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Hydrolytic Properties of a Hybrid Xylanase and Its Parents

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Abstract The hydrolytic properties of a hybrid xylanase (ATx) and its parents (reAnxA and reTfxA) were studied using xylans and xylooligosaccharides as substrates. Analysis of reaction mixtures by high-performance liquid chromatograph revealed that xylotriose (X3) was the main product released from birchwood xylan and wheat bran insoluble xylan by ATx and reAnxA, respectively. Xylobiose (X2) was the main product separately released from birchwood xylan and wheat bran insoluble xylan by reTfxA. Xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) could be hydrolyzed by ATx, which showed no activity on X2 and X3. Therefore, X4 might be the minimum oligomer hydrolyzed by ATx. X2–X6 could be hydrolyzed by reAnxA and reTfxA, respectively. All of ATx, reAnxA, and reTfxA showed transglycosylation activity.

Keywords Xylanase · Hydrolysis · Transglycosylation · HPLC

Abbreviations

ATx	a hybrid xylanase whose parents are *Thermomonospora fusca* xylanase A and *Aspergillus niger* xylanase A
HPLC	high-performance liquid chromatography

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X	xylose
X2	xylobiose
X3	xylotriose
X4	xylotetraose
X5	xylopentaose
X6	xylohexaose
AFM	atomic force microscope
DP	degree of polymerization
XOs	xylooligosaccharides

Introduction

Biomass from plant material is the most abundant and widely spread renewable raw material for the production of high-value bioproducts. Xylan, a major component of the plant cell wall, consists of a backbone of β-(1,4)-linked D-xylosyl residues with substitutions of arabinosyl, acetyl, and glucuronosyl residues [1–3]. The hydrolysis of the xylan backbone involves several enzymes, and the most important one is the *endo*-β-(1,4)-xylanase (EC 3.2.1.8). Based on sequence similarities and hydrophobic cluster analysis, endoxylanases have been grouped into families 10 and 11 of glycosyl hydrolases [4–6]. To date, more than 200 *endo*-β-(1,4)-xylanase have been identified, and the great majority of these originate from bacteria and fungi [7]. In recent years, xylanases have attracted considerable research interest because of their industrial applications, and one of the special attentions has been given to the use of xylanases in production of xylo-oligosaccharides (XOs) [8–12]. XOs, as high value-added ingredients for functional foods, have various physiological important actions such as maintaining gastrointestinal health, improving intestinal mineral absorption, and reducing cholesterol [10, 12]. Considered as food ingredients, XOs show favorable technological features, including stability in acidic media, resistance to heat, and ability for offering lower available energy and for achieving significant biological effects at low daily intakes. With the increasing health consciousness among consumers, the functional food market is growing rapidly. Besides biological effects concerning human health, XOs have been employed for phytopharmaceutical and feed applications [13, 14].

XOs can be produced from xylan-rich materials by chemical methods, direct enzymatic hydrolysis of a susceptible substrate [13, 15] or a combination of chemical and enzymatic treatments [9, 16]. Among these methods, enzymatic hydrolysis is more desirable because it does not produce undesirable byproducts or high amounts of monosaccharides and does not require special equipment. Although many wild xylanases have been used in industrial applications, each has one or two desired characteristics, and they still cannot meet the requirements of industry application. A thermostable hybrid xylanase, ATx, was constructed by substituting the N terminus of the *Thermomonospora fusca* xylanase A (TfxA) for its corresponding region of the *Aspergillus niger* xylanase A (AnxA) in our previous studies [17]. Biochemical properties of the xylanase ATx have been discussed, but the action pattern of this enzyme is not assessed [17]. In this study, xylan degradation by the hybrid xylanase ATx was detected by high-performance liquid chromatography (HPLC), and hydrolytic properties of ATx and its parents were compared using birchwood xylan, wheat bran insoluble xylan, and standard xylooligosaccharides (X2–X6) as

substrates. Furthermore, the structure change of xylan during the hydrolysis by ATx was investigated by atomic force microscopy (AFM).

