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BIOCATALYSIS

A novel neutral xylanase with high SDS resistance from *Volvariella volvacea*: characterization and its synergistic hydrolysis of wheat bran with acetyl xylan esterase

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Abstract A neutral xylanase (XynII) from *Volvariella volvacea* was identified and characterized. Unlike other modular xylanases, it consists of only a single GH10 catalytic domain with a unique C-terminal sequence (W-R-W-F) and a phenylalanine and proline-rich motif (T-P-F-P-P-F) at N-terminus, indicating that it is a novel GH10 xylanase. XynII exhibited optimal activity at pH 7 and 60 °C and stability over a broad range of pH 4.0–10.0. XynII displayed extreme highly SDS resistance retaining 101.98, 92.99, and 69.84 % activity at the presence of 300 mM SDS on birchwood, soluble oat spelt, and beechwood xylan, respectively. It remained largely intact after 24 h of incubation with proteinase K at a protease to protein ratio of 1:50 at 37 °C. The kinetic constants K_m value towards beechwood xylan was 0.548 mg ml^{-1}, and the k_{cat}/K_m ratio, reflecting the catalytic efficiency of the enzyme, was 126.42 ml mg^{-1} s^{-1} at 60 °C. XynII was a true endo-acting xylanase lacking cellulase activity. It has weak activity towards xylotriose but efficiently hydrolyzed xylans and xylooligosaccharides larger than xylotriose mainly to xylobiose. Synergistic action with acetyl xylan esterase (AXEI) from *V. volvacea* was observed for de-starched wheat bran. The highest degree of synergy (DS 1.42) was obtained in sequential reactions with AXEI digestion preceding XynII. The high SDS resistance and intrinsic stability suggested XynII may have potential applications in various industrial processes especially for the detergent and textile industries and animal feed industries.

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Keywords *Volvariella volvacea* · Xylanase · SDS resistance · Synergistic action · Acetyl xylan esterase

Abbreviations

GH	Glycoside hydrolase family
PNP-glycoside	ρ-Nitrophenyl-glycosides
PNPG	ρ-Nitrophenyl-glucopyranoside
PNPX	ρ-Nitrophenyl-xylopyranoside
PNPA	ρ-Nitrophenyl-arabinofuranoside
CMC	Carboxymethylcellulose
XOS	Xylooligosaccharides
X1	Xylose
X2	Xylobiose
X3	Xylotriose
X4	Xylotetraose
X5	Xylopentaose
X6	Xylohexaose
EST	Expressed sequence tag
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HPAEC-PAD	High-performance anion exchange chromatography with pulsed amperometric detection

Introduction

Xylan is the major constituent of hemicellulose and is the second most abundant renewable resource with a high potential for degradation to useful end-products. Unlike cellulose, xylan generally contains heterogeneous substituents such as L-arabinose, *O*-acetyl, ferulic (4-hydroxy-3-methoxycinnamic) acid, ρ-coumaric (4-hydroxycinnamic)

acid and 4-*O*-methyl-D-glucuronic acid. Complete biodegradation of this structurally complex polymer requires the cooperation of xylanases and β-xylosidases along with several accessory enzymes, including α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and ferulic/coumaric acid esterases (EC 3.1.1.73) [8]. Of these xylanolytic enzymes, xylanases are of particular significance because they can randomly attack the internal β-1,4-glycosidic bonds within the polymer backbone and catalyze the initial breakdown of xylan [31]. Based on amino acid sequence homologies and hydrophobic cluster analysis, xylanases have been classified into two main groups in glycoside hydrolase family (GH)10 and GH11, although a few characterized xylanases have recently been ascribed to GH8, 30, and 43 [33] (http://www.cazy.org/Glycoside-Hydrolases.html). Enzymes from these families have different molecular structures, molecular weights, and catalytic properties.

The commercial application of xylanases began in the 1980s in the preparation of animal feed, and later expanded to the food, textile, and paper industries. Since then, the biotechnological use of these enzymes has increased dramatically, covering a wide range of industrial sectors [5, 30, 34]. Stability in the conditions of industrial processes is a usual requisite for an enzyme to be successfully applied in biotechnology. Stabilization becomes even more difficult for an enzyme when toxic reagents and extreme conditions are employed in industrial processes. One approach to meet the requirements of enzymes for various industrial processes is to make specific modifications of the structure of the enzymes and their functions by protein engineering, but this demands a detailed understanding of enzyme structure and function.

Volvariella volvacea, the edible straw mushroom, is grown on an industrial scale in many tropical and subtropical regions of Southeast Asia. *V. volvacea* grows naturally on rice straw, a prominent component of which is arabinoxylan. *V. volvacea* is an atypical white rot basidiomycetous fungus, which has complete cellulolytic and hemicellulolytic systems but with very low lignolytic activity [37]. Here, a novel family ten xylanase (XynII) was identified from *V. volvacea*. Its enzymatic action modes and synergism with acetyl xylan esterase (AXEI) from *V. volvacea* in hydrolysis of wheat bran were carefully studied. This enzyme exhibited maximum activity at pH 7.0, wide pH stability, and extremely high SDS and proteolysis resistance, suggesting it may be used as a model protein for structure–function relationship studies of GH10 xylanase and an alternative to potential industrial applications, especially for the detergent, textile, and animal feed industries.

