

# Biochemical and kinetic characterization of GH43 $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase and GH30 $\alpha$ -L-arabinofuranosidase/ $\beta$ -D-xylosidase from rumen metagenome

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**Abstract** The present study focuses on characterization of two hemicellulases, RuXyn1 and RuXyn2, from rumen bacterial metagenome and their capabilities for degradation of xylans. Glycosyl hydrolase (GH) family 43  $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase RuXyn1 can hydrolyze *p*-nitrophenyl- $\beta$ -D-xylopyranoside (pNPX), *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (pNPA), and xylo-oligosaccharide substrates, while GH30 1,5- $\alpha$ -L-arabinofuranosidase/ $\beta$ -D-xylosidase RuXyn2, the first  $\alpha$ -L-arabinofuranosidase assigned to this GH family, shows activities towards 1,5- $\alpha$ -L-arabinobiose and pNPX substrates but no activity for pNPA. Kinetic analysis for aryl-glycosides revealed that RuXyn2 had higher catalytic efficiency than RuXyn1 toward pNPX substrate. RuXyn1 shows high synergism with endoxylanase, elevating by 73% the reducing sugars released from brichwood xylans, and converted most intermediate xylo-oligosaccharide hydrolysate into xylose. The high xylose conversion capability of RuXyn1 suggests it has potential applications in enzymatic production of xylose and improvement of hemicellulose saccharification for production of biofuels. RuXyn2 shows no obviously synergistic effect in the endoxylanase-coupled assay for enzymatic saccharification of xylan. Further cosmid DNA sequencing revealed a neighboring putative GH43  $\alpha$ -L-arabinofuranosidase RuAra1 and two putative GH3  $\beta$ -xylosidase/arabinosidases, RuXyn3 and RuXyn5, downstream of RuXyn2, indicating that this

hemicellulase gene cluster may be responsible for production of end-product, xylose and arabinose, from hemicellulose biomass.

**Keywords** Metagenome · Xylosidase ·  $\beta$ -D-Xylosidase/ $\alpha$ -L-arabinofuranosidase · Xylan · Synergistic

## Introduction

Xylans, the major heteropolysaccharides of plant cell walls and especially abundant in secondary cell walls, consist of a linear  $\beta$ -D-(1,4)-linked xylopyranoside backbone that is variously substituted with (1,2)- or (1,3)-linked  $\alpha$ -L-arabinofuranosyl,  $\alpha$ -D-glucuronic acid, O-2- and/or O-3-linked acetate groups, and other substituents depending on the biomass source [27]. Complete degradation of hemicellulose requires concerted action of several hemicellulases, including backbone depolymerizing enzymes [endoxylanases (EC 3.2.1.8),  $\beta$ -xylosidases (EC 3.2.1.37), and glucuronoxylan hydrolases (EC 3.2.1.136)] and enzymes capable of hydrolyzing substitutions on the xylan backbone [ $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterases (EC 3.1.1.72), feruloyl esterases (EC 3.1.1.73), and  $\alpha$ -glucuronidases (EC 3.2.1.39)] [2, 5, 21].

$\beta$ -Xylosidases are exo-type glycosidases that successively remove  $\beta$ -xylosyl residues from nonreducing termini of xylobiose and xylo-oligosaccharides, which is essential for relieving the end-product inhibition of endoxylanase during complete hydrolysis of xylan.  $\beta$ -Xylosidases are currently classified into seven different families (GH family 3, 28, 30, 39, 43, 52, and 54), and their reaction proceeds via either inversion (GH family 43) or retention (GH family 3, 28, 30, 39, 52, and 54) mechanism of stereochemical configuration at the anomeric carbon [8, 21].

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Based on their overall similar folds, the GH family 43 is classified as part of the GH-F clan, together with GH family 62, which share a five-bladed  $\beta$ -propeller catalytic domain [8]. GH family 43  $\beta$ -xylosidases exhibit no transglycosylation activity even at high substrate concentrations, and the most catalytically efficient xylosidases fall into this family [11]. D-Xylopyranose and L-arabinofuranose are spatially similar, such that the glycosidic bonds and hydroxyl groups can be overlaid, thereby rationalizing the bifunctional  $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase activity of most  $\beta$ -xylosidases [11, 14, 17]. Arabinofuranosidases remove the arabinofuranosyl units from the xylan backbone, thus facilitating endoxylanase hydrolysis, and the bifunctional capacity of xylosidase/arabinofuranosidase allows for more complete hydrolysis of plant cell matter [26]. The family 30 glycoside hydrolases are clan GH-A enzymes utilizing the retaining mechanism. Enzyme activities currently assigned to GH family 30 include glucosylceramidase (EC 3.2.1.45),  $\beta$ -glucosidase (EC 3.2.1.21),  $\beta$ -xylosidase (EC 3.2.1.37), and endo- $\beta$ -1,6-glucanase (EC 3.2.1.75) [23]. According to the CAZy database (<http://www.cazy.org>), three putative  $\beta$ -xylosidases were assigned to this GH family. Only a single publication deals with  $\beta$ -xylosidase from *Bifidobacterium breve*, which hydrolyzes a single xylose moiety from the saponin ginsenoside Ra1 derived from ginseng root [22].

In this study, we constructed a metagenome library from Chinese yak rumen samples to screen for  $\beta$ -xylosidase-encoding genes. A GH43  $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase gene (*RuXyn1*) and GH30 1,5- $\alpha$ -L-arabinofuranosidase/ $\beta$ -D-xylosidase gene (*RuXyn2*) were identified after functional screening and sequencing. We also investigated the enzymatic properties of these two hemicellulases and their potential applications in saccharification of hemicellulose.

## Materials and methods

### Construction of metagenome library

Rumen samples were collected from Chinese yaks (*Bos grunniens*) of the Qinghai–Tibet Plateau at Xining City, Qinghai Province. Community DNA of the total microorganism was extracted as previously described by An et al. [1]. DNA fragments in the size range 30–50 kb were blunted by an End Repair kit (Epicentre, USA), and then ligated into cosmid vector pWEB. Ligation products were packaged with MaxPlax lambda packaging extract kit (Epicentre) and transfected *Escherichia coli* EPI100. Transformants formed were replicated in 96-well plates and stored at  $-80^{\circ}\text{C}$ .