Materials and Methods

Materials

Birchwood xylan was from Sigma Chemical Company. The standard xylooligosaccharides (X2–X6) were from Megazyme Company. Xylose was from Merck. The wheat bran insoluble xylan was kindly provided by Dr. Shang-Wei Chen (Southern Yangtze University). The purified ATx and its parents (reAnxA, reTfxA) were stored at 4 °C in our laboratory. Xylanase activities of ATx, reAnxA, and reTfxA were 633, 175, and 117 U/mg, respectively. The optimum temperature and pH for ATx, reAnxA, and reTfxA were 60, 50, 60 °C, and pH 5.0, 5.0, 6.0, respectively [17]. All other chemicals were of analytical grade.

Hydrolysis Products of Birchwood Xylan and Wheat Bran Insoluble Xylan by ATx and Its Parents

The 1.0% (*w*/*v*) birchwood xylan and wheat bran insoluble xylan were incubated with ATx, reAnxA, and reTfxA at 40 °C with constant shaking (250 rpm), respectively. In the action mixtures, Xylanase ATx, reAnxA, and reTfxA with the same enzyme activities (1.5 U) were added, and the substrates (birchwood xylan and wheat bran insoluble xylan) were excessive. The aliquots at different time intervals (6, 12, 18, and 24 h) were analyzed by HPLC with Sugar-pak™ 1 column (300 mm length and 6.5 mm diameter, Waters), pure water as mobile phase (0.5 ml/min), and injection volumes of 10 μl. The column was maintained at 85 °C. Sugar peaks were screened using Waters 2410 refractive index detector. Xylose (X) and standard xylooligosaccharides (X2–X6) were resolved in pure water, and then they were diluted to different times. Samples (X–X6) were analyzed by HPLC separately. The areas of sugar peaks from HPLC results combined with the concentrations of sugars to obtain the standard concentration curves of each sugar (X–X6) were obtained. The hydrolytic products of xylan were quantified on standard curves.

Changes in Structure of Birchwood Xylan Hydrolyzed by ATx

One percent birchwood xylan was incubated with ATx in water bath at 40 °C for 12 h. The mixture was heated at 100 °C for 5 min, and then, it was centrifugated. The 1% birchwood xylan mixed with inactive ATx (as control) was also boiled and centrifuged. Before being scanned by AFM (SPM-9500J3, Japan), the supernatants were diluted 50 times with pure water.

Enzymatic Hydrolysis of Standard Xylooligosaccharides by ATx and Its Parents

The mode of action of ATx and its parents were determined using standard xylooligosaccharides (XOs) as substrate. The standard xylooligosaccharides solutions (pure water, pH 7.0) were incubated with purified ATx, reAnxA, and reTfxA at 40 °C, respectively. The samples at different time intervals were analyzed by HPLC.

Results and Discussion

Degradation of Birchwood Xylan by ATx and Its Parents

The hydrolysis products from birchwood xylan by ATx were X–X5 with X3 as major product (Figs. 1A and 2A). As the hydrolysis progressed, the concentrations of X, X2, and X3 increased with the simultaneous decreased concentrations of X4 and X5. After 24 h of incubation, about 22.6% and 45.6% of the total hydrolysis products were X2 and X3 with the concentration 0.620 and 1.240 mg/ml, respectively.

The hydrolysis products from birchwood xylan by reAnxA were X–X5 with X3 as major product (Figs. 1B and 2B). After 24 h of incubation, about 39.2% and 52.4% of the total hydrolysis products were X2 and X3 with the concentration 0.807 and 1.080 mg/ml, respectively.

The hydrolysis products from birchwood xylan by reTfxA were X–X6 with X2 as major product (Figs. 1C and 2C). After 24 h of incubation, about 43.2% and 38.5% of the total hydrolysis products were X2 and X3 with the concentration 1.078 and 0.960 mg/ml, respectively.