Materials and methods

Strains, culture conditions, vectors, and chemicals

Volvariella volvacea V14 was obtained from the culture collection of the Centre for International Services to Mushroom Biotechnology located at The Chinese University of Hong Kong (accession no. CMB002). *Escherichia coli* DH5α was used for cloning and *Pichia pastoris* KM71H was used for protein expression. The pGEM-T vector (Promega, Madison, WI, USA) was used to subclone DNA fragments for sequencing. The pPICZαB vector (Invitrogen, Carlsbad, CA, USA) was used for construction of yeast expression vector. For extraction of RNA, the fungus was cultured in rice straw compost as described previously [10]. The xylans from beechwood, birchwood, and oat spelt, ρ-nitrophenyl-glucopyranoside (PNPG), ρ-nitrophenyl-xylopyranoside (PNPX), ρ-nitrophenyl-arabinofuranoside (PNPA) carboxymethylcellulose (CMC), locust bean gum, and chitin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), xylohexaose (X6) were purchased from Megazyme (Bray, Ireland). Soluble and insoluble xylan from oat spelt xylan were prepared as described by Moraïs et al. [25]. De-starched wheat bran was prepared according to the method as described by Lamsal et al. [22].

Cloning of xylanase II (XynII) gene

An expressed sequence tag (EST) was obtained previously through EST sequencing of a *V. volvacea* cDNA library constructed from mRNA isolated from mycelia of strain V14 grown on rice straw compost. Based on a BLAST search against recent GenBank releases, this EST sequence showed homology to xylanase. The gene-specific primer 5′-GCGCTTGGCGAGGGCAACAATTTCGT-3′ based on the EST sequence was designed to generate the 5′- end DNA fragment coding for XynII by rapid amplification of cDNA ends (RACE)—PCR using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). The 5′- cDNA end fragment was subcloned into the pGEM-T vector and sequenced. The full-length cDNA of *xynII* was then generated by 3′- RACE using the gene-specific primer 5′-GGGAGGCATCACCCAGCTTCTTCG TACC-3′ designed from the sequence of the extreme 5′- end of *xynII*, and sequenced as above.

Expression and purification of XynII in *Pichia pastoris*

The transformation and expression of *xynII* in *P. pastoris* was carried out using the expression plasmid pPICZαB

according to the manufacturer's protocol (Invitrogen). The cDNA fragment encoding mature XynII was flanked by EcoRI/XbaI restriction sites at the 5′ and 3′ ends, respectively, and amplified by PCR using the following oligonucleotides:

5′-TGCCGGAATTCCGACGCCATTCCCCCCTTTCAA-3′ and 5′-GCTCTAGATCAATGATGATGATGATGATGGAACCAACGCCATTTATTGA-3′ and Pfu polymerase (Agilent-Stratagene; La Jolla, CA, USA). After digestion, the fragment was ligated at the EcoRI/XbaI sites of pPICZαB Pichia expression vector to yield the construct pPICZαB-xynII in which *xynII* was under the transcriptional control of the AOX1 (alcohol oxidase) promotor. This construct was transferred into *E.coli* DH5α and the gene insert was confirmed by DNA sequencing. The pPICZαB-xynII plasmid was then linearized by SacI and transformed into *P. pastoris* KM71H competent cells by electroporation with a Genepulser II apparatus (Bio-Rad, Hercules, CA, USA). Transformants of *P. pastoris* containing the *xynII* cDNA were selected on the basis of Zeocin resistance using yeast extract-peptone-dextrose (YPD) agar plates supplemented with 1 M sorbitol and 100 μg/ml Zeocin (Invitrogen). Screening of the most efficient XynII-producing transformants under the methanol-inducible AOX1 promotor was carried out according to the manufacturer's protocol (Invitrogen). Twenty-two Transformants were grown in 50 ml of BMGY medium contained in 250-ml flasks at 30 °C and 250 rpm for 16–24 h, after which time the cell density reached an OD_{600} value of between three and four. Yeast cells from a portion of the culture suspension were harvested by centrifugation and resuspended in 25 ml of BMMY medium to a final OD_{600} value of 30. Following an additional 2 days of induction at 30 °C and 250 rpm, xylanase activity in culture supernatants was determined. The *xynII* transformant exhibiting the highest level expression was selected for production of recombinant XynII. The recombinant XynII with the 6 × His-tag was purified by affinity chromatography using Ni–NTA Agarose gel (Qiagen, Valencia, CA, USA) according to the manufacturer's manual. Enzyme homogeneity and the molecular weight of purified XynII were estimated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [10 % (w/v)].

Biochemical characterization of XynII

Xylanase standard assay was performed in reaction mixtures containing 1.0 ml 0.1 M phosphate sodium citrate buffer (pH 7.0), 100 μl of appropriately diluted purified enzyme (0.028 U), and 0.4 ml of 0.5 % (w/v) beechwood xylan. The reaction mixtures were incubated for 10 min at 60 °C, then reducing sugar released was determined by the Somogyi–Nelson method. Optimal pH and temperature values were determined using the standard assay over the ranges pH 3.0–12.0 (Universal buffer: 50 mM H_3PO_4, 50 mM CH_3COOH, 50 mM H_3BO_3, pH adjusted with 0.2 M NaOH at 25 °C) and 30–80 °C, respectively. Thermal stability was monitored by assessing the residual activity using the standard methods after pre-incubating the enzyme at 40–60 °C without substrate for specific time as needed. The pH stability was estimated by measuring the residual activity after incubating the enzyme in Universal buffer with pHs of 3.0–11.0 at room temperature for 1 h. The effect of various metal ions, sodium azide, and EDTA on enzyme activity was determined using beechwood xylan as substrate under the standard assay conditions.