### Library screening for $\beta$ -xylosidases

Metagenome libraries were cultured in 96-well plate containing 200  $\mu\text{l}$  lysogeny broth (LB) supplemented with ampicillin (100  $\mu\text{g}/\text{ml}$ ) at  $37^{\circ}\text{C}$  overnight. The cells were harvested by centrifugation, resuspended in 20  $\mu\text{l}$  lysis buffers (50 mM phosphate/citrate buffer, pH 5.0), and ruptured by the freezing and thawing method [4]. For screening of  $\beta$ -xylosidase, 20  $\mu\text{l}$  0.1 mM 4-methylumbelliferyl- $\beta$ -D-xylopyranoside (MuX) was added to each well, and the plates were then incubated at  $37^{\circ}\text{C}$  for 30 min. The reactions were stopped by adding 40  $\mu\text{l}$  1.0 M  $\text{Na}_2\text{CO}_3$  per well, and the plates were observed under ultraviolet excitation. Clones emitting fluorescence were selected and sequenced.

### Bioinformatic analysis

Homology searching was carried out in the GenBank database, and modular architecture was analyzed using SMART on the Swiss ExPasy server (<http://www.expasy.ch>). The phylogenetic analysis was conducted using the software DNAMAN (Lynnon Biosoft, Canada) based on the neighbor-joining tree method [20].

### Expression and purification of recombinant enzymes

The DNA fragment of *RuXyn1* was amplified by polymerase chain reaction (PCR) using primers 5'-GAATTC GCTGATAAAGTTAAGAAACGCT-3' and 5'-CTCGAG CTCATCCATGCCTTCGATGGTGCGG-3', followed by digestion with *EcoRI* and *XhoI*, and then cloned into plasmid pET21a. The DNA fragment of *RuXyn2* was amplified using primers 5'-GGATCCATGAGGAATTGG ATGATATTACTCG-3' and 5'-CTCGAGAAGCACCAG CGTATTGATCGAGCCG-3', and then cloned into pET21a using *BamHI* and *XhoI*. The constructed plasmids were transformed into *E. coli* BL21 (DE3) to express the recombinant enzymes. Protein purification was conducted by utilizing Ni-NTA resin (Invitrogen, USA) as described by You et al. [28], and elution fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [13]. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard [3].

### Enzyme characterization

The  $\beta$ -xylosidase assay was carried out in 100  $\mu\text{l}$  reaction mixture containing 50 mM phosphate/citrate buffer pH 7.0 and 5 mM of the substrate *p*-nitrophenyl- $\beta$ -D-xylopyranoside (pNPX). After preincubation of the mixture at  $40^{\circ}\text{C}$  for 5 min, 2  $\mu\text{l}$  purified enzyme was added, and then

reaction proceeded at 40°C for 5 min and was stopped by adding 100  $\mu$ l 1 M Na<sub>2</sub>CO<sub>3</sub>. The released *p*-nitrophenol was immediately measured at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme (mg) that releases 1  $\mu$ M *p*-nitrophenol per minute.

The pH optimum was determined in 50 mM phosphate/citrate buffer covering a pH range from 3.5 to 9.0 at 40°C. The temperature optimum was measured from 10°C to 60°C for 10 min of incubation in 50 mM phosphate/citrate buffer. Thermostabilities were examined by measuring the residual activity after the enzyme was treated at the indicated temperature in 50 mM phosphate/citrate buffer. Denaturation half-lives ( $t_{1/2}$ ) were then calculated using the equation  $t_{1/2} = \ln 2/k_{\text{inact}}$ , where  $k_{\text{inact}}$  is the inactivation rate constant obtained from the slope of log(residual activity/initial activity) against time [25].

Michaelis–Menten constants for the substrates pNPX and *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (pNPA) were determined according to the Lineweaver–Burk method [15]. The reactions were carried out in 50 mM phosphate/citrate buffer at optimal conditions for 5 min, with increasing substrate concentration from 0.75 to 10 mM.

The inhibition constants were measured at concentrations of 0, 10, 100, 250, and 500 mM, with 1–10 mM pNPX or pNPA in 50 mM phosphate/citrate.  $K_i$  values were calculated from plots of slopes/intercepts of Lineweaver–Burk plots against inhibitor concentrations [7].

#### Hydrolysis of hemicellulosic substrates

Hydrolysis of xylo-oligosaccharide or arabinobiose was detected by utilizing thin-layer chromatography (TLC). For measuring the hydrolysis of xylo-oligosaccharides (xylobiose, xylotriose; Wako, Japan) and arabinobiose (Megazyme, Ireland), 0.8 mg substrate (1% final concentration) was prepared in 50 mM phosphate/citrate buffer [pH 7.0 (RuXyn1) or 5.5 (RuXyn2)], and reactions were initiated by addition of 0.1 U RuXyn1 or RuXyn2. Following incubation at 37°C for 4 h, 1.5  $\mu$ l hydrolysate was spotted on silica gel 60 F254 plate (Merck, Germany) and developed in butanol–ethanol–H<sub>2</sub>O (5:3:2, v/v/v) twice, then visualized by spraying the plates with a mixture of ethanol (95% v/v) and sulfuric acid (5% v/v), and then the plates were heated at 100°C for 10 min.