The conversion of birchwood xylan to products by ATx, reAnxA, and reTfxA were about 33.99%, 25.76%, and 31.19%, respectively. ATx inherited some properties from its parents. The two catalytic amino acid residues of ATx were from xylanase AnxA. The major hydrolysis product of birchwood xylan by ATx was X3, which was the same as that of reAnxA. Meanwhile, there were some differences between ATx and its parents, reTfxA. reTfxA hydrolyzed birchwood xylan to yield predominantly X2. X6 was not detected in the hydrolysis products released by ATx, whereas it existed in hydrolysis products by reTfxA.

Hydrolysis Products of Wheat Bran Insoluble Xylan by ATx and Its Parents

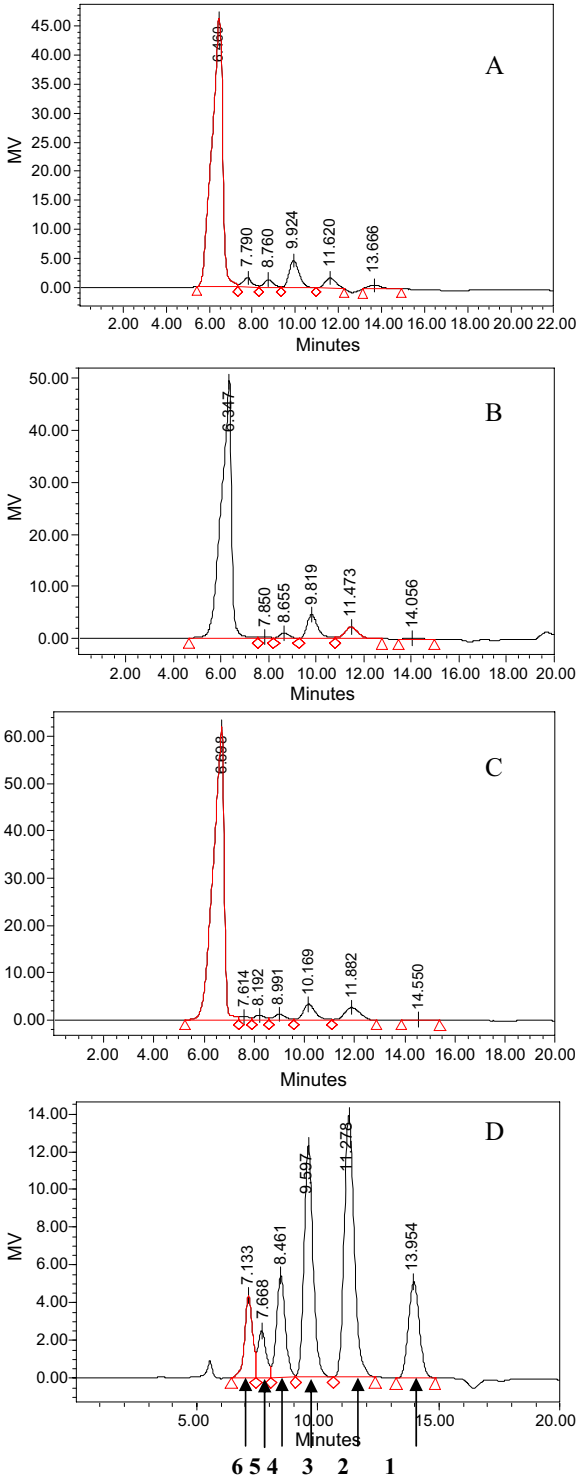
The hydrolysis products released by ATx from wheat bran insoluble xylan were X–X6, and the major hydrolysis product was X3 (Fig. 3A). After 24 h of incubation, about 27.2% and 27.3% of the total hydrolysis products were X2 and X3 with concentrations of 0.356 and 0.357 mg/ml, respectively.

Hydrolysis products released by reAnxA from wheat bran insoluble xylan were X–X6, whose concentrations increased during the hydrolysis (Fig. 3B). After 24 h of incubation, about 21.6% and 23.3% of the total reaction products were X2 and X3 with concentrations of 0.202 and 0.218 mg/ml, respectively.

Hydrolysis products released by reTfxA from wheat bran insoluble xylan were X–X6 with X2 as main product (Fig. 3C). After 24 h of incubation, about 33.2% and 22.4% of the hydrolysis products were X2 and X3 with concentrations of 0.577 and 0.389 mg/ml, respectively.