Substrate specificity and kinetic parameters

The substrate specificities of XynII were determined at standard condition by measuring the amount of reducing sugar equivalents released in reaction mixtures containing one of the following substrate solutions/suspensions: 0.5 % (w/v) beechwood xylan, or 1 % (w/v) birchwood xylan, insoluble and soluble oat spelt xylan, CMC, locust bean gum, and chitin. Specificity towards ρ-nitrophenyl-glycosides (PNP-glycoside) was determined by measuring the amount of ρ-nitrophenol released in reaction mixtures containing 20 mM PNP-glycoside according to the method of Rättö and Poutanen [26]. One unit (U) of xylanase activity for xylans or PNP-glycosides was defined as the amount of enzyme required to release 1 μmol of reducing sugar or ρ-nitrophenol, respectively, per minute under standard assay conditions. The kinetic constants (V_{max} and K_m) were determined at 50 or 60 °C by reaction for 5 min using beechwood xylan at concentrations 0.1–1.5 mg ml^{-1}. The V_{max} and K_m values were calculated by GraphPad Prism 5.0 software (http://www.graphpad.com/prism/) using nonlinear regression.

SDS and proteolytic resistance

The effect of SDS on the xylanase activity of XynII was assayed under the standard assay conditions except that SDS was added at a concentration from 0 to 300 mM. The reaction was performed at 50 °C with 0.5 % (w/v) beechwood xylan or 1 % (w/v) birchwood xylan, insoluble and soluble oat spelt xylan as substrate. The SDS resistance of XynII was also assayed by comparing the migration on gel of boiled and unboiled samples containing SDS [24]. The proteolytic resistance of XynII was studied using proteolysis by proteinase K (Takara Bio, Shiga, Japan). The protein samples (5 μg) were incubated with different concentrations of proteinase K at 37 °C for 24 h, denatured by boiling in 4 × SDS-PAGE loading dye (containing SDS

and 2-mercaptoethanol), and subjected to SDS-PAGE. Gels were stained using Coomassie blue.

Analysis of the hydrolysis products

Hydrolysis of different xylans by XynII was carried out at 50 °C for up to 2 h under the same conditions used for the activity assay. After hydrolysis, the mixture was heated for 5 min in a boiling water bath to terminate the reaction, and centrifuged at 11,000 × *g* for 10 min. For enzymatic hydrolysis of different xylooligosaccharides (XOS), including X3, X4, X5, and X6, each xylooligosaccharide (50 μg) was incubated with XynII (0.028 U) in 0.1 M phosphate sodium citrate buffer (1.5 ml, pH 7.0) at 50 °C for 24 h. The hydrolysis products in supernatants were analyzed at 30 °C using a Carbo-Pac PA200 column (3 × 250 mm) fitted to an ICS-3000 high-performance anion exchange chromatography system (Dionex, Sunnyvale, CA, USA) with pulsed amperometric detection (HPAEC-PAD). A dual mobile-phase system (A, 100 mM NaOH; B, 500 mM sodium acetate) was applied, and saccharides were eluted using a linear sodium acetate gradient (B: 0–24 % in 40 min; 0.3 ml/min), followed by elution with 100 mM NaOH (15 min; 0.3 ml/min) as previously described [37]. X1, X2, X3, X4, X5, and X6 were used as external standards.

Synergistic interactions between XynII and acetyl xylan esterase

The recombinant acetyl xylan esterase I (AXEI) from *V. volvacea* was produced and purified by heterologous expression as previously described [10]. The synergistic interactions between XynII and recombinant AXEI were carried out in simultaneous or sequential reactions of both enzymes as described by Raweesri et al. [28]. Twenty milligrams of de-starched wheat bran was incubated with two enzymes, XynII and AXEI, in 1 ml phosphate sodium citrate buffer (pH 7.0) at 50 °C. In simultaneous reactions, the reaction mixture contained both enzymes and was incubated for 2 h. The mixture was then boiled for 10 min to inactivate the enzyme activity and incubated for another 2 h under same conditions. In sequential reactions, the first reaction containing either individual enzyme, as indicated in the results, was incubated for 2 h. The mixture was then boiled for 10 min to inactivate the enzyme activity. Thereafter, the second reaction was carried out by adding the other enzyme which was not present in the first reaction and incubating for another 2 h. Control experiments were conducted with each enzyme alone (2 h). Varying dosage ratios of the two enzymes as indicated were used in the synergistic reaction. The reducing sugars in supernatants were analyzed by Somogyi–Nelson method with xylose as standard. The monosaccharides and oligosaccharides in supernatants were analyzed by HPAEC-PAD.

Nucleotide sequence accession number

The nucleotide sequence of the XynII from *V. volvacea* has been submitted to GenBank with accession No. KC492049.

Results

Cloning and sequence analysis of *xynII*

The full-length cDNA of *xynII* consisted of 1,202 bp encoding 350 amino acids with a putative signal peptide of 20 amino acids and a mature protein of 330 amino acids. Two *N*-glycosylation sites were detected, at positions N90 and N130, using NetNGlyc 1.0 Server. Alignment of the deduced amino acid sequence of *xynII* with xylanase genes of other organisms showed the highest sequence similarities to hypothetical GH10 xylanase from *Coprinopsis cinerea* okayama7#130 (68 %), beta-1,4-endoxylanase from *Gloeophyllum trabeum* (62 %), endo-1,4-beta xylanase from *Agaricus bisporus* (60 %), GH10 protein from *Schizophyllum commune* H4–8 (58 %), and endo-1,4-B-xylanase A from *Phanerochaete chrysosporium* (57 %) (Fig. 1). Two putative catalytic glutamate residues and six/seven conserved residues near the catalytic center highly conserved among GH10 members were identified in this sequence (Fig. 1). It is noteworthy that, on the basis of multiple sequence alignment with other GH10 xylanases in GenBank, the XynII contains a unique C-terminal Trp-Arg-Trp-Phe (W–R–W–F) extension, which is not found in other xylanases. The mature protein of XynII also contains unconserved phenylalanine and proline-rich motif Thr-Pro-Phe-Pro-Pro-Phe (T-P-F-P-P-F) at the N-terminus. The family 10 xylanase from *Coprinopsis cinerea* okayama7#130 has an extended Pro-Val-Arg-Arg (P-V-R-R) motif at the C-terminus, but lacks phenylalanine and proline-rich motif in the N-terminus.