Alkali-extractable xylans from rice straw were prepared as described previously [19]. Recombinant endoxylanase xynR8 (0.4 U) [16] alone or with 1 U RuXyn1 was incubated with 1 ml 2% (w/v) birchwood xylan (Sigma) or rice straw xylan in 50 mM phosphate/citrate buffer at pH 6.5 and 40°C for 3 h. Hydrolysis of xylans by endoxylanase coupled with RuXyn2 was conducted under the same substrate concentrations and units of enzymes unless the reaction condition was set at pH 5.5 and 40°C. Hydrolysate

(50  $\mu$ l) was taken every 30 min to determine the reducing sugars using the Nelson–Somogyi assay [18]. After 16 h of digestion, oligosaccharide composition in the reaction mixture was profiled by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a Dionex system (Dionex, USA) equipped with a 250  $\times$  4.5 mm CarboPac PA-1 column (Dionex). The column was equilibrated with 100 mM NaOH, and elution was performed with a linear gradient of 0–250 mM sodium acetate in 100 mM NaOH over 30 min with flow rate of 1 ml/min.

#### Nucleotide sequence accession

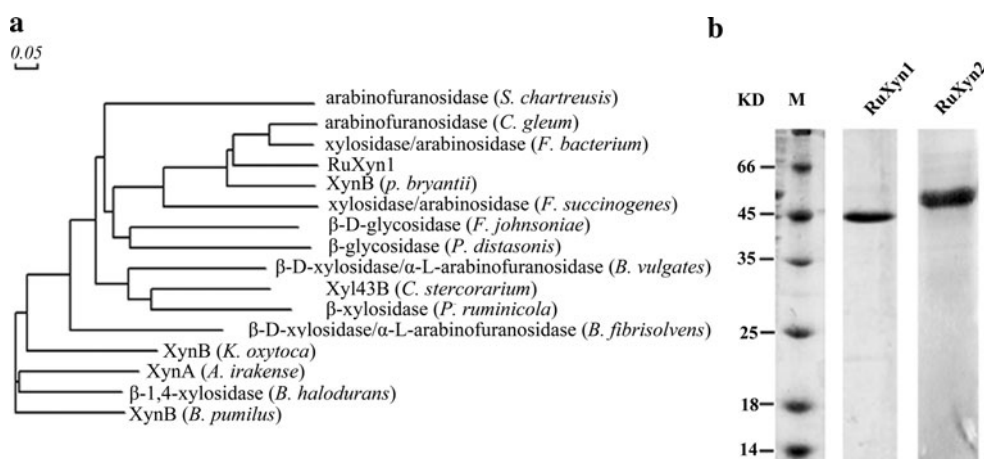
The nucleotide sequences coding for RuXyn1 and RuXyn2 were deposited in the National Center of Biotechnology Information (NCBI) GenBank database under accession nos. GU573895 and GU573896, respectively.

## Results

#### Cloning and expression of RuXyn1 and RuXyn2

The metagenomic cosmid library of yak ruminal microorganisms was screened for  $\beta$ -xylosidase activity by utilizing the fluorescent substrate MuX. After separately subcloning and DNA sequencing of positive clones, two xylosidase coding genes, *RuXyn1* and *RuXyn2*, were obtained. *RuXyn1* is 999 bp in length and shows 72% similarity to a GH family 43  $\beta$ -xylosidase/ $\alpha$ -L-arabinofuranosidase from *Xanthomonas campestris* (AM920689). The amino acid sequence of RuXyn1 shows homology to GH family 43 members ranging from 27% to 67%, and exhibits the highest homology to  $\beta$ -xylosidase/ $\alpha$ -L-arabinofuranosidase xylB2 (ABR37770) from *Bacteroides vulgatus*. SMART analysis revealed that RuXyn1 comprises a single GH43 domain. Phylogenetic analysis showed that RuXyn1 is closely related to the ortholog from the marine bacterium *Flavobacteriaceae bacterium*, and clusters together with other GH family 43 members from ruminal bacteria including *Prevotella bryantii* and *Fibrobacter succinogenes* (Fig. 1a).

*RuXyn2* has a coding sequence of 1,392 bp in length and no homologous nucleotide sequence in the GenBank database. However, amino acid sequence of RuXyn2 exhibits 52% homology to GH family 30 glucosylceramidase (EEH66246.1) from *Actinomyces urogenitalis* and 50% homology to  $\beta$ -glycosidase (ZP\_05284511) from *Bacteroides* sp. Domain analysis revealed that RuXyn2 contained a 16-residual signal peptide at the N-terminus and a unique Glyco\_hydro\_30 domain at amino acid residues 12–463.



**Fig. 1** Characterization of RuXyn1 and RuXyn2. **a** Molecular phylogenetic tree for the amino acid sequences of β-xylosidase belonging to glycoside hydrolase family 43. The scale bar corresponds to genetic distance of 0.05 substitutions per position. Accession numbers of proteins: BAA90772.1, exo-α-1,5-L-arabinofuranosidase from *Streptomyces chartreusis*; ZP\_03852842.1, α-L-arabinofuranosidase from *Chryseobacterium gleum*; ACU08175.1, xylosidase/arabinosidase from *Flavobacteriaceae bacterium*; CAA89208.1, β-xylosidase (XynB) from *Prevotella bryantii*; ACX75586.1, β-xylosidase/α-L-arabinofuranosidase from *Fibrobacter succinogenes*; ABQ05058.1, candidate β-D-glycosidase/α-L-glycosidase from *Flavobacterium johnsoniae* 3519-10; ABR44771.1, candidate β-glycosidase

from *Parabacteroides distasonis*; ABR39655.1, β-xylosidase/α-L-arabinofuranosidase from *Bacteroides vulgatus*; BAC87941.1, β-xylosidase (Xyl43B) from *Clostridium stercorarium*; BAA78558.1, β-xylosidase from *Prevotella ruminicola*; AAA63610.1, β-xylosidase/α-L-arabinofuranosidase from *Butyrivibrio fibrisolvens*; AAQ62864.1, β-xylosidase B (XynB) from *Klebsiella oxytoca*; AAF66622.1, β-xylosidase/α-L-arabinofuranosidase (XynA) from *Azospirillum irakense*; BAB07402.1, β-1,4-xylosidase from *Bacillus halodurans* C-125; CAA29235.1, β-xylosidase (XynB) from *Bacillus pumilus* IPO. **b** SDS-PAGE of recombinant RuXyn1 and RuXyn2 purified from extraction of engineered *E. coli* strain. M protein marker, lane 1 recombinant RuXyn1, lane 2 recombinant RuXyn2

Recombinant RuXyn1 and RuXyn2 fused to 6× His at the C-terminus were expressed in *E. coli* and purified using Ni-NTA resin. Both of the purified recombinant enzymes formed a single band with purity of at least 95%, and the indicated molecular masses of 42 and 50 kD were consistent with the calculated molecular weights of RuXyn1 and RuXyn2, respectively (Fig. 1b).