Wheat bran is composed of hemicellulose-rich substrates, among which xylan represents about 40% of dry matter [18]. ATx and its parents hydrolyzed wheat bran insoluble xylan to a mixture of xylooligosaccharides with X2 and X3 being the major product. This might make ATx and its parents potentially suitable for production of xylooligosaccharides from wheat bran. The conversion efficiency of wheat bran insoluble xylan to products by ATx, reAnxA, and reTfxA were about 16.35%, 11.70%, 21.72%, respectively.

Fig. 1 HPLC analysis of hydrolytic products from birchwood xylan by ATx and its parents. **A**–**C** HPLC analysis of hydrolytic products from birchwood xylan by ATx, reAnxA, and reTfxA (1.5 U, at 40 °C and optimum pH) after 6 h reaction, respectively. **D** The standard xylooligosaccharides analyzed by HPLC. The positions of xylose (*1*), xylobiose (*2*), xylotriose (*3*), xylotetraose (*4*), xylopentaose (*5*), and xylohexaose (*6*) are shown


A
MV
Minutes
6.460
7.790
8.760
9.924
11.620
13.666
B
6.347
7.850
8.655
9.819
11.473
14.056
C
6.698
7.614
8.192
8.991
10.169
11.882
14.550
D
7.133
7.668
8.461
9.597
11.278
13.954
6 5 4 3 2 1