Purification and characterization of XynII

The gene encoding XynII from *V. volvacea* was expressed in *P. pastoris*. The recombinant protein with a 6 × His-tag at its C terminus was purified by affinity chromatography in a one-step procedure using Ni–NTA agarose gel. SDS-PAGE analysis revealed that the purified XynII migrated as one band with a molecular mass of 39 kDa, the same as the theoretical molecular mass (Fig. 2a). Optimal pH and temperature for XynII were 7.0 and 60 °C, respectively (Fig. 3a, b). The XynII was stable at a pH ranging from 4.0 to 10.0, retaining more than 80 % of the initial activity

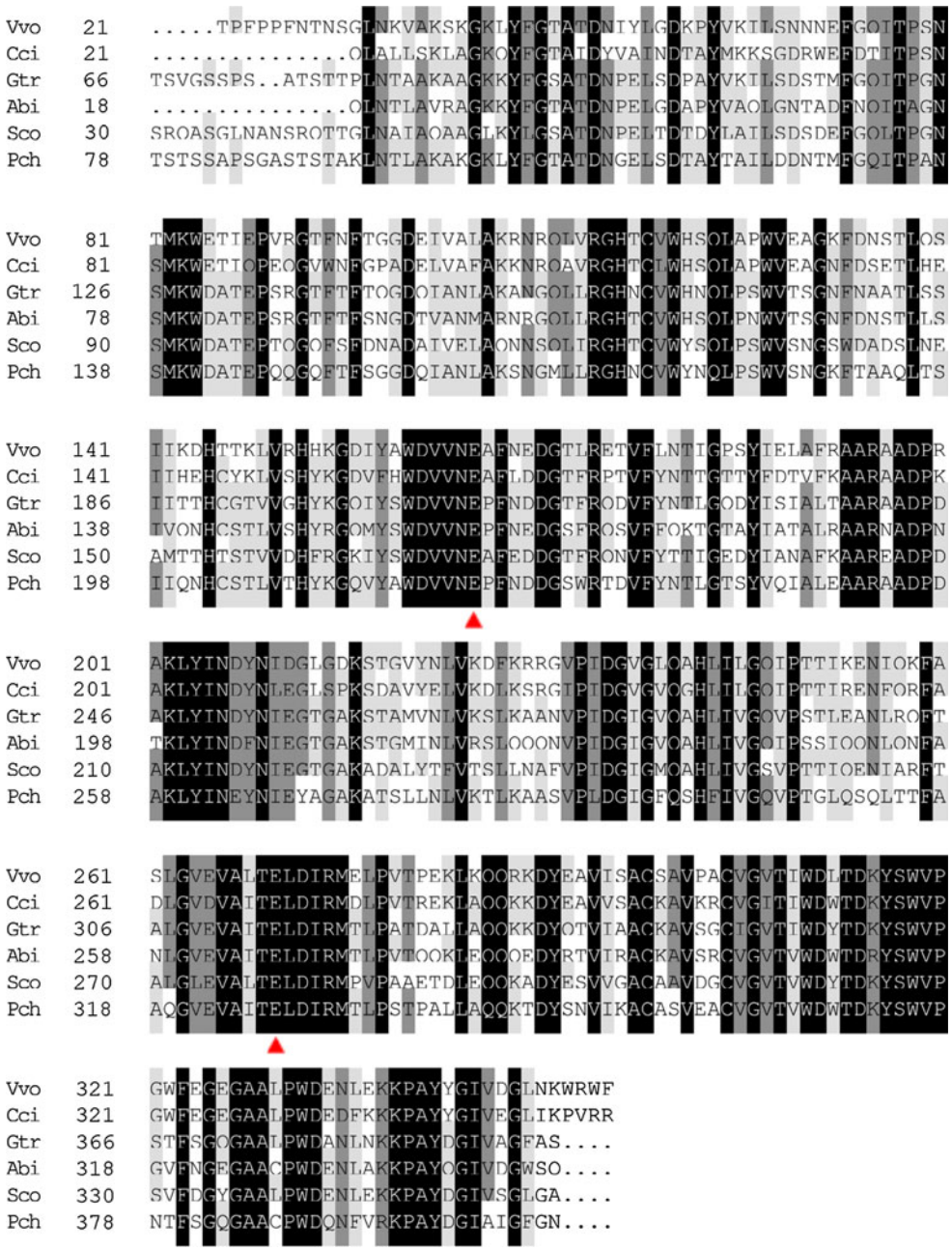

Fig. 1 Alignment of the deduced amino acid sequence of XynII with those of other GH10 xylanases. Sequences (GenBank accession numbers) of *C. cinerea* okayama7# 130C (Cci) GH 10 xylanase (XP_001838887), *G. trabeum* (Gtr) beta-1,4-endoxylanase (AEJ35165.1), *A. bisporus* (Abi) endo-1,4-beta xylanase (CAB05665.1), *S. commune* H4-8 (Sco) GH 10 protein (XP_003027315.1), *P. chrysosporium* (Pch) endo-1,4-B-xylanase A (AAG44992.1). Identical residues are *shaded in black* and conserved residues are *shaded in gray*. The two conserved catalytic residues (Glu) are marked with *red triangles*

after incubation for 1 h at room temperature (Fig. 3c). XynII was stable at 45 °C, more than 90 % of the activity was retained after 1.5 h incubation at 45 °C, however, the activity rapidly decreased at temperature above 55 °C (Fig. 3d).

