Multifunctional Properties of Glycoside Hydrolase Family 43 from *Paenibacillus curdlanolyticus* Strain B-6 Including Exo-β-xylosidase, Endo-xylanase, and α-L-Arabinofuranosidase Activities

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The glycoside hydrolase family 43 from *Paenibacillus curdlanolyticus* strain B-6 (GH43B6) exhibited multifunctional properties, including exo-β-xylosidase, endo-xylanase, and α-L-arabinofuranosidase enzymatic activities. GH43B6 released xylose as a hydrolysis product from the successive reduction of xylooligosaccharides as a result of exo-β-xylosidase activity. Moreover, GH43B6 also predominantly released xylose from low-substituted xylan derived from birchwood. