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Characterization of a recombinant multifunctional glycoside hydrolase family 3 β-xylosidase/α-L-arabinofuranosidase/β-glucosidase from *Cellulosimicrobium cellulans* sp. 21

Ye Yuan[a], Yanbo Hu[a], Han Zhang[a], Jiayi Leng[a], Fan Li[a], Xuesong Zhao[c], Juan Gao[a,b,*], Yifa Zhou[a,*]

[a] *Jilin Province Key Laboratory on Chemistry and Biology of Natural Drugs in Changbai Mountain, School of Life Sciences, Northeast Normal University, Changchun 130024, PR China*
[b] *School of Biological Science and Technology, University of Jinan, Jinan 250022, PR China*
[c] *College of Mining Industry, Liaoning Technical University, Fuxin 123000, PR China*

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ABSTRACT

A multifunctional β-xylosidase/α-L-arabinofuranosidase/β-glucosidase gene (*ccxyl3a*) belonging to glycoside hydrolase family 3 (GH3) was cloned from *Cellulosimicrobium cellulans* sp. 21 and expressed in *Escherichia coli* BL21 (DE3). The molecular mass of recombinant CcXyl3A was estimated to be approximately 95 kDa. With *p*-nitrophenyl-β-D-xyloside (*p*NPβXyl) as a substrate, the purified protein presented an optimal pH of 8.5 and an optimal temperature of 45 °C. Moreover, CcXyl3A was activated in the presence of the metals K^+ and Na^+. Purified CcXyl3A demonstrated multifunctional activities on *p*NPβXyl, *p*-nitrophenyl-β-D-glucoside (*p*NPβGlc), and *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPαAraf). The greatest catalytic activity were found on *p*NPβXyl followed by *p*NPα*Araf* and *p*NPβGlc, respectively. Using xylooligosaccharides as substrate, CcXyl3A completely hydrolyzed xylobiose, xylotriose, xylotetraose and xylohexaose, xylose was the sole product. In addition, CcXyl3A synergistically acted with *Thermomyces lanuginosus* xylanase in the degradation of beechwood xylan, released xyloses from intermediate xylooligosaccharides produced by *T. lanuginosus* xylanase. To date, this is the first report to demonstrate the cloning and characterization of a multifunctional GH3 enzyme in *C. cellulans* that may have applications in hemicellulose degradation.

1. Introduction

Hemicellulose is the second most common polysaccharides in nature, including xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan. These polysaccharides contain pentoses such as xylose and arabinose, and hexoses such as glucose and mannose [1,2]. Owing to the heterogeneity in composition and structure, the complete degradation of hemicellulosic biomass requires the synergetic actions of different enzymes [3,4]. It is reported that many microorganisms produced a serial of divergent enzymes to degrade hemicellulose. In order to achieve efficiently and completely degradation, some microorganisms have evolved multifunctional hemicellulolytic enzymes, such as β-xylosidase/α-L-arabinofuranosidase and β-glucosidase/β-xylosidase/α-arabinosidase, which could hydrolyze a diverse range of fibre substrates [5,6]. Identification and utilization of novel multifunctional hemicellulolytic enzymes is of potentially useful for the conversion of hemicellulosic biomass into biofuels, as well as for the preparation of other useful chemicals [7,8].

Glycoside hydrolase family 3 (GH3) is the main GH family involving exoacting β-D-glucosidases, α-L-arabinofuranosidases, and β-D-xylosidases (www.cazy.org). The α-L-arabinofuranosidases and β-D-xylosidases from GH3 are involved in the degradation of hemicellulosic xylan and arabinoxylan. Many of the GH3 enzymes have dual or broad substrate specificities with respect to monosaccharide residues, linkage position, and chain length of the substrate [9,10]. Previously, several bifunctional α-L-arabinosidases/β-xylosidases and multifunctional β-glucosidases/β-xylosidases/α-arabinosidases belonging to GH family 3 have been characterized [11–13].

* Corresponding authors.
E-mail addresses: gaoj199@nenu.edu.cn (J. Gao), zhouyf383@nenu.edu.cn (Y. Zhou).

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Cellulosimicrobium cellulans, previously designated as *Oerskovia xanthineolytica*, is an actinobacteria with yeast-lytic glucanases activity [14,15]. Previously, *C. cellulans* was reported to produce hemicellulose-degradation enzymes in culture medium when using corn cob as sole carbon source [16]. In this study, a novel multifunctional β-xylosidase/α-L-arabinofuranosidase/β-glucosidase gene belonging to GH family 3 was cloned from *C. cellulans* sp.21, the recombinant protein, CcXyl3A, was expressed, purified and the enzymatic properties were investigated. Additionally, the utility of CcXyl3A for the degradation of xylan was investigated by examining its enzymatic properties in combination with xylanase.

2. Materials and methods

2.1. Strains and reagents

Strain *C. cellulans* sp. 21 was isolated from the soil in Changbai Mountain (Jilin Province, China), and stored in China General Microbiological Culture Collection Center (collection number CGMCC 7587) [18]. *E. coli* BL21 (DE3) and pET-28a (+) were used as host and expression vector, respectively (Novagen, Madison, WI, USA). *p*-Nitrophenyl-β-glucopyranoside (*p*NPβGlc), *p*NP-α-glucopyranoside (*p*NPαGlc), *p*NP-β-galactopyranoside (*p*NPβGal), *p*NP-α-galactopyranoside (*p*NPαGal), *p*NP-β-mannopyranoside (*p*NPβMan), *p*NP-α-mannopyranoside (*p*NPαMan), *p*NP-β-xylopyranoside (*p*NPβXyl), *p*NP-α-L-arabinofuranoside (*p*NPαAraf) and *T. Lanuginosus* xylanase (X2753-10G) was from Sigma (St. Louis, MO, USA). Genomic DNA isolation, DNA purification, and plasmid isolation kits were from Tiangen Biotech (Beijing, China). All of other chemicals and reagents were analytical grade.