The substrate specificity of XynII was determined by assaying its activity towards various substrates (Table 1). XynII showed the highest activity on beechwood xylan, followed by soluble and insoluble oat spelt xylan, and birchwood xylan. XynII had no detectable activity against synthetic ρ-nitrophenyl derivatives of xylose, arabinofuranose and glucopyranose, CMC, locust bean gum, and chitin, indicating it is true *endo*-acting xylanase. Kinetic constants K_m values towards beechwood xylan were 0.478 and 0.548 mg ml^{-1}, and V_{max} values were 52.42 and 109.89 U mg^{-1} at 50 and 60 °C, respectively. The k_{cat}/K_m ratios, reflecting the catalytic efficiency of the enzyme, were 69.14 and 126.42 ml mg^{-1} s^{-1} at 50 and 60 °C, respectively.

The effects of metal ions on XynII activity were tested by using beechwood xylan as the substrate. The significant inhibition (56.89 and 35.58 % inhibition, respectively) was observed in the presence of 5 mM Cu^{2+} and Fe^{3+}, but other metal ions had only slight or no inhibition effects

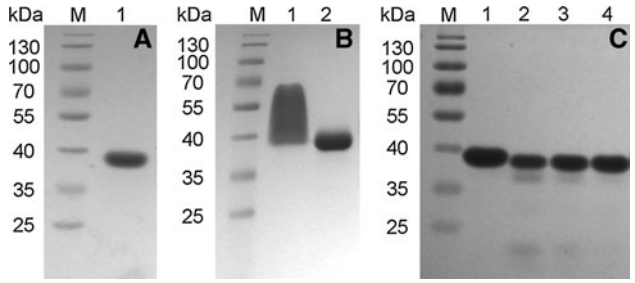

Fig. 2 **a** SDS-PAGE of purified XynII. *Lane M* molecular weight markers; *lane 1* purified XynII. **b** SDS-PAGE analysis of SDS-resistance of XynII. *Lane M* molecular weight markers; *lanes 1 and 2* unboiled and boiled XynII containing SDS, respectively. **c** SDS-PAGE analysis of proteolytic resistance of XynII. *Lane M* molecular weight markers; *lane 1* untreated XynII; *lanes 2–4* XynII were pre-incubated for 24 h at 37 °C with proteinase K using a protease/protein ratio of 1:50, 1:100 and 1:200, respectively

(Table 2). The putative inhibitors such as sodium azide at 100 mM and EDTA at 1 mM had little or no effect on this XynII activity (Table 2).

SDS and proteolytic resistance

XynII displayed extremely high SDS resistance; SDS at 300 mM had little or no effect on the enzyme activity on birchwood xylan or soluble oat spelt xylan. It also retained approximately 93 and 70 % of its activity on beechwood xylan at 200 and 300 mM of SDS, respectively (Table 3). However, nearly all the activity was lost when assaying the activity using de-starched wheat bran (data not shown) and insoluble oat spelt xylan as substrates, in the presence of 50 and 300 mM SDS, respectively. The SDS resistance of XynII was also assayed by comparing the migration on gel of boiled and unboiled samples containing SDS (Fig. 2b). The unboiled XynII exhibited a slower and smear migration in the gel, indicating unboiled XynII was at least partially resistant to SDS-induced denaturation and/or has a lesser overall negative charge of the SDS-protein complex in heterogeneous manner compared to boiled proteins. As shown in Fig. 2c, XynII was also strongly resistant to proteinase K digestion; it remained largely intact after 24 h of incubation with proteinase K at a protease-to-protein ratio of 1:50 at 37 °C. The proteinase K is a broad-spectrum and aggressive serine protease, and the strong resistance to proteases in vitro suggests XynII as a good candidate for animal feed supplement.

Enzymatic mode and synergism with acetyl xylan esterase in hydrolysis of wheat bran and xylans

To explore the enzymatic action of XynII, the hydrolysis products of three xylan substrates and XOS by XynII were determined by HPAEC-PAD. XynII had very weak activity to X3, and only a tiny amount of X3 was hydrolyzed into X1 and X2 even after 24 h of hydrolysis (Fig. 4a). However, it completely hydrolyzed X4, X5, and X6. X4 was rapidly degraded into X2 as dominant product with trace amounts of X3 and X1 (Fig. 4b). X5 was degraded into mainly X2 and X3 (Fig. 4c). X6 was also hydrolyzed to mainly X2 and X3, in which the amount of X2 was much higher than that of X3 (Fig. 4d). The hydrolysis of X4 into

