity (%)	87.4	92.5	90.7	87.9	98.7	96.6	99.5	97.1	5.6

[a]The concentration of each reagent was 1 mM in the assay buffer.

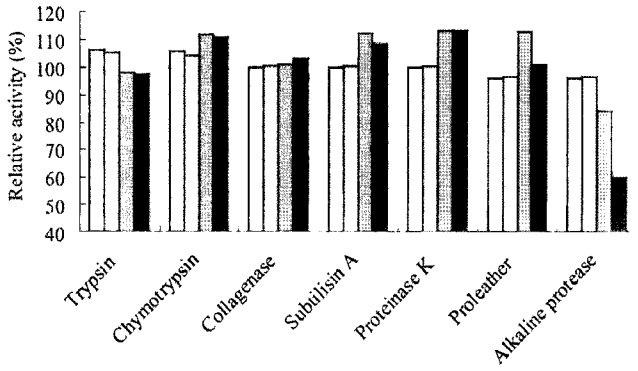

Fig. 4. Effect of proteases on SfXyn10 stability.
The purified SfXyn10 was incubated with proteases under specific conditions for each protease for 1 h (▒) or 2 h (■), and the residual activity measured in a McIlvaine buffer (pH 7.8) at 60°C. The purified SfXyn10 was also incubated without proteases under the same conditions for 1 h (□) or 2 h ( ), and the residual activity measured as the control sample. The purified SfXyn10 was not incubated as 100% activity.

fradiae var. k11 was heavily inoculated into a medium that contained both feathers and xylan as the inducing substrates, protease activity and xylanase activity were simultaneously detected in the supernatant after shaken cultivation at 37°C for 12 h. Moreover, both of these activities increased over the cultivation time (data not shown).

SfXyn10 isolated from *S. fradiae* var. k11 exhibited a very strong resistance to neutral and alkaline proteases. Fernández-Abalos *et al.* [5] previously demonstrated that the xylanase Xys1L (45 kDa) from *S. halstedii* JM8 could be specifically processed extracellularly to produce Xys1S (33.7 kDa), which retained a catalytic activity yet no cellulose-binding capacity, by *Streptomyces* serine proteases, whereas Xys1L was completely and nonspecifically degraded by commercial serine proteases from different sources, even after short incubation periods at low protease concentrations. In contrast, SfXyn10 still showed the molecular mass of 47 kDa after incubation with high concentrations of trypsin and collagenase for 1 h (data not shown). Fontes *et al.* [6] investigated the resistance of cellulases and xylanases to proteolytic inactivation and showed that five recombinant xylanases derived from mesophilic and thermophilic organisms displayed a complete resistance to bovine α-chymotrypsin, porcine pancreatin, and duodenal juice inactivation. However, these researchers did not examine the resistance to microbial proteases, which are widely used in industrial processes. The tertiary structures of enzymes from thermophilic organisms include strong interactions between amino acid residues, and this type of tight structure could confer such enzymes with an inherent protease resistance [6]. In addition, the surface charge and hydrophobicity that influence proteases to approach enzymes may also be important for enzyme resistance to proteases. After treatment with pepsin for 30 min, the XYNA from *S. olivaceoviridis* still retained over 95% activity [35]. However, the activity of SfXyn10 was completely lost after incubation with pepsin (Sigma), even within very short time periods. Thus, at a low pH (pH 2.0), it is possible that the surface charge or tertiary structure of SfXyn10 was changed, allowing the pepsin more easy access to the hydrolysis sites. Yet, for a better answer to why certain enzymes exhibit resistance to certain proteases, further analysis is needed of the enzymatic tertiary structure, surface charge, glycosylation sites [31], hydrophobicity, and primary structure.

In conclusion, SfXyn10 showed a wide pH stability and strong protease resistance, allowing potential application in bread making, cotton scouring, and improving animal nutrition. In bread making systems, alpha-amylases, xylanases, and proteases have a softening effect on the texture of transglutaminase-supplemented pan breads, leading to a better shape and greater specific volume and void fraction in loaves [3]. As regards the cotton scouring process, the ecofriendly, energy-saving, and clean process of compound enzymatic scouring (including neutral cellulase, neutral and alkaline proteases, and xylanase) could substitute for the conventional alkali scouring process [21, 32]. Finally, to improve animal nutrition, transgenic animals could be developed that secrete cellulases and/or xylanases into the small intestine [9]. These enzymes could then hydrolyze the gel-like structures formed by structural polysaccharides to release the trapped nutrients [22]. Yet, to be effective in the small intestine, the enzymes should be resistant to the resident proteases. SfXyn10 has a strong protease resistance, suggesting that it could be an important candidate for protease-resistant mechanistic research.

Acknowledgments

This work was supported by the Chinese Program on Research for Public Good (No. 2005DIB4J038) and National Key Technologies R&D Programs of China (No. 2004BA-522B07-8).

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