2.2. Cloning and expression of ccxyl3a gene

Genomic DNA of *C. cellulans* sp. 21 was isolated using Tiangen genomic DNA isolation kit. The primers having restriction sites for *Nde*I (5′-GGAATTCCATATGACCGACGTCGTCCCTGCCGT-3′) and *Bam*HI (5′-CGGGATCCTCATCATGCGCGCTCCCCCGCCT-3′) were used for gene ccxyl3a amplification. PCR was performed using Phusion polymerase and the following program: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 68 °C for 45 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR product and pET-28a (+) were digested with *Nde*I and *Bam*HI, the ccxyl3a gene was ligated with pET-28a (+) to generate the recombinant plasmid pET28a-ccxyl3a. For CcXyl3A expression, *E. coli* BL21 (DE3) cells harboring pET28a-ccxyl3a were grown in 200 ml of LB broth with 50 μg/ml kanamycin at 37 °C. When the OD_{600nm} reached 0.5, the culture was induced with 0.5 mM IPTG and then grown for 20 h at 25 °C.

2.3. Purification of recombinant CcXyl3A by Ni sepharose fastflow column

Cells were harvested by centrifugation at 4 °C and 14,000*g* for 10 min, suspended in 20 ml of lysis buffer (0.1 M NaCl, 20 mM Tris-HCl, pH 7.5) and then disrupted by sonication on ice (3 s pulse with 3 s interval for 5 min). Cell debris were removed by centrifugation (14,000*g*, 4 °C, 30 min). The crude extract containing CcXyl3A was loaded onto Ni sepharose fastflow column (GE healthcare). The column of 5 ml was pre-equilibrated by binding buffer (10 mM imidazole, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5), washed with 20 ml of washing buffer (20 mM imidazole, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5) and eluted with 20 ml of eluting buffer (250 mM imidazole, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5). The active fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.5). The purified CcXyl3A was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% separating gel [17]. Protein concentrations were determined by the method of Bradford using bovine serum albumin (BSA) as a standard.

2.4. Enzymatic characterization

The specific activity measurement was carried out in 200 μl of 50 mM phosphate buffer (pH 8.0) containing 40 mM substrate and 2 μg recombinant CcXyl3A. After incubated at 30 °C for 10 min, the reaction was stopped by adding 100 μl NaOH (0.2 M), and the released *p*-nitrophenol was measured at 405 nm by BioTek ELx808 microplate reader (Winooski, VT, USA) [18]. The reaction mixture without enzyme was used as a blank. The enzymatic activities of CcXyl3A on sophorose, laminaribiose, laminaritriose, cellobiose, cellotriose, gentiobiose, xylobiose and xylotriose were measured by determining the reducing sugers with dinitrosalicylic acid (DNS) reagent at 520 nm [19]. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 μmol/min of *p*-nitrophenyl/reducing sugar under assay conditions.

The effect of pH on CcXyl3A activity was determined at different pHs ranging from 2.0 to 10.5 at 30 °C for 10 min using *p*NPβXyl (40 mM) as a substrate. The pH stability was investigated under standard assay conditions after incubation of the purified CcXyl3A for 24 h at 4 °C in the buffers without substrate. The optimum temperature was determined by measuring the enzymatic activity at optimal pH in the temperatures ranging from 20 to 90 °C. Thermostability was performed by incubating the enzyme at different temperatures (30–50 °C) for up to 1 h. The residual activities were assayed using *p*NPβXyl as a substrate at 30 °C for 10 min. The initial activity was defined as 100%.

The effects of metals and other chemicals on CcXyl3A activity were determined. CcXyl3A was pre-incubated in 20 mM Tris-HCl buffer (pH 7.0) in the presence of metals or reagents (5 or 50 mM, final concentration) at 30 °C for 1 h. Then the residual activity was determined using *p*NPβXyl as a substrate (final concentration 40 mM) as described before.

The effect of sugars (glucose, xylose and arabinose) on CcXyl3A activity was carried out by pre-incubating CcXyl3A for 1 h at optimal pH and temperature with 5, 10, 25, 50 or 100 mM of each sugar. The retained activity of CcXyl3A was then measured with *p*NPβXyl as a substrate and compared to that in the absence of sugar.

2.5. Determination of kinetic parameters

The kinetic parameters of CcXyl3A were determined by measuring the initial reaction velocity at different substrate concentrations ranging from 0.1 mM to 5 mM at optimal pH and temperature. The K_m and V_{max} values were calculated from GraphPad Prism V5.

2.6. Xylo-oligosaccharides degradation by CcXyl3A

The hydrolytic activity of CcXyl3A on xylo-oligosaccharides was determined. The reaction mixture of 200 μl composed of 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0), 10 mg/ml xylobiose (X2), xylotriose (X3) or xylo-oligosaccharides mixture (XOS-70, Shandong Longli Co.) and 10 μg/ml purified CcXyl3A was incubated at 30 °C for 10 min. The hydrolytic products of xylobiose and xylotriose were determined by thin layer chromatography (TLC). The TLC plates were developed using ethyl acetate/methanol/water (16/6/1, v/v/v) as developing solvent. Products were visualized by spraying the plates with sulphuric acid/ethanol (5/95, v/v) followed by heating for 5 min at 105 °C in an oven. The hydrolytic products of xylo-oligosaccharides mixture were determined by Inertsil NH_2 column (DIKMA 4.6 × 250 mm, 5 μm) connected to an HPLC system (Shimadzu, Japan), eluted at the flow rate of 1.0 ml/min for 15 min with 65% acetonitrile (in distilled water, v/v), monitored by the refractive index detector.

2.7. Synergistic action of CcXyl3A with xylanase during the hydrolysis of beechwood xylan

Hydrolysis of xylan was performed by incubating 10 mg/ml beechwood xylan with *T. lanuginosus* xylanase (Xyn, final concentration 10 μg/ml), or 10 μg/ml CcXyl3A, or both in 50 mM phosphate buffer (pH 8.0), at 37 °C and 150 rpm for 8 h. The amount of reducing sugars released from the reaction at various time points (0, 1, 2, 4, 8 h) was measured by DNS method, and comparing it to a standard curve of xylose. To identify the hydrolysis products, aliquots of hydrolysate were removed at 0, 1, 2, 4 and 8 h, respectively, and analyzed by TLC method as described before.