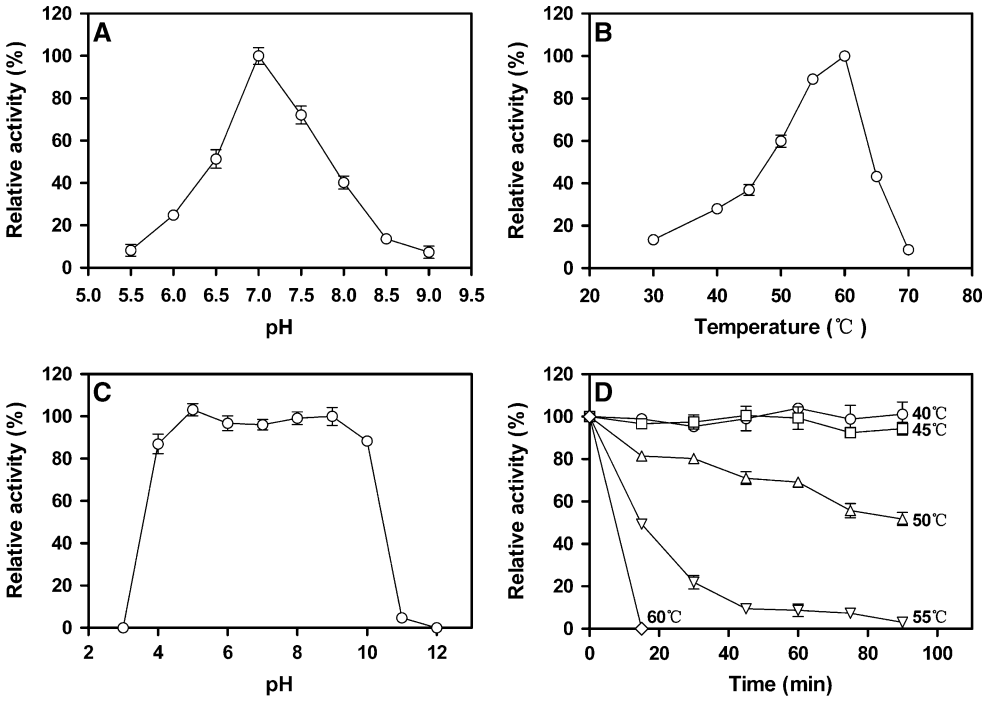

Fig. 3 Effects of pH (**a**) and temperature (**b**) on the activity of XynII and effects of pH (**c**) and temperature (**d**) on the stability of XynII. Values shown are the means of the results of triplicate experiments ± standard errors of the means (SE)

Table 1 Biochemical properties of XynII

Substrate	Specific activity (U mg^{-1})
Beechwood xylan	67.27 ± 0.79
Birchwood xylan	37.94 ± 1.90
Insoluble oat spelt xylan	59.17 ± 0.91
Soluble oat spelt xylan	64.27 ± 2.10
CMC	ND
Locust bean gum	ND
Chitin	ND
PNPG	ND
PNPX	ND
PNPA	ND

The values shown represent the means ± standard errors of the results of triplicate experiments

ND not detected

Table 2 Effect of metal ions and chemical reagents on the xylanase activity of XynII

Metal ions and chemical agents	Relative activity (%)	
	1 mM	5 mM
Ca^{2+}	87.15 ± 1.24	96.48 ± 1.49
Mn^{2+}	97.12 ± 1.32	87.88 ± 0.85
Mg^{2+}	91.37 ± 1.18	94.92 ± 1.35
Cu^{2+}	91.18 ± 0.96	43.11 ± 1.47
Zn^{2+}	91.56 ± 1.65	107.23 ± 1.59
Fe^{3+}	86.00 ± 1.38	64.42 ± 1.77
Li^{+}	100.88 ± 1.73	97.86 ± 1.44
Ni^{2+}	89.97 ± 1.55	89.68 ± 1.28
Co^{2+}	98.64 ± 1.79	102.82 ± 1.12
NH_4^{+}	96.20 ± 1.22	97.08 ± 1.46
EDTA (0.1 mM)	95.74 ± 1.22	
EDTA (0.5 mM)	94.42 ± 1.55	
EDTA (1 mM)	93.90 ± 1.60	
Sodium azide (10 mM)	99.34 ± 2.63	
Sodium azide (50 mM)	94.72 ± 1.67	
Sodium azide (100 mM)	93.95 ± 1.96	
2-Mercaptoethanol (l0 mM)	82.76 ± 1.88	
Control[a]	100 ± 0.98	

The values shown represent the means ± standard errors of the results of triplicate experiments

Beechwood xylan was used as the substrate

[a] The activity of XynII without any reagent was taken as control (100 %)

X2 as almost the only end-product and presence of much higher concentrations of X2 than X3 in X6 reaction mixture suggested that the XynII cleaves the internal β-1,4-xylosidic bonds of the XOS with a preference for cleavage sites at the second β-1,4-xylosidic bond from the reducing end.

XynII displayed typical *endo*-activity against xylan from beechwood, birchwood, and soluble oat spelt, i.e., a mixture of XOS, mainly X2, a minor amount of X3, and a trace amount of X4, X5, and X6, was generated after hydrolysis (Fig. 5a–c). X2 was also the dominant hydrolytic product of de-starched wheat bran, but a minor amount of X1 and trace X3 and X4 was generated as end reaction products (Fig. 5d).

The synergistic action between XynII and AXEI was investigated by comparing sequential actions of individual enzymes and simultaneous actions of both enzymes to those of predicted contributions from XynII alone and AXEI alone. A moderate but significant degree of synergy (DS) was observed for both of sequential and simultaneous action of de-starched wheat bran, and the highest DS (1.42) was obtained when AXEI (6 U) was used as the first enzyme and XynII (1 U) was used as the second enzyme (Table 4). Conversely, no synergy was observed when XynII was used as the first enzyme and AXEI was used as the second enzyme (after heat inactivation of XynII) (data not shown). In both cases, the degrees of synergy were increased as the amount of added AXEII increased from 2 to 6 U. These results clearly confirmed the necessity of removing the *O*-acetyl groups from the xylose residues before hydrolysis of the wheat bran by the xylanases. However, the hydrolysis product profiles from xylans and de-starched wheat bran remained same whether XynII worked alone or synergistic action with AXEI (Fig. 5).

Discussion

XynII is non-modular xylanase that consists of only a single GH10 catalytic domain without additional carbohydrate-binding module. Unlike members of this family typically having a high molecular mass and a low p*I* [7], XynII has a very high p*I* (7.67). Furthermore, XynII has two remarkable features: a unique C-terminal W-R-W-F extension and T-P-F-P-P-F motif at N-terminus, suggesting it is a novel GH10 xylanase.

Xylanases with good broad pH stability and optimal activity at neutral-alkaline pH, were reported previously from bacteria [27, 39], but rarely from fungi. XynII has broad pH stability and neutral pH optimum; this characteristic was probably due to the preference of *V. volvacea* to grow on a neutral-alkaline condition in nature. Generally, cations play roles as cofactors or ligands, facilitating binding of substrates to amino acid residues, and so enhancing enzyme activity [14, 32]. EDTA is a chelating agent that removes ions from the enzymes thereby inhibiting many xylanase activities [6, 27, 32]. In this paper, XynII is not sensitive to EDTA, indicating that XynII does not depend on cations as cofactors.