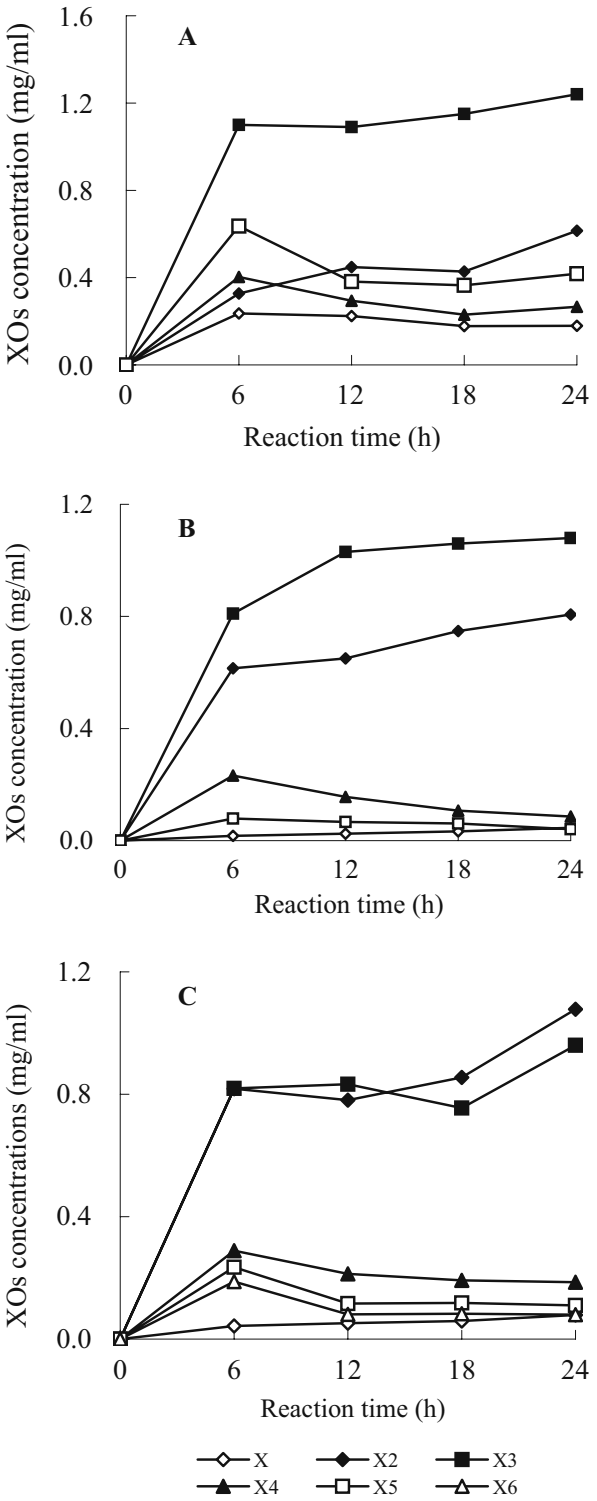

A
XOs concentration (mg/ml)
1.6
1.2
0.8
0.4
0.0
0
6
12
18
24
Reaction time (h)
B
XOs concentration (mg/ml)
1.2
0.8
0.4
0.0
0
6
12
18
24
Reaction time (h)
C
XOs concentrations (mg/ml)
1.2
0.8
0.4
0.0
0
6
12
18
24
Reaction time (h)
X
X2
X3
X4
X5
X6

Fig. 2 Changes in the concentrations of hydrolytic products from birchwood xylan by ATx and its parents. Three xylanases (1.5 U) were separately incubated with 1% birchwood xylan at 40 °C and optimum pH for different time. **A**–**C** HPLC analysis of hydrolytic products from birchwood xylan by ATx, reAnxA, and reTfxA, respectively

Changes in Structure of Birchwood Xylan Hydrolyzed by ATx

AFM that belongs to scanning probe microscopy (SPM) is a relatively mature tool for the detailed structural investigation of biological specimens [19]. Košikobá et al. developed a scanning electron microscopy (SEM) procedure for investigating the supermolecular structure of lignin–saccharidic complex [20]. The changes in lignin–saccharidic complex during xylooligosaccharides production from corncob were investigated by SEM, and SEM micrographs showed that the structure of lignin–saccharidic complex of corncob had been changed, and xylan was almost removed from lignin after steaming and the enzymatic hydrolysis [9].

In this study, the AFM photographs showed detailed structure changes of birchwood xylan during hydrolysis by ATx. The untreated birchwood xylan showed its characteristic and well-arranged surface (Fig. 4A). After 12 h hydrolysis, the surface of birchwood xylan was altered significantly, and it had a much smoother appearance. The degradation led to a thinner and more diluted interface of birchwood xylan, and the compact surface became relaxed (Fig. 4B).

Degradation of Standard Xylooligosaccharides by ATx and Its Parents

The hydrolytic properties of ATx and its parents (reAnxA and reTfxA) were evaluated using xylooligosaccharides as substrates. Enzyme activity increased as the degree of polymerization (DP) of the xylooligosaccharides became larger. As the hydrolytic ability of xylanases was varied on xylooligosaccharides with different length, the digestion time was adjusted [21, 22].

reAnxA and reTfxA could hydrolyze X2–X6 and had very low activity on X2 and X3 (Fig. 5B1–5 and C1–5). The hybrid xylanase ATx could hydrolyze X4–X6 and showed no hydrolysis activity on xylobiose (X2) and xylotriose (X3; Fig. 5A1–5). This indicated that X4 might be the minimum oligomer hydrolyzed by ATx, which was different from most characterized xylanases [23–26]. X2 and X3 could be further hydrolyzed by the reAnxA and reTfxA, so the minimum hydrolysis oligomer among them was X2.

X2 was the major product released from X4 by ATx, reAnxA, and reTfxA (Fig. 5). Trace amount of xylohexaose (X6) was detected among the hydrolysis products from X4 by ATx, which might be the result of transglycosylation reaction. The patterns of transglycosylation reaction were as follows: X4+E=E(X4); E(X4)=2×2+E; E(X4)+X2=X6+E, where X4 represents xylotetraose, X2 represents xylobiose, X6 represents xylohexaose, E represents free ATx, and E(X4) represents ATx–xylotetraose complex. X3 was among the hydrolysates of X4 by Atx, but X was not detected, suggesting that X3 was directly from X6 rather than from X4.

X2–X4 were the products released from X5 by ATx and its parents with X3 as main product (Fig. 5A4, B4 and C4). No xylose (X) was detected in the reaction mixture, which suggested that the formation of X4 might be the result of transglycosylation action. ATx and its parents showed transglycosylation activity.