2.8. Nucleotide sequence accession numbers

The sequences for the 16 S rRNA and CcXyl3A genes from *C. cellulans* sp. 21 were deposited in GenBank under accession numbers KR349463 and KT833270, respectively.

3. Results and discussion

3.1. Sequence analysis

GH3 enzymes such as β-xylosidase, α-L-arabinofuranosidase and β-glucosidase are proved to be key enzymes for the degradation of hemicellulosic biomass. *C. cellulans* was reported to produce hemicellulose-degradation enzymes in culture medium when using corn cob as sole carbon source [16]. However, the genes encoding for GH3 enzymes in *C. cellulans* have not yet been well characterized. In this study, a GH3 gene ccxyl3a from *C. cellulans* was cloned and overexpressed in *E. coli*. Gene ccxyl3a consisted of 2547 bp, encoded a predicted protein of 848 amino acids with theoretical molecular mass of 88.6 kDa and *p*I value of 4.92 (http://web.expasy.org/compute_pi/). Signal sequence of CcXyl3A was analyzed by the SignalP4.1 server (http://www.cbs.dtu.dk/services/SignalP/), the lack of a signal sequence suggests CcXyl3A is an intracellular enzyme. Analysis of CcXyl3A with BlastP and Pfam identified the protein as a member of glycoside hydrolases family 3. Similar to some GH3 enzymes, a protein domain at the carboxyl-end portion named fibronectin type III-like domain was found in CcXyl3A, the function of which is still not well defined.

The multiple amino acid sequence alignment indicated CcXyl3A had 77, 73, 69, and 60% identity to the putative GH3 enzymes from *Cellulomonas carbonis* T26 (Genbank KGM11479), *Isoptericola variabilis* 225 (Genbank AEG45495), *Microbacterium hydrocarbonoxydans* (Genbank WP_045258019), and *Jonesia denitrificans* (Genbank WP_015772335), respectively.

CcXyl3A is the first enzyme which exhibits xylosidase, arabinofuranosidase and glucosidase functionality in *C. cellulans*. Previously, several GH3 bifunctional β-xylosidases/β-glucosidases and multifunctional β-xylosidases/β-glucosidases/α-L-arabinofuranosidases from different sources were characterized [11–13]. We also compared CcXyl3A with these previously published enzymes (Fig. 1). The results indicated that CcXyl3A exhibited low similarities to enzyme Bgxa1 from the rumen contents of a grass/hay-fed dairy cow (AGN51317, 29% identity), RubGX1 from yak rumen metagenome (ADD17009, 30% identity), and BglX-V-Ara from *Caulobacter crescentus* (WP_010920890, 37% identity), which suggested that CcXyl3A is a novel trifunctional β-xylosidase/α-L-arabinofuranosidase/β-glucosidase among family 3 glycoside hydrolases.

3.2. Overexpression, purification, and molecular mass determination of CcXyl3A

To systematically study the function of CcXyl3A, the gene was cloned into the expression vector pET-28a and overexpressed in *E. coli*. After induction with 0.5 mM IPTG at 25 °C for 20 h, the recombinant enzyme CcXyl3A accounted for approximately 50% of the total soluble cellular protein (data not shown). The concentration of total protein was 167.5 mg/l culture and the specific activity of recombinant CcXyl3A was 1.9 U/mg. CcXyl3A was purified by Ni sepharose fastflow column with a final purification of 2.33-fold, a yield of 69.4%, and a specific activity of 4.42 U/mg. The detailed purification results from a representative 200-ml culture are summarized in Table 1. The expressed protein analyzed by SDS-PAGE showed a single band after one-step affinity chromatography, with a molecular mass of approximately 95 kDa (Fig. 2). The molecular weight of CcXyl3A is similar to that of some GH3 bifunctional or multifunctional enzymes, such as Bgxa1 (81.7 kDa), RubGX1 (80 kDa) and BglX-V-Ara (95 kDa) [11–13]. Compared with other non-secretory proteins which are difficult to be purified, recombinant CcXyl3A can be easily purified, involving in only one-step affinity chromatography.

3.3. Characterization of recombinant CcXyl3A

The effect of pH on CcXyl3A activity was examined ranging from 2.0 to 10.5 with *p*NPβXyl as a substrate. Our results showed that the maximum activity was observed at pH 8.5 (Fig. 3a), which was similar to that of the GH3 multifunctional enzyme BgxA1 (optimal pH 8.5 for *p*NPβXyl), but different from BglX-V-Ara, the optimal pH of which was observed at 6.0. CcXyl3A was stable within the pH range from 6.0 to 9.5. The enzyme retained more than 50% of the initial activity after pre-incubation up to 24 h in buffers ranging from pH 6.0–9.5 (Fig. 3b). The alkali-stability of CcXyl3A makes it possible to collaborate with alkaline xylanase, this would be useful in xylan degradation. The effect of temperature on the enzyme activity was investigated at optimal pH, and the maximum activity was observed at 45 °C (Fig. 4a), which was in accordance with other GH3 multifunctional enzymes (50 °C for BglX-V-Ara and RubGX1, 40 °C for BgxA1) [11–13]. The thermostability of CcXyl3A was investigated ranging from 30 to 50 °C at a constant pH of 8.5. As showed in Fig. 4b, approximate 71.7% of its activity remained after treatment at temperature at 35 °C for 1 h, however, the activity significantly decreased above 40 °C, and completely lost at 50 °C after 15-min incubation.

The effects of metal ions and reagents on the enzymatic activity of CcXyl3A were investigated. As shown in Table 2, CcXyl3A activity was strongly inhibited by 5 mM Cu^{2+}, Hg^{2+}, Fe^{3+}, and reagents SDS and DTT. It is noticed that Mn^{2+} was reported to be an enzyme enhancer for some β-xylosidases [20]. However, the activity of CcXyl3A was strongly inhibited by Mn^{2+} at the concentration of 5 mM. CcXyl3A was almost completely inhibited by Cu^{2+}, how-

Table 1
Summary of purification of recombinant CcXyl3A.