#### Enzymatic characterizations

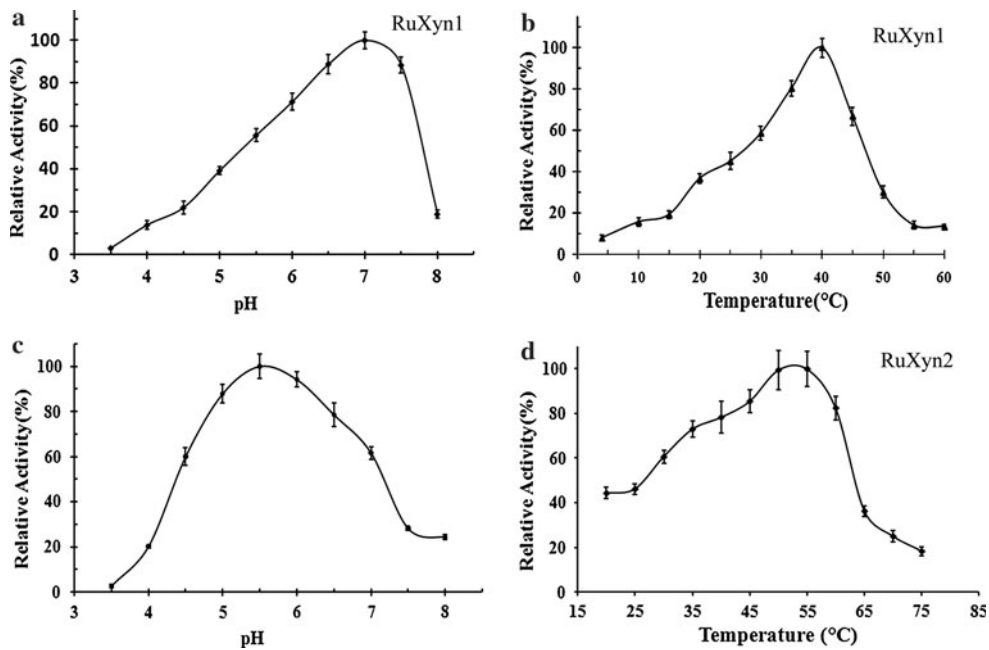
To determine the pH optima of recombinant RuXyn1 and RuXyn2, 50 mM phosphate/citrate buffers ranging from pH 3.5 to 8.0 at intervals of 0.5 were applied. RuXyn1 exhibited the highest activity for pNPX substrate between pH 6.5 and 7.5 and retained over 72% of optimal activity at pH 6.0, at least 40% at pH 5.0, and more than 10% even at pH 4.0 (Fig. 2a). However, RuXyn1 activity decreased dramatically at pH 8.0, and only 20% of its activity remained. Therefore, RuXyn1 could appropriately be classified as a neutral hemicellulase active over a broad pH range in acidic buffer. The maximum enzyme activity of recombinant RuXyn2 was observed at pH 5.5 (Fig. 2c). Over the pH range 4.5–7.0, 60% of RuXyn2 activity for pNPX substrate remained. The optimum temperature of RuXyn1 was 40°C (Fig. 2b), while the thermal optimum of the recombinant RuXyn2 was nearly 10°C higher than that of RuXyn1 at pH 5.5 (Fig. 2d).

Thermal stability assays revealed that RuXyn1 was relatively stable at 40°C, but lost 76% of its activity after 10 min at 45°C, and 97% of activity at 50°C (Fig. 3a). The irreversible thermal denaturation half-time ( $t_{1/2}$ ) of RuXyn1 was calculated to be 112 min at 40°C, and 5.8 min at 45°C. RuXyn2 was stable at 50°C, and its  $t_{1/2}$  was 87.7 min at 50°C, but it lost activity drastically at temperatures above 60°C (Fig. 3b).

#### Substrate specificity and kinetic studies

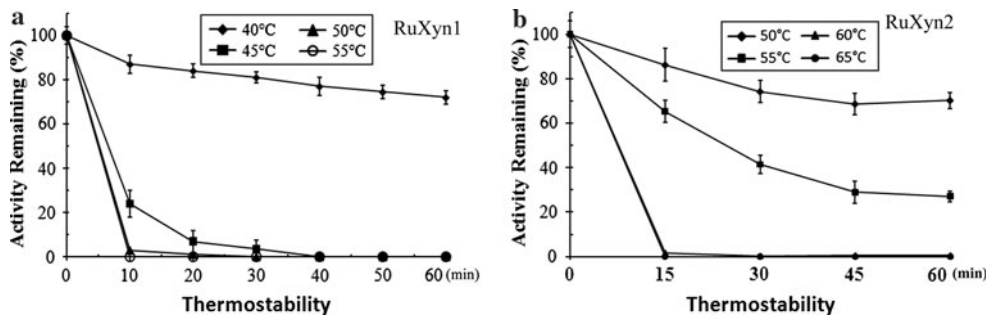
Both RuXyn1 and RuXyn2 can efficiently hydrolyze pNPX. RuXyn1 also exhibited significant activity on pNPA with 14.2 IU/mg, while RuXyn2 did not. Neither RuXyn1 nor RuXyn2 displayed activity on *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, rice straw xylan or birchwood xylan. The  $K_m$  value of RuXyn1 was 3.43 mM for pNPX and 2.23 mM for pNPA at 40°C, demonstrating a definite preference of RuXyn1 for pNPA (Table 1). However, the turnover number  $k_{cat}$  for pNPX was significantly higher than that of pNPA, and the catalytic efficiency constant  $k_{cat}/K_m$  for pNPX was 3.24-fold that of pNPA. RuXyn2 has similar substrate affinity ( $K_m$  value 3.42 mM) towards pNPX as RuXyn1, but the values  $k_{cat}$  and  $k_{cat}/K_m$  were 1.43-fold that of RuXyn1, indicating that RuXyn2 had higher hydrolysis efficiency.

The  $K_i$  calculated for RuXyn1 was 76.0 mM ( $R^2 = 0.99$ ) for xylose on pNPX and 20.8 mM ( $R^2 = 0.98$ ) for arabinose



**Fig. 2** Temperature and pH activity profiles. **a** Optimal pH for activity, determined by incubating RuXyn1 in 50 mM phosphate/citrate buffer containing 1.25 mM pNPX, varying pH from 3.5 to 8.0 at 40°C for 10 min. **b** Effect of temperature on activity of RuXyn1. Activity was measured in 50 mM phosphate/citrate buffer pH 7.0 containing 1.25 mM pNPX at indicated temperature for 10 min. **c** Optimal pH of RuXyn2. Enzyme activity was assayed in 50 mM

phosphate/citrate buffer containing 1.25 mM pNPX, varying pH from 3.5 to 9.0 at 50°C for 10 min. **d** Temperature optimum of RuXyn2. Reactions were carried out in 50 mM phosphate/citrate buffer pH 5.5 containing 1.25 mM pNPX for 10 min at temperatures shown. The specific activity of RuXyn1 and RuXyn2 for pNPX substrate corresponding to 100% relative activity was 36.3 U/mg and 42.1 U/mg, respectively



**Fig. 3** Thermostabilities of recombinant enzymes. **a** Thermostability of RuXyn1 versus time. Activity assays determined the residual activity of RuXyn1 incubated at different temperatures for 0–60 min in 50 mM phosphate/citrate buffer pH 7.0 containing 1.25 mM pNPX at 50°C for 10 min. Initial activity was defined as 100%.

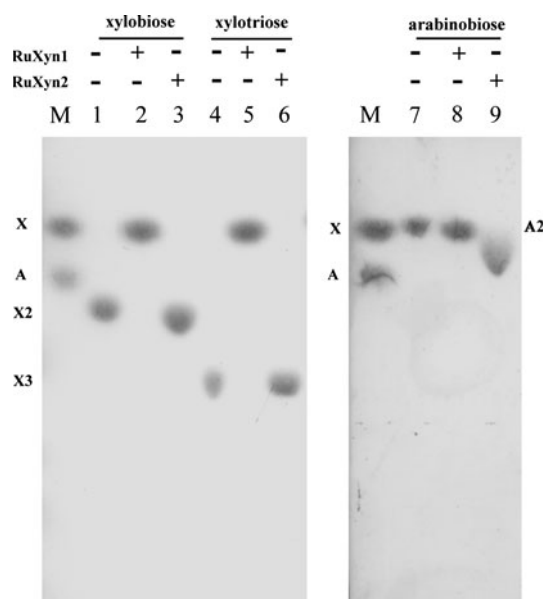
**b** Thermostability of RuXyn2. Recombinant RuXyn2 was treated at indicated temperature for 0–60 min, and its remaining activities was analyzed in 50 mM phosphate/citrate buffer pH 5.5 containing 1.25 mM pNPX at 50°C for 10 min

**Table 1** Kinetic parameters of RuXyn1 and RuXyn2

	Substrate	$K_m$ (mM)	$k_{cat}$ (1/s)	$k_{cat}/K_m$ (mM/s)	$K_i$ (mM)	Activity (IU/mg)
RuXyn1	pNPX	3.43	27.6	8.1	76.0	36.3
	pNPA	2.23	5.6	2.5	20.8	14.2
RuXyn2	pNPX	3.42	40.1	11.7	10.6	42.1

Assays for RuXyn1 were carried out in 50 mM phosphate/citrate buffer pH 7.0 at 40°C for 5 min. Kinetic parameters of RuXyn2 were determined in 50 mM phosphate/citrate buffer pH 5.5 at 50°C. Specific activities were determined by measuring the amount of *p*-nitrophenyl released





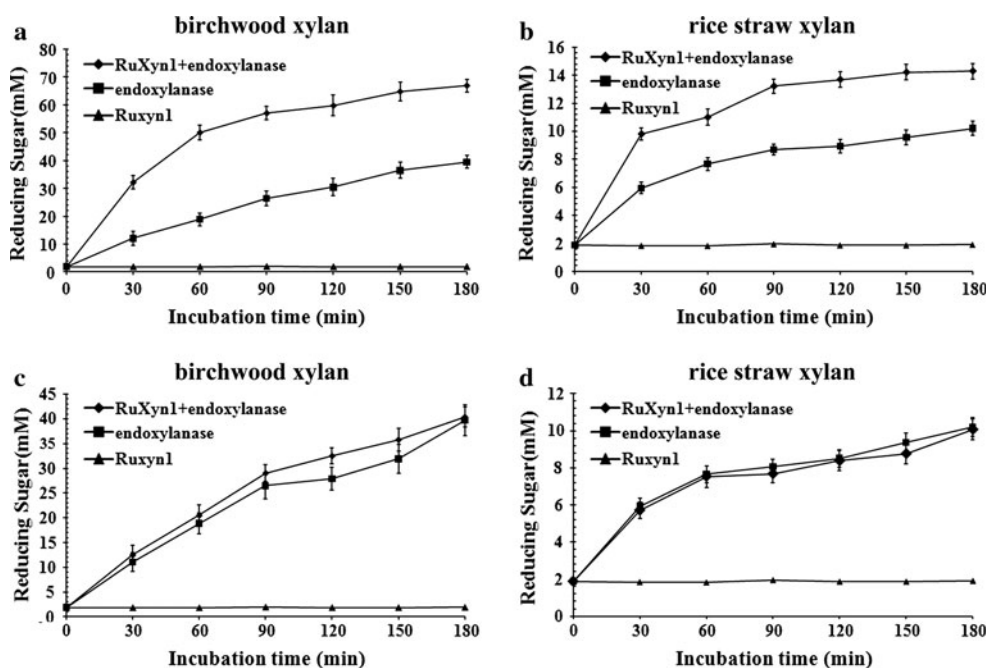
**Fig. 4** Thin-layer chromatography of hydrolysis products obtained from xylobiose, xylotriose, and arabinobiose. Substrate (1%, 80  $\mu$ l) was incubated with 0.1 U enzyme in 50 mM phosphate/citrate buffer [pH 7.0 (RuXyn1) or 5.5 (RuXyn2)] at 37°C for 4 h, then products were resolved by TLC. A arabinose, A2 arabinobiose, X xylose, X2 xylobiose, X3 xylotriose

on pNPA, consistent with a shared affinity of the active site of RuXyn1 for binding of the arabinofuranosyl substrate. The  $K_i$  values for xylose on RuXyn2 activity was 10.6 mM ( $R^2 = 0.98$ ), appearing to be more readily inhibited than GH family 43  $\beta$ -xylosidase RuXyn1.

#### Hydrolysis of hemicellulosic substrates

The activities of purified RuXyn1 and RuXyn2 were further assayed against natural substrates xylobiose, xylotriose, and arabinobiose. RuXyn1 could degraded the xylo-oligosaccharide substrates, xylobiose and xylotriose, into xylose, but exhibited no activity for 1,5- $\alpha$ -L-arabinobiose substrate (Fig. 4). By contrast, RuXyn2 could degrade 1,5- $\alpha$ -L-arabinobiose to arabinose and showed no apparent activity on these two xylo-oligosaccharide substrates, indicating that RuXyn2 was a 1,5- $\alpha$ -L-arabinofuranosidase.

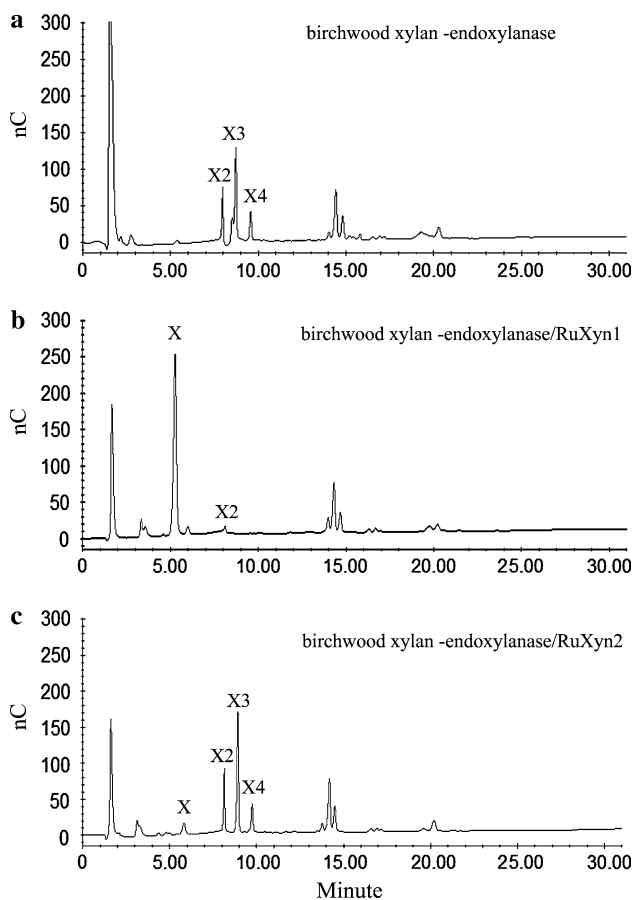
To evaluate the synergistic effect of  $\beta$ -xylosidase RuXyn1 or RuXyn2 with endoxylanase and their capability to yield xylose from xylans, RuXyn1 and RuXyn2, either alone or individually in combination with endoxylanase xynR8, were incubated with birchwood xylan or rice straw xylan substrate, respectively. No reducing sugars were released in the presence of RuXyn1 or RuXyn2 alone from



**Fig. 5** Time course of xylan enzymatic degradation with endoxylanase and  $\beta$ -xylosidase. **a** Reducing sugars released from birchwood xylans. Birchwood xylan (2%) was incubated with 1 U RuXyn1 and 0.4 U endoxylanase xynR8 per milliliter, alone or in combination, in 50 mM phosphate/citrate buffer pH 6.5 at 40°C for the indicated time. **b** Reducing sugars released from rice straw xylans. Rice straw xylan (2%) was incubated with same units of RuXyn1 and endoxylanase

under the same conditions stated above. **c** Time course of birchwood xylan enzymatic degradation with RuXyn2 and xynR8. Birchwood xylan (2%) was incubated with 1 U RuXyn2 or combined with 0.4 U endoxylanase xynR8 per milliliter, respectively, in 50 mM phosphate/citrate buffer pH 5.5 at 40°C for 0–180 min. **d** Reducing sugars released from rice straw xylans by xynR8 or mixtures of RuXyn2 and endoxylanase xynR8

either birchwood xylans or rice straw xylans (Fig. 5), suggesting that RuXyn1 and RuXyn2 have no endoxylanase activity. When RuXyn1 was combined with xynR8, the amount of released reducing sugar from birchwood xylan and from rice straw was 65.1 and 12.4 mM, which was 173% and 142% of that released by endoxylanase alone after 3 h hydrolysis, respectively (Fig. 5a, b), indicating that endoxylanase degradation of xylans was dramatically promoted by adding RuXyn1. Analysis of hydrolysis product by HPAEC showed that xylobiose, xylotriose, and xylotetraose were the main products released from birchwood and rice straw xylan by endoxylanase (Figs. 6a, 7a). Addition of RuXyn1 led to further degradation of endoxylanase's hydrolysates to release xylose (Fig. 6b), and RuXyn1 could almost completely hydrolyze xylobiose and xylotriose as well as xylotetraose. Similar results were also obtained when using rice straw xylan as substrate (Fig. 7b).