X6 was rapidly hydrolyzed by ATx and its parents, and generated xylooligosaccharides mixture (DP<6) with X3 as the major product (Fig. 5A5, B5, and C5).

X2 and X3 were the main hydrolysis products from X4, X5, and X6 by ATx and its parents, suggesting that the three xylanases were typical endo-acting enzymes that

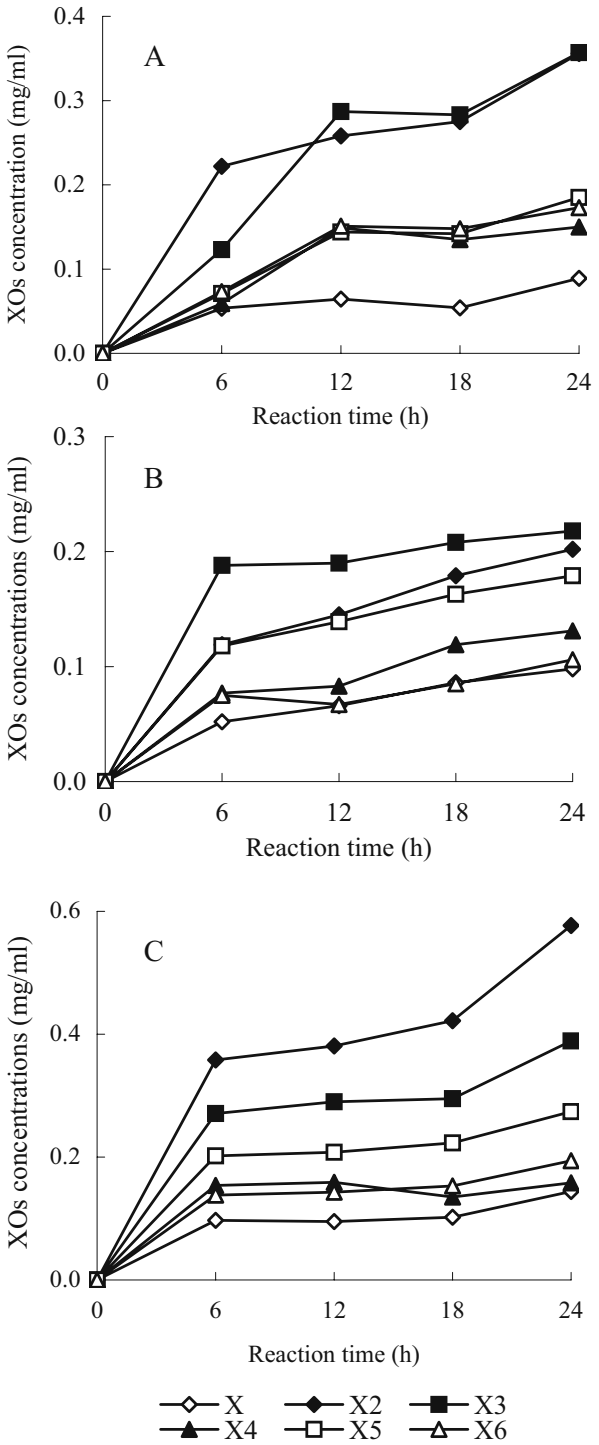

A
XOs concentration (mg/ml)
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
Reaction time (h)
B
XOs concentrations (mg/ml)
0.3
0.2
0.1
0.0
0
6
12
18
24
Reaction time (h)
C
XOs concentrations (mg/ml)
0.6
0.4
0.2
0.0
0
6
12
18
24
Reaction time (h)
X
X2
X3
X4
X5
X6

Fig. 3 Changes in the concentrations of hydrolytic products from wheat bran insoluble xylan by ATx and its parents. Three xylanases (1.5 U) were separately incubated with 1% wheat bran insoluble xylan at 40 °C for different time. **A**–**C** HPLC analysis of hydrolytic products from wheat bran insoluble xylan by ATx, reAnxA, and reTfxA, respectively

preferentially cleaved the internal glycosidic bonds of the xylooligosaccharides. Endo-mode enzyme showed low susceptibility of substrate of DP=2, such as xylobiose, chitobiose, and maltose [27]. The degradation of xylobiose might be the result of transglycosylation reactions [26].