Purification step	Volume (ml)	Total Protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme extract	15.0	33.5	63.65	1.90	100	1.0
Ni sepharose fastflow column	12.5	10.0	44.2	4.42	69.4	2.33

[a] Protein was quantified according to the Bradford method using bovine serum albumin (BSA) as standard.
[b] The activity was reported as activity on *p*NPβXyl (40 mM).

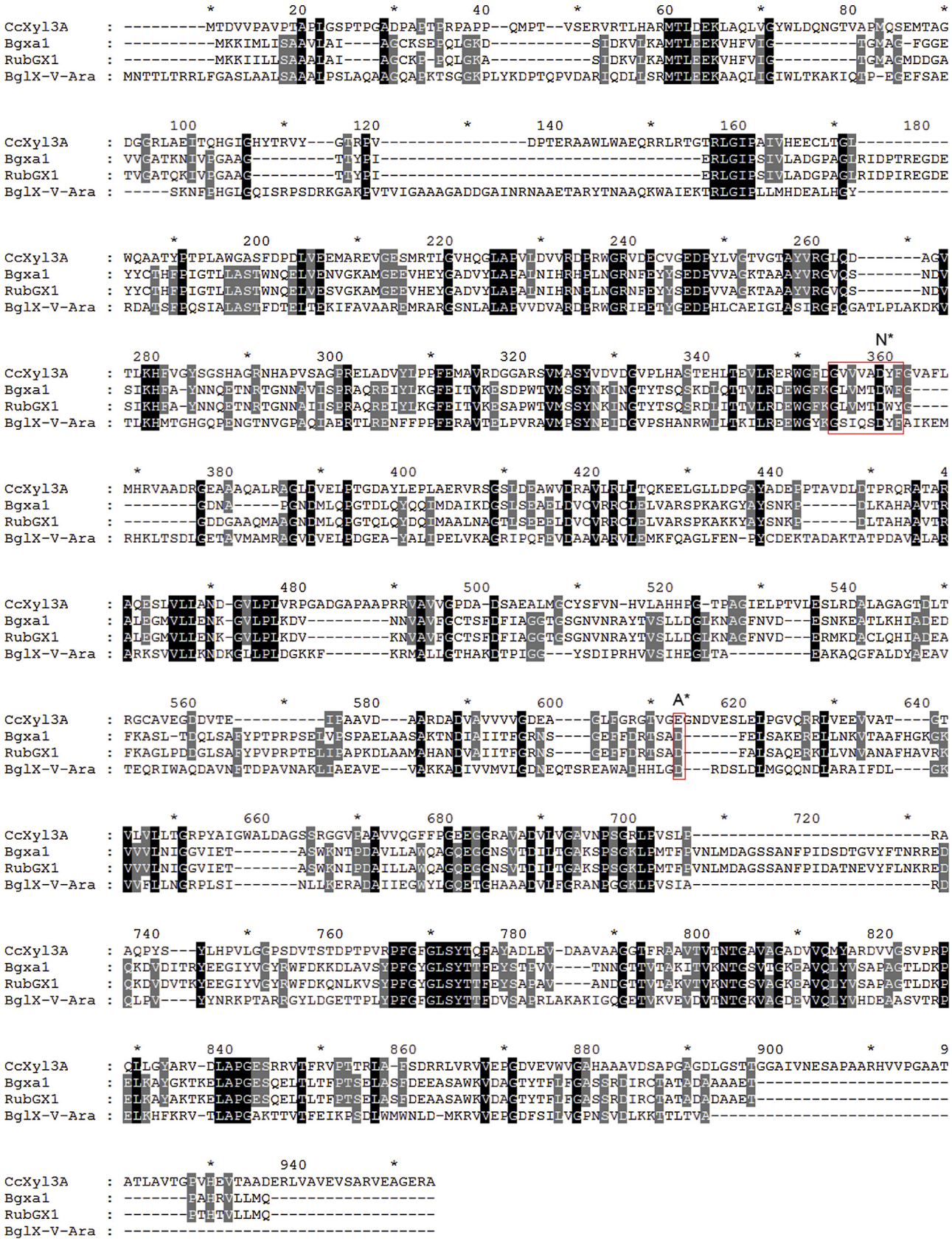

Fig. 1. Multiple amino acid sequence alignment of CcXyl3A with previously reported GH3 multifunctional enzymes Bgxa1 (AGN51317), RubGX1 (ADD17009) and BglX-V-Ara (WP_010920890). Dark shading indicates highly conserved residues in all sequences, lighter shading indicated conserved residues in 3 of the 4 sequences. Alignment was generated using Clustal Omega and formatted with Genedoc. Putative catalytic residues are labelled, in which N* as nucleophile and A* as acid/base catalyst.

ever, Cu^{2+} did not strongly affect the enzyme activity of BgxA1, in which only 17.7% of the activities was lost at the concentration of 5 mM [11]. At the concentration of 50 mM, most metal ions and reagents we tested inhibited the activity of CcXyl3A. However, K^+ and Na^+ significantly activated the enzymatic activity of CcXyl3A. The enzyme activity of CcXyl3A was increased 132.2% and 132.6%, respectively, in the presence of K^+ and Na^+ at concentrations of 50 mM.

Previously, Huy et al. reported the β-xylosidase activity of GH3 bifunctional xylosidase/arabinofuranosidase rPcXyl was inhibited by xylose or arabinose at concentrations 10–100 mM, the inhibition mechanism was deduced to be competitive by preventing recognition between enzyme and substrate [21]. To assess the effect of sugars on CcXyl3A activity, we examined the β-xylosidase activity of CcXyl3A in the presence of various concentrations of glucose, xylose and arabinose (Fig. 5). Similar to rPcXyl, xylose and arabinose exhibited inhibition effect on CcXyl3A activity at concentrations of 10 mM or above. However, glucose exhibited no significant effect on the β-xylosidase activity of CcXyl3A, which is different from that of rPcXyl. At the concentration of 100 mM, the enzyme activity was 50.9 ± 1.2%, 94.7 ± 2.1% and 48.4 ± 1.6% with the presence of xylose, glucose or arabinose, respectively.

3.4. Substrate specificity of CcXyl3A on different substrates

The specificity of CcXyl3A on various substrates was investigated. Using *p*-NP compounds as substrates, the hydrolysis order was *p*NPβXyl » *p*NPαAraf > *p*NPβGlc. Other *p*NP-β-glycosides and *p*NP-α-glycosides we tested were not substrates for CcXyl3A (Table 3). This result suggested that CcXyl3A was a multifunctional β-xylosidase/α-L-arabinofuranosidase/β-glucosidase. Using *p*NPβXyl as a substrate, the kinetic parameters of purified recombinant CcXyl3A were determined. The K_m, V_{max}, and k_{cat} values were 4.0 ± 0.1 mM, 10.1 ± 0.4 μmol/min/mg, and 15.4 ± 0.1 s^{-1}, respectively. The catalytic efficiency k_{cat}/K_m was determined to be 3.85 ± 0.1 mM^{-1} s^{-1}. In previous reports, two multifunctional β-xylosidase/β-glucosidase/α-L-arabinofuranosidase BgxA1 and BglX-V-Ara belonging to GH3 were characterized [11,13]. Both BgxA1 and BglX-V-Ara were reported to show highest activity on *p*NPβGlc, followed by *p*NPβXyl and finally *p*NPαAraf, which was different from that of CcXyl3A. The catalysis efficiency of CcXyl3A (3.85 ± 0.1 mM^{-1} s^{-1} for *p*NPβXyl) was higher than BgxA1 (3.0 ± 0.3 mM^{-1} s^{-1} for *p*NPβXyl) and BglX-V-Ara (0.14 mM^{-1} s^{-1} for *p*NPβXyl). In our previous study, a novel GH1 β-glucosidase gene *ccbgl1a* from *C. cellulans* was cloned and overexpressed in *E. coli* [18]. Enzymatic analysis showed that CcBgl1A exhibited highest activity on *p*NPβGlc, followed by *p*NPβXyl and finally *p*NPαAraf, the hydrolysis order was different from that of CcXyl3A. The catalysis efficiency of CcBgl1A for *p*NPβXyl was 0.19 ± 0.02 mM^{-1} s^{-1}, which was lower than that of CcXyl3A.

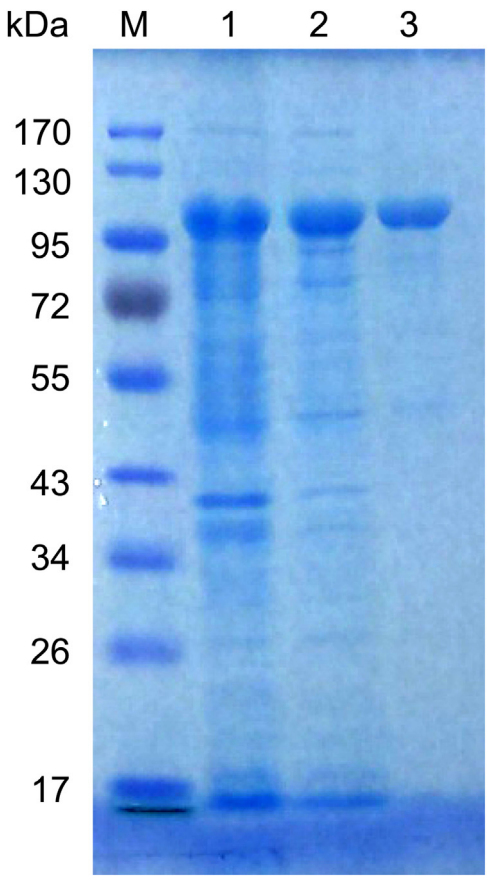

Fig. 2. SDS-PAGE analysis of recombinant CcXyl3A on 10% resolving gel. Lane 1, culture lysate of *E. coli* BL21-pET28a-ccxyl3a after IPTG induction; lane 2, supernatant of the culture lysate of *E. coli* BL21-pET28a-ccxyl3a after IPTG induction; lane 3, CcXyl3A purified from Ni sepharose fastflow column; M, molecular weight marker (PageRuler Prestained Protein Ladder, Thermo Scientific).