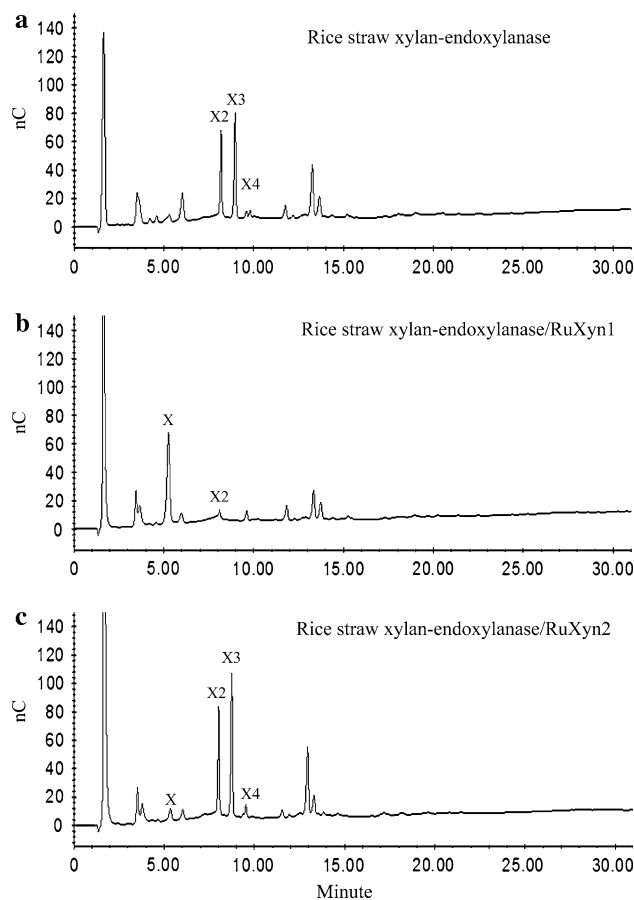


**Fig. 6** HPAEC elution profile of birchwood xylan hydrolysates. Hydrolysis was carried out with 2% birchwood xylan buffered in 50 mM phosphate/citrate buffer for 16 h. Aliquots were removed for analysis by HPAEC. **a** Hydrolysate of birchwood xylan by endoxylanase xynR8. **b** Hydrolysate of birchwood xylan by mixture of endoxylanase xynR8 and RuXyn1. **c** Hydrolysate of birchwood xylan by the mixtures of endoxylanase xynR8 and RuXyn2. Peaks: X xylose, X2 xylobiose, X3 xylotriose, X4 xylotetraose

However, in the case of RuXyn2, addition of RuXyn2 in endoxylanase degradation of birchwood xylan or rice straw xylan procured no obvious improvement in the amount of reducing sugar (Fig. 5c, d). Analysis of birchwood and rice straw endoxylanase hydrolysate by HPAEC also revealed that RuXyn2 could barely further degrade the endoxylanase's hydrolysates including xylobiose, xylotriose, and xylotetraose (Fig. 7).

## Discussion

Bacterial species inhabiting the rumen play a central role in the nutrition of ruminant animals. Most ruminal bacteria are responsible for depolymerizing hemicelluloses into soluble sugars, and then end-products of fermentation are utilized by the mammalian host as the major source of energy [6], leaving no doubt a series of cellulose degradation enzymes must be produced by these microorganisms. However, the rumen environment is anaerobic and at



**Fig. 7** HPAEC assays of rice straw xylan hydrolysates. **a** Hydrolysate of rice straw xylan by endoxylanase xynR8. **b** Hydrolysate of rice straw xylan by mixtures of endoxylanase xynR8 and RuXyn1. **c** Hydrolysate of rice straw xylan by combining endoxylanase xynR8 and RuXyn2

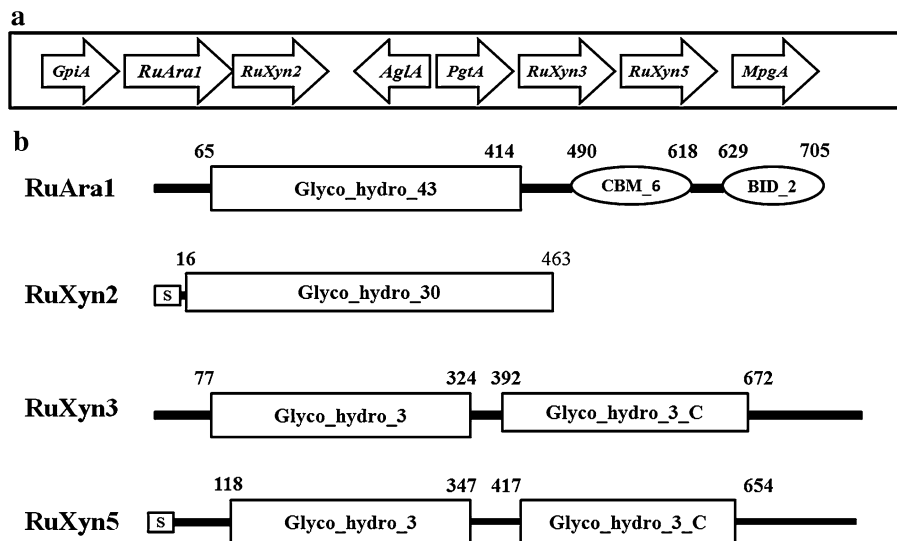
constant temperature and pH, which limits reculture of these microorganisms in vitro and cloning of cellulose and hemicellulose degradation enzymes. Metagenomic technologies provide access to the prokaryotic genomes available in environmental samples, independent of culturability [24]. In this study, two glycosidases,  $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase RuXyn1 and  $\alpha$ -L-arabinofuranosidase/ $\beta$ -D-xylosidase RuXyn2, were cloned and characterized by using metagenomic technology. These two glycosidases belong to different glycosyl hydrolases families, exhibiting distinct enzymatic properties and different capabilities for oligomer degradation.