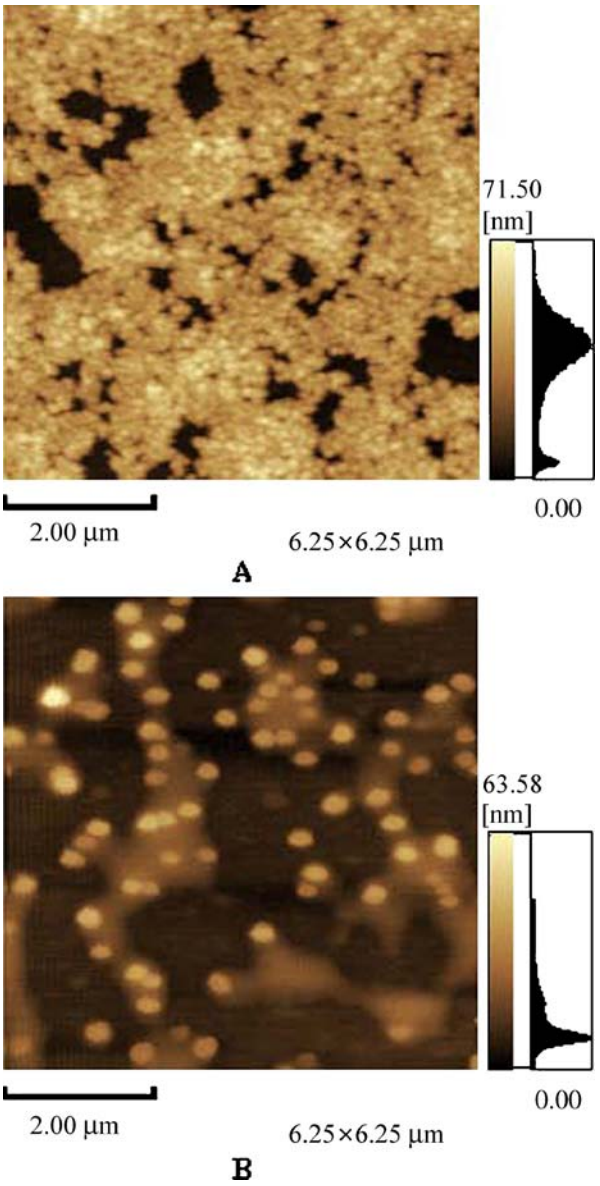


Fig. 4 AFM photographs of birchwood xylan hydrolyzed by ATx. **A** Used as control that the 1% birchwood xylan was mixed with inactive ATx. **B** The result that birchwood xylan was hydrolyzed by ATx for 12 h