Table 3 Effect of SDS on the xylanase activity of XynII

Substrate	Relative activity (%)[a]			
	50 mM[b]	100 mM	200 mM	300 mM
Beechwood xylan	102.02 ± 1.34	95.58 ± 4.51	92.52 ± 2.33	69.84 ± 2.92
Birchwood xylan	141.27 ± 2.40	125.06 ± 1.64	125.80 ± 5.00	101.98 ± 4.17
Soluble oat spelt xylan	116.58 ± 4.54	114.50 ± 1.55	104.15 ± 1.86	92.99 ± 1.28
Insoluble oat spelt xylan	32.49 ± 1.65	26.09 ± 2.80	9.84 ± 1.22	5.16 ± 0.70

The values shown represent the means ± standard errors of the results of triplicate experiments

[a] The activity of XynII without SDS was taken as 100 %

[b] The final SDS concentration in the reaction system

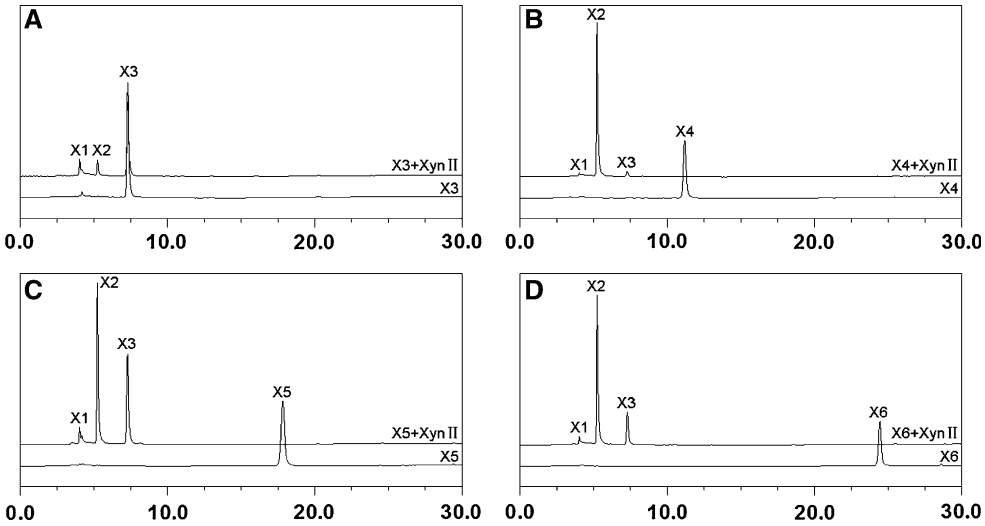

Fig. 4 HPAEC-PAD analysis of hydrolysis products generated from xylotriose (**a**), xylotetraose (**b**), xylopentaose (**c**), and xylohexaose (**d**) following treatment with XynII for 24 h. *X1* xylose, *X2* xylobiose, *X3* xylotriose, *X4* xylotetraose, *X5* xylopentaose, *X6* xylohexaose

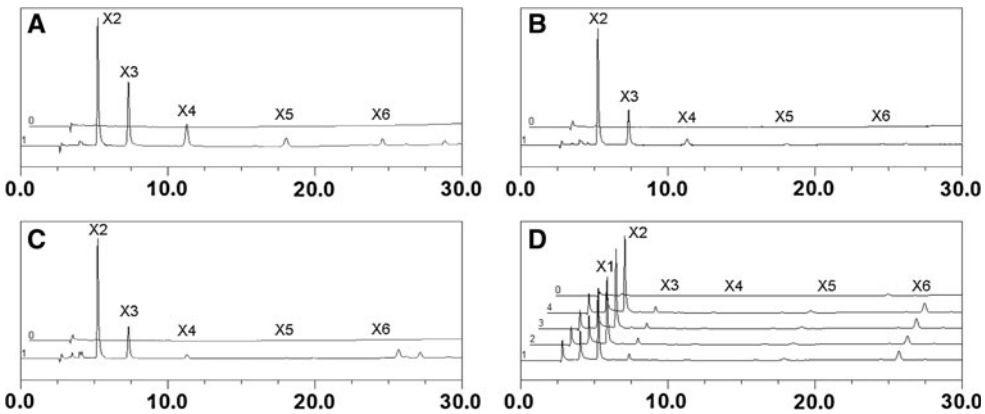

Fig. 5 HPAEC-PAD analysis of oligosaccharides released from beechwood xylan (**a**), birchwood xylan (**b**), soluble oat spelt xylan (**c**), and de-starched wheat bran (**d**), after incubation with no enzyme (*0*), XynII only (*1*), sequential reactions of XynII and AXEI (*2*), sequential reactions of AXEI and XynII (*3*), and simultaneous reaction of XynII and AXEI (*4*). *X1* xylose, *X2* xylobiose, *X3* xylotriose; *X4* xylotetraose, *X5* xylopentaose, *X6* xylohexaose

Sodium dodecyl sulphate (SDS) is a strong protein denaturant that inactivates most xylanases even at low concentration of SDS [1, 35]. There are few enzymes in the literature that could retain their activity in the presence of a high concentration of SDS. For example, xylanase BSX from alkalophilic *Bacillus* sp. NG-27, GH11 xylanase from symbiotic *Streptomyces* sp. TN119 and GH 10 xylanase XynAHJ3 from *Lechevalieria* sp. HJ3, have been reported to retain over 100 % activities in the presence of 100 mM SDS [2, 38, 39]. Two endochitinases, designated as ASCHI5 3 and ASCH from *Aeromonas schubertii* retained, respectively, 65 and 75 %, of their chitinase activity in the presence of 5 % SDS [23]. It has been proposed that SDS resistance is a common property of kinetically stable proteins, which have limited unfolded conformations susceptible to proteolytic attack and own strong proteolytic resistance [24]. Both the high resistances to SDS and proteinase K of XynII confirmed the good correlation