The optimal pH of RuXyn1 is around 7.0 (Fig. 2), which is not consistent with ruminal pH (6.0–6.2). As almost all bacterial  $\beta$ -xylosidases are cell associated and are considered to occur in the cytosol in soluble form [12], it seems reasonable to suppose that the optimal pH and pH stability may be consistent with the original intracellular environment, because no predicted signal peptide was observed at the N-terminus of RuXyn1. The mature form of RuXyn2 may be a secretory protein due to a 16-aa signal peptide at its amino-terminus, and the optimal pH of RuXyn2 is around pH 5.5–6.0. RuXyn2 showed similar affinity for pNPX substrate to that of RuXyn1, while a high rate of turnover resulted in high catalytic efficiency (Table 1). In contrast, the xylose inhibition constant  $K_i$  of RuXyn2 is lower than that of RuXyn1, resulting in the catalytic rate being more readily inhibited by xylose. Both RuXyn1 and RuXyn2 exhibit bifunctional  $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase activity toward pNPX and pNPA substrates.

Bifunctional activities of xylosidase and arabinosidase are of interest for utilization in substituted xylans [26]. Such bifunctional properties are common in GH family 3 and 43 xylosidases. However, xyl1 from *Trichoderma koningii* G39 belonging to GH family 54 displays similar bifunctional  $\beta$ -D-xylosidase/ $\alpha$ -L-arabinofuranosidase activity [10].

GH family 43 enzymes include  $\beta$ -xylosidase (EC 3.2.1.37),  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55), arabinase (EC 3.2.1.99), xylanase (EC 3.2.1.8), and galactan 1,3- $\beta$ -galactosidase (EC 3.2.1.145). The xylosidase RuXyn1 characterized in this study shows significant identity with family 43 glycosyl hydrolases classified in the Carbohydrate Active Enzymes database (<http://www.cazy.org>). The GH family 43  $\beta$ -xylosidases are the unique ones that operate an inverted mechanism, and almost all catalytically efficient xylosidases characterized to date fall into this GH family [6]. Reaction of inverting glycosyl hydrolases is irreversible once the linkage is cleaved with the addition of a water molecule, which benefits GH-catalyzed hydrolysis [9]. In our study, RuXyn1 converted the xylo-oligosaccharides produced by endoxylanase into xylose efficiently, suggesting that RuXyn1 is advantageous for biomass hydrolysis and for production of xylose from hemicellulosic material. However, the catalytic rate was inhibited at high concentrations of xylose, indicating that there might be further details yet to discover concerning the relationship between catalytic mechanism and catalytic efficiency of glycosyl hydrolases.

While much kinetic and mechanistic data has been collected for GH family 30 glucosylceramidases, very little



**Fig. 8** Orientation of the open reading frames (ORFs) detected in the 31B4 cosmid insertion. **a** Scale representation of the gene assembly of hemicellulose degradation and carbohydrate metabolism enzyme genes from the 31B4 cosmid. *GpiA* glucose-6-phosphate isomerase, *RuAra1* glycosyl hydrolase family 43  $\alpha$ -N-arabinofuranosidase, *RuXyn2* glycosyl hydrolase family 30  $\beta$ -xylosidase, *PgtA* peptidoglycan

glycosyltransferase, *AglA* glycosyl hydrolase family 31  $\alpha$ -glucosidase, *RuXyn3* glycosyl hydrolase family 3  $\beta$ -xylosidase, *RuXyn5* glycosyl hydrolase family 3  $\beta$ -xylosidase/arabinosidase, *MpgA* mannose-1-phosphate guanylyltransferase. **b** Conserved architecture of 31B4 cosmid hemicellulase gene cluster. *S* Predicted signal peptide, *CBM\_6* cellulose binding domain type IV, *BID\_2* bacterial Ig-like domain 2



is known regarding the properties and catalytic mechanism of  $\beta$ -xylosidases belonging to this family. Identification of RuXyn2 in this study can provide meaningful information about GH family 30 xylosidase. With regard to the capability of the xylosidases for yielding xylose from xylan, the GH30 xylosidase RuXyn2 showed poor efficiency, and no synergy with endoxylanase in hydrolysis of xylan. However, RuXyn2 exhibited relatively high hydrolysis activity towards 1,5- $\alpha$ -L-arabinobiose substrate, yielding arabinose monosaccharides. To our knowledge, this is the first 1,5- $\alpha$ -arabinofuranosidase belonging to the GH30 family.

Further sequencing was performed on *RuXyn2* and adjacent sequences to determine whether an operon existed that would provide clues to the enzyme's function and relevant substrates. Sequencing of the 31B4 cosmid clone revealed a hemicellulase gene cluster arranged on a 25-kb fragment (Fig. 8). A gene just upstream of *RuXyn2* was annotated as a putative GH family 43  $\alpha$ -L-arabinofuranosidase gene *RuAra1* (GU573894). Based on the short intergenic space (80 nucleotides) between *RuXyn2* and *RuAra1*, it is possible that these two genes are expressed as an operon encoded by a single polycistronic messenger RNA (mRNA). At 14 kb downstream of *RuXyn2*, there are two putative family 3  $\beta$ -xylosidase/arabinosidase genes: *RuXyn3* (GU573897) and *RuXyn5* (GU573898). Interestingly, all four of these hemicellulases participate in yielding monosaccharide end-product from hemicellulose. It is unclear whether these hemicellulases function synergistically to hydrolyze xylan; however, studies of substrate specificity and characterization of these proteins are underway in our laboratory.

In conclusion, this study characterized two hemicellulose-degrading enzymes from uncultured ruminal bacteria. GH43 RuXyn1 displayed high catalytic efficiency in degrading xylo-oligosaccharide intermediates produced by endoxylanase into end-products, and may have potential applications for xylose production and improvement of hemicellulose saccharification for production of biofuels. RuXyn2 is the first 1,5- $\alpha$ -L-arabinofuranosidase belonging to the GH30 family, and showed high activity towards aryl  $\beta$ -xyloside substrates.

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