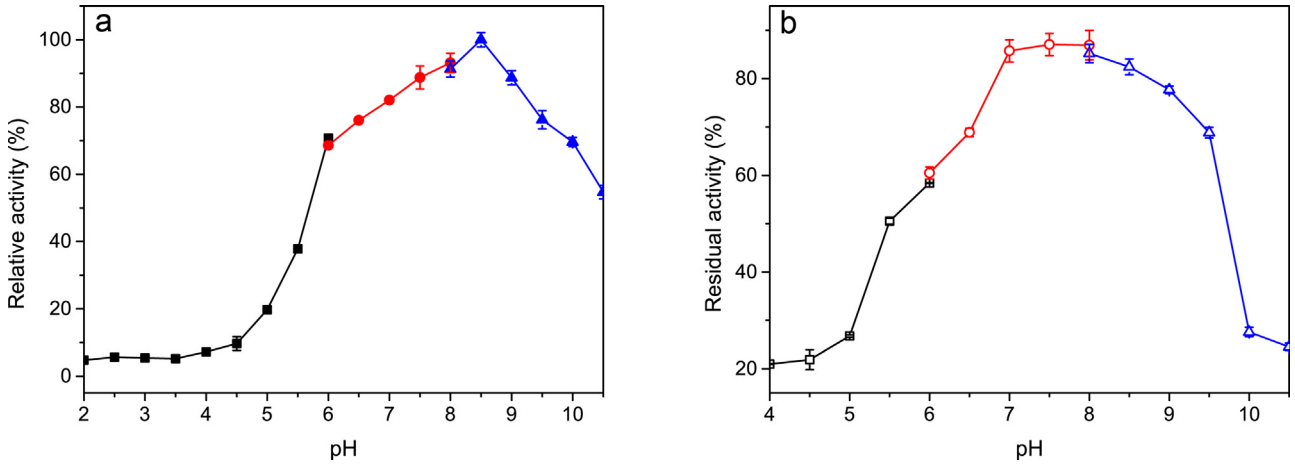

Fig. 3. Effect of pH on activity (a) and stability (b) of CcXyl3A using *p*NPβXyl as substrate. The optimal pH of CcXyl3A was determined ranging from pH 2.0–10.5 using following buffers: sodium acetate buffer, pH 2.0–6.0; Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0–8.0; Glycine-NaOH buffer, pH 8.0–10.5. The maximum activity obtained was defined as 100%. The pH stability of CcXyl3A was determined by pre-incubating CcXyl3A in different pH for 24 h at 4 °C, then determining the percentage of residual activity under standard assay conditions. The activity of CcXyl3A without pre-incubating was defined as 100%. Results are presented as means ± standard deviations (n = 3).

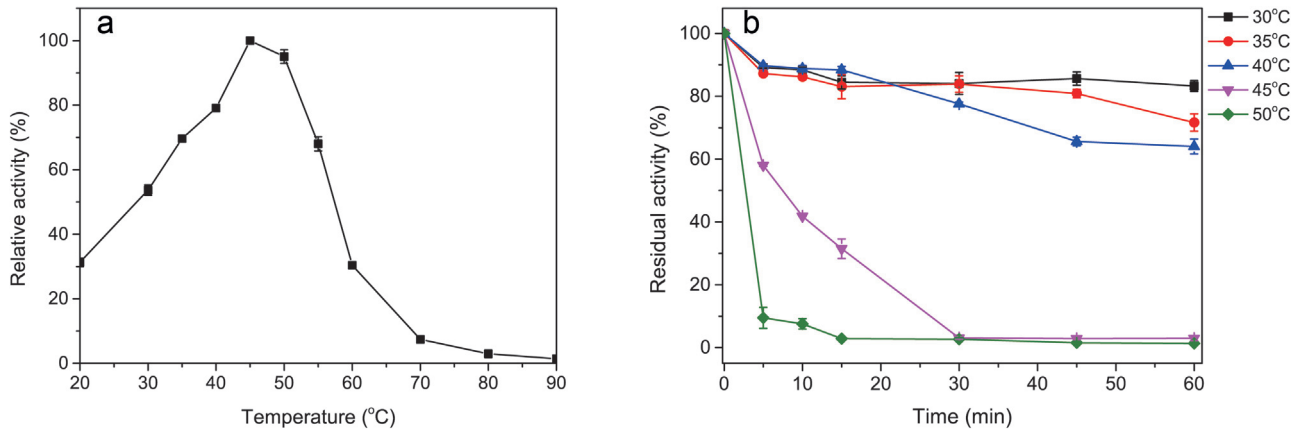

Fig. 4. Effect of temperature on activity (a) and stability (b) of CcXyl3A using *p*NPβXyl as substrate. The optimal temperature was determined at different temperatures ranging from 20 to 90 °C. The maximum activity obtained was defined as 100% activity. Thermal stability was determined by incubating the enzyme for 1 h at different temperatures. The activity of the enzyme before incubation was defined as 100%. Results are presented as means ± standard deviations (n = 3).

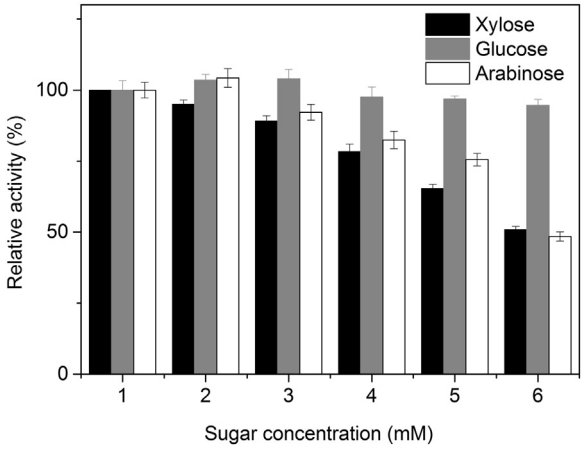

Fig. 5. Effect of sugar concentration on the β-xylosidase activity of recombinant CcXyl3A. CcXyl3A was pre-incubated with respective concentrations (5, 10, 25, 50 and 100 mM) of each sugar in 100 μl of 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 8.0) for 1 h at 37 °C. Then reactions were carried out by adding *p*NPβXyl to a final concentration of 40 mM for 10 min. The residual activity in the absence of sugar was defined as 100%. Results are presented as means ± standard deviations (n = 3).

Table 3
Relative activity of CcXyl3A on different substrates.[a]

Substrate[b]	Relative activity (%)[c]	Substrate	Relative activity (%)
*p*NPβGlc	9.6 ± 0.2	xylobiose	100 ± 2.5
*p*NPβXyl	100 ± 0.0	xylotriose	100 ± 2.4
*p*NPβMan	0	cellobiose	0
*p*NPβGal	1.4 ± 0.1	cellotriose	0
*p*NPαAra*f*	14.4 ± 2.1	sophorose	19.9 ± 2.0
*p*NPαGlc	1.5 ± 0.3	laminaribiose	17.5 ± 0.8
*p*NPαGal	0.3 ± 0.1	laminaritriose	14.4 ± 1.2
*o*NPβGlc	6.4 ± 0.1	gentiobiose	0
*p*NPαMan	0.4 ± 0.1		

[a] Reactions were performed with 40 mM substrate, pH 8.0, at 37 °C for 10 min.
[b] Absorption caused by released *p*-nitrophenol was measured at 405 nm. The relative activity on *p*NPβXyl was defined as 100%.
[c] The data are reported as means ± standard errors from the mean for three independent experiments.

Cello- and xylo-oligosaccharides are common moieties found in plant cell walls, which could be used in biomass conversion for biofuel production. Therefore, the hydrolytic activity of CcXyl3A on cello- and xylo-oligosaccharides were determined. The results showed that CcXyl3A had no activity on cello-oligosaccharides

Table 2
Effects of metal ions and chemical agents on the activity of CcXyl3A.

Metal ions or reagents	Relative activity (%)[a]	
	5 mM	50 mM
KCl	107.7 ± 2.2	132.2 ± 3.7
$MgCl_2$	108.0 ± 4.7	70.0 ± 3.8
$CaCl_2$	95.0 ± 3.3	20.3 ± 0.5
NaCl	106.3 ± 3.8	132.6 ± 2.4
$CuCl_2$	2.5 ± 0.1	0.4 ± 0.0
$FeCl_3$	21.4 ± 1.9	–[b]
$HgCl_2$	1.3 ± 0.0	–
$BaCl_2$	78.0 ± 2.8	31.8 ± 2.8
$MnCl_2$	59.5 ± 4.4	16.0 ± 0.8
DTT	56.2 ± 1.5	49.2 ± 1.1
EDTA	72.6 ± 1.1	1.2 ± 0.1
SDS	6.3 ± 0.3	–

[a] The activity assayed in the absence of cations or reagents was taken as 100%. Results are presented as means ± standard deviations (n = 3).
[b] Not detected.

(Table 3), but release xylose from xylobiose and xylotriose (Table 3 and Fig. 6a). When using a xylo-oligosaccharides mixture as the substrate, the enzyme showed considerable hydrolytic activity. As shown in Fig. 6b, the xylo-oligosaccharides mainly containing xylobiose, xylotetraose and xylohexaose were completely converted after 10 min incubation, xylose was the sole product.

3.5. Synergistic effect between CcXyl3A and xylanase in degradation of xylan

β-Xylosidase catalyzes the breakdown of β-1,4-linked xylooligosaccharides, which are produced from degradation of xylan by xylanases. To examine the ability of CcXyl3A in the saccharification of lignocellulosic xylan, the enzyme was incubated with beechwood xylan on its own and in combination with a commercial available xylanase. During the reaction, the percentage of residual activity of CcXyl3A (pH 8.0 and 37°C) was measured with pNPβXyl as a substrate. The result showed that approximate 65% of initial hydrolytic activity was reserved after 8 h incubation. During the hydrolysis process, the amount of released reducing sugars was determined (Fig. 7a), the hydrolytic products were analyzed by TLC (Fig. 7b). Although CcXyl3A completely hydrolyzed the xylooligosaccharides to xylose with good activity, it showed very poor hydrolytic activity on beechwood xylan. After incubated with CcXyl3A for 8 h, only a few amount of xylose was released from beechwood xylan. The beechwood xylan was partially degraded after incubated with xylanase for