Table 4 Synergistic interactions between XynII and acetyl xylan esterase I (AXEI) on de-starched wheat bran

Enzyme added		Reducing sugar (μg ml^{-1})[a]	DS[b]
First reaction	Second reaction		
Control reaction			
XynII (1 U)	No enzyme	112.48 ± 1.94	
No enzyme	XynII (1 U)	132.87 ± 5.70	
AXEI (2, 4, 6 U)	No enzyme	ND	
Sequential reaction			
AXEI (2 U)	XynII (1 U)	158.32 ± 1.05	1.19
AXEI (4 U)		170.40 ± 2.11	1.28
AXEI (6 U)		188.25 ± 4.78	1.42
Simultaneous reaction			
AXEI (2 U) + XynII (1 U)	No enzyme	127.78 ± 2.90	1.14
AXEI (4 U) + XynII (1 U)	No enzyme	140.43 ± 3.61	1.25
AXEI (6 U) + XynII (1 U)	No enzyme	150.14 ± 3.26	1.33

The values shown represent the means ± standard errors of the results of triplicate experiments

ND not detected

[a] This value was calculated by taking the amount of liberated reducing sugar in supernatant

[b] The degree of synergy values were calculated as the activity divided by the sum of the activities of the control reactions

between kinetic stability and SDS resistance, and suggests this enzyme may be used as a model protein for structure–function relationship studies. SDS is mainly used in detergents for laundry with many cleaning applications. Thus far, enzymes, such as proteases [20], amylases [12], and lipases [16], are normally used to increase the efficiency of detergents in the detergent industry. For an enzyme to be used as a detergent additive, it should be stable and active in the presence of such detergent components. Supplementation of xylanases enhancing the cleaning ability of detergents has already been proven [18]. Hence, XynII with such favorable properties, might be potential candidates in the detergent and textile industries.

It is interesting to note that SDS resistance is also partially associated with substrate solubility. It retained only 5.16 % activity on insoluble oat spelt xylan compared with 92.99 % on soluble xylan in the presence of 300 mM SDS. The GH10 xylanases all have very similar structures, comprising $(\alpha/\beta)_8$-barrels as well as additional helices and loops that are arranged in a basic triosephosphate isomerase (TIM)-barrel structure forming the active site cleft [17]. The cleft forms deep grooves consistent with the *endo*-mode of action, and comprises a series of subsites, each one tailored towards the binding of a single xylose moiety [13]. Although GH10 xylanases display the same gross fold, the topology of their substrate-binding clefts is not conserved [19]. Probably, the SDS might induce substrate-binding cleft change and affect the formation of enzyme-substrate complex, and therefore influence its activities on insoluble oat spelt xylans.

It is not clear what chemical-physical property is responsible for this resistance. The N-terminal to C-terminal motifs play special roles in protein folding and function [21]. Proteins have a dominant tendency to bring their N- and C-terminal elements together and the docking of their terminal element is the first step in folding [15]. Bhardwaj et al. [2] demonstrated that the N- and C-terminal contact through aromatic interactions was critical for SDS stability of BSX. Further investigation is necessary to elucidate the significance of the N- and C-terminal sequences in the structural stability of XynII.

Wheat bran is an abundant industrial by-product and represents an interesting biomass for further applications such as XOS or fermentable sugars. Enzymatic hydrolysis was thought to be a more environmentally friendly process for converting wheat bran into value-added products such as XOS or fermentable hydrolysate comparing to acid treatment. However, *endo*-1,4-xylanases alone did not hydrolyze wheat bran efficiently due to the complexity and heterogeneity of the wheat bran arabinoxylan structure, both side-group cleaving and depolymerizing enzyme activities are required for full enzymatic degradation into monosaccharides [29]. The acetyl substitutions on the xylan backbone may impede the action of *endo*-1,4-β-xylanases and thus retard the enzymatic degradation of the xylan [3]. Acetyl xylan esterases (EC 3.1.1.72) hydrolyze specifically the ester linkages of the acetyl groups in position 2 and/or 3 of the xylose moieties of natural xylan and play a role in enhancing the accessibility of xylanases to the xylan backbone and subsequent hydrolysis of xylan

[11]. Synergism between xylanases and acetyl xylan esterases on chemically acetylated xylan and a few insoluble lignocellulosic materials has been demonstrated by a number of groups [4, 9, 36]. The DS is largely dependent on the sources of lignocellulosic materials and content of residual acetyl groups in xylan. For example, the hydrolysis degree of xylans in the pretreated wheat straw and giant reed were enhanced by 15.3 and 3.8 %, respectively, when AXE from *Trichoderma reesei* was added to xylanase (XYL) from *Thermoascus aurantiacus* comparing with XYL alone [36]. The role of acetyl xylan esterases on wheat bran enzymatic hydrolysis is still poorly understood. Our data clearly demonstrated that XynII had synergistic actions significantly with acetyl xylan esterase in enzymatic hydrolysis of de-starched wheat bran.

In conclusion, we reported a novel neutral GH10 xylanse (XynII) with extremely high SDS resistance from *V. Volvacea*. Thus, it will represent an ideal model for studying the structure–function relationship for GH10 xylanases. Excellent hydrolytic properties, stability, and synergistic effect of this xylanase with acetyl xylan esterase also offer great opportunities for potential applications, especially in the detergent, textile, and animal feed industries.

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