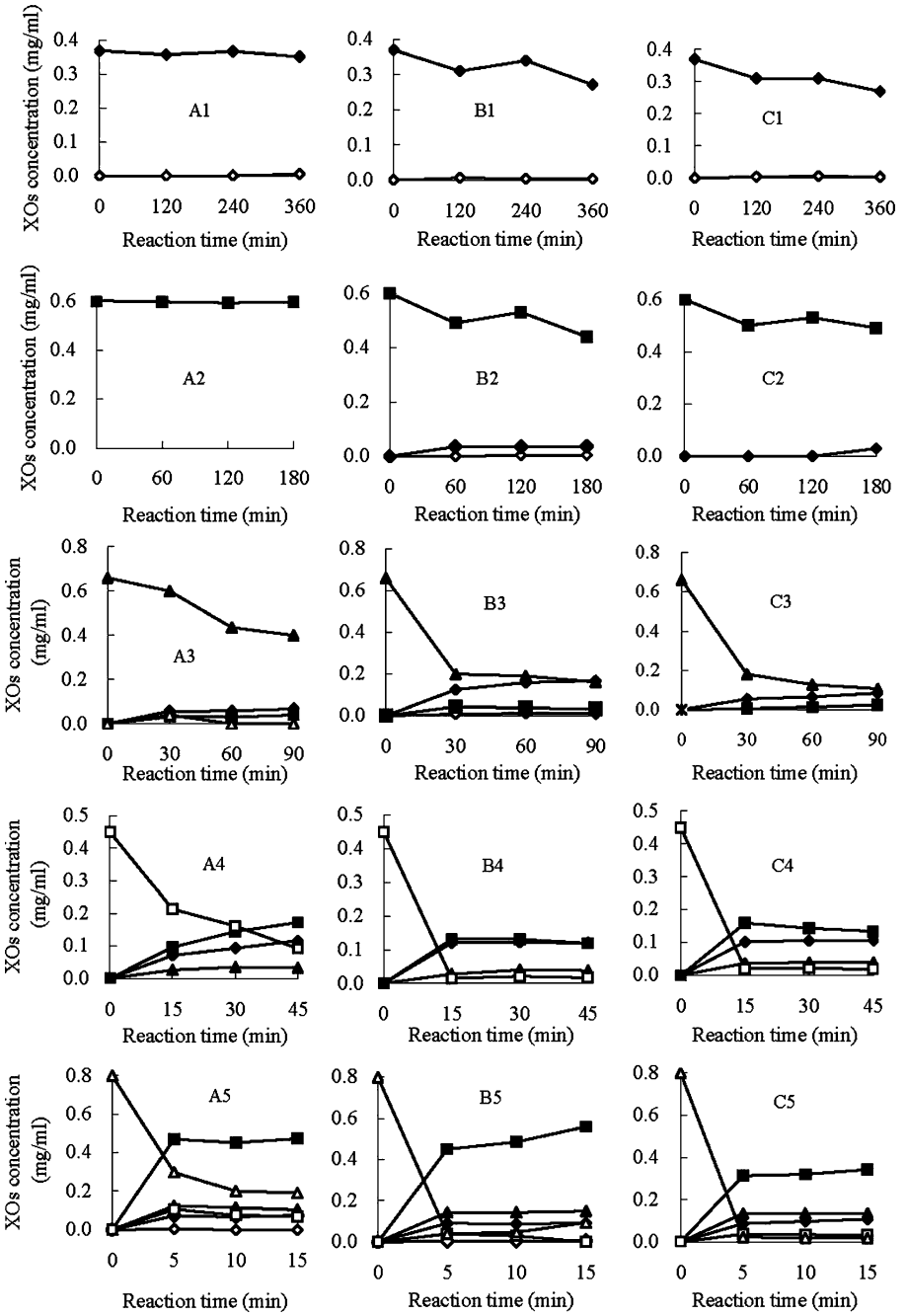


Fig. 5 Changes in the concentrations of hydrolytic products from XOs by ATx and its parents. Xylanases (0.5 U) were separately incubated with XOs (X2–X6) at 40 °C for a different time. At regular time intervals, aliquots of the reactions were analyzed by HPLC for xylose (*open diamond*), xylobiose (*filled diamond*), xylotriose (*filled square*), xylotetraose (*filled triangle*), xylopentaose (*open square*), and xylohexaose (*open triangle*), respectively. *1–5* Hydrolytic products from X2, X3, X4, X5, and X6, respectively. **A**–**C** ATx, reAnx, and reTfx, respectively. X–X6 means xylose to xylohexaose, respectively

Conclusions

As a hybrid xylanase, ATx inherited some hydrolytic properties from its parents, and it was an endo-acting xylanase. The major hydrolysis product of birchwood xylan and wheat bran insoluble xylan by ATx was X3, which was the same as that of reAnxA. Meanwhile, there were some differences between ATx and its parents. The hybrid xylanase ATx showed no hydrolysis activity on xylobiose (X2) and xylotriose (X3), and X4 might be the minimum oligomer hydrolyzed by it, which was different from most characterized xylanases. All of ATx, reAnxA, and reTfxA showed transglycosylation activity.

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