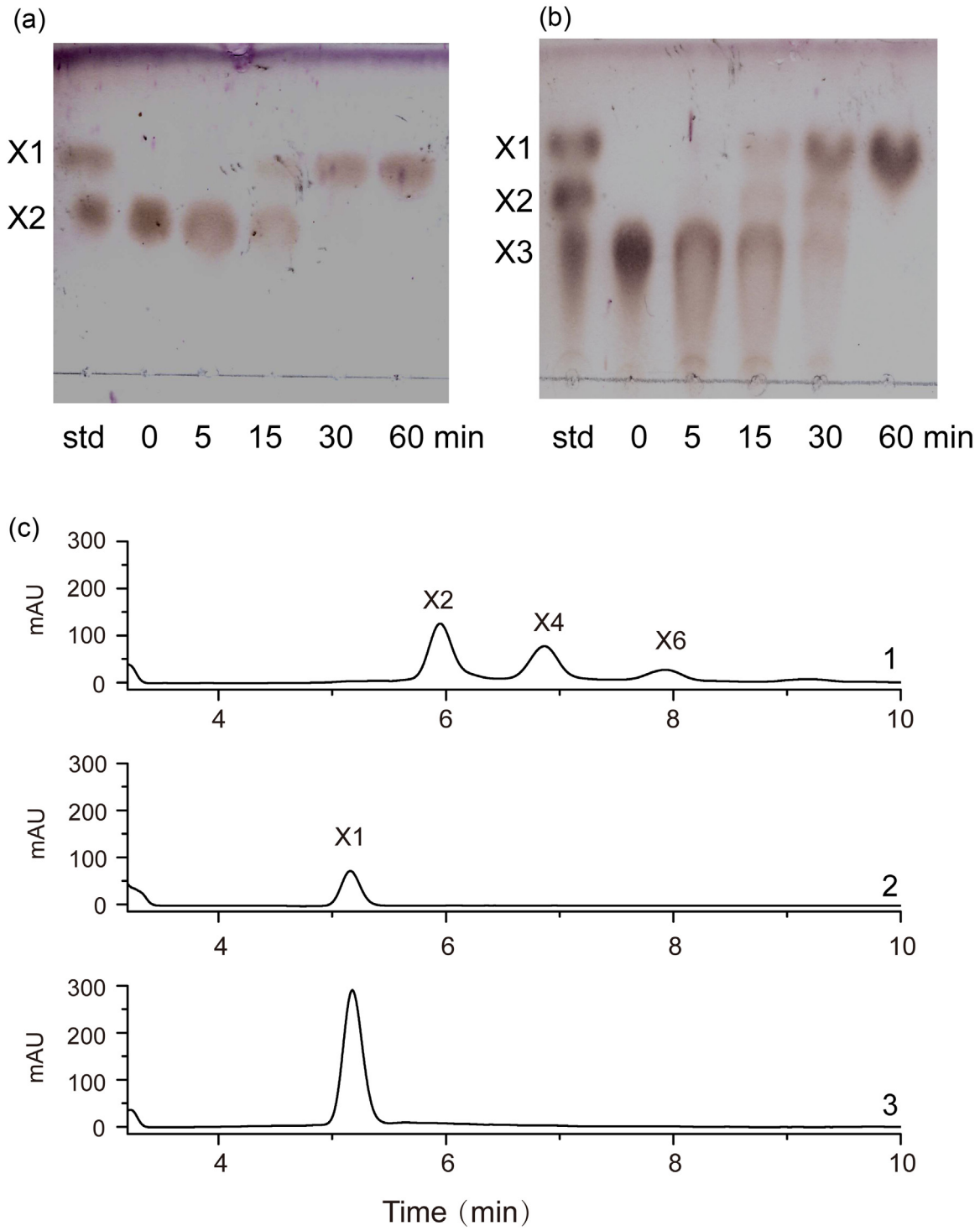

Fig. 6. TLC and HPLC analysis of the hydrolysis products of xylo-oligosaccharides by CcXyl3A. (a) TLC analysis of the hydrolysis process of xylobiose by CcXyl3A. Std: standards xylose (X1) and xylobiose (X2). (b) TLC analysis of the hydrolysis process of xylotriose by CcXyl3A. Std: standards xylose (X1), xylobiose (X2) and xylotriose (X3). (c) HPLC analysis of the hydrolysis process of xylooligosaccharides by CcXyl3A. Curve 1, transformed substrate containing xylobiose (X2), xylotetraose (X4) and xylohexaose (X6); curve 2, standard xylose (X1); curve 3, hydrolysis product.

8 h, with xylobiose and xylooligosaccharides as the main products. The combination of CcXyl3A and xylanase in the reaction mixture significantly improved the liberation of the reducing sugars from beechwood xylan. At the ratio of 1:1 (CcXyl3A/xylanase), the amount of released reducing sugars was 1.7-fold relative to that of endoxylanase alone. After 8-h incubation, most of the xylobiose and xylooligosaccharides was hydrolyzed and a significant amount of xylose was accumulated. These results suggested that CcXyl3A functioned as a β-xylosidase in saccharification of xylans, which promoted the xylan degradation by the synergistic action with xylanase.

4. Conclusions

In conclusion, we have identified, cloned and characterized the β-xylosidase/α-arabinosidase/β-glucosidase CcXyl3A from

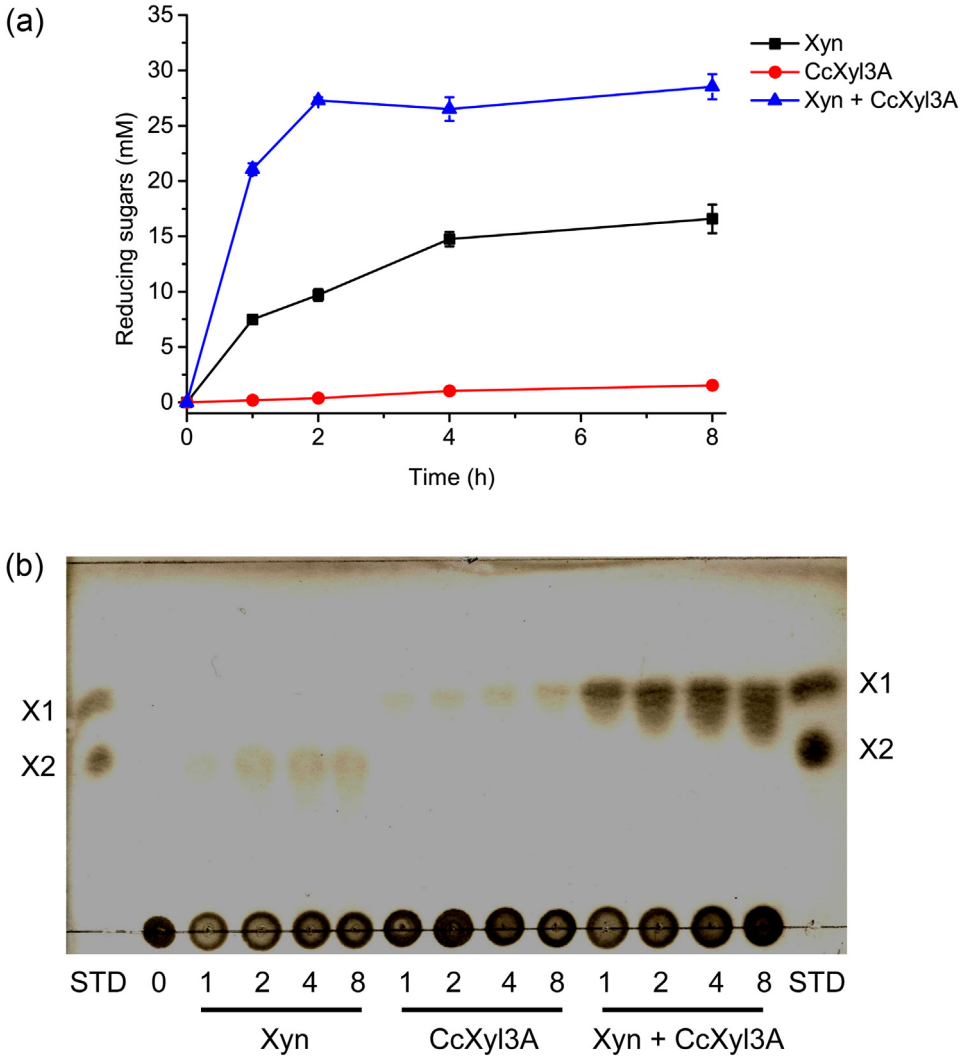

Fig. 7. Time-course analysis of xylan hydrolysis by the combination of recombinant CcXyl3A and *T. lanuginosus* xylanase (Xyn). (a) The amount of released reducing sugars during xylan hydrolysis were determined by DNS reagent; (b) The hydrolysis of xylan was analyzed by TLC method. STD: standards xylose and xylobiose, lane 0, 1, 2, 4, 8 means different reaction times.

C. cellulans sp. 21, which was the first multifunctional GH3 enzyme reported in this strain. CcXyl3A can effectively hydrolyze xylooligosaccharides to release xylose and exhibited a synergistic effect with endoxylanase in xylan degradation. The discovery and characterization of the multifunctional β-xylosidase/α-arabinosidase/β-glucosidase CcXyl3A would be helpful for the application of *C. cellulans* in the saccharification of lignocellulosic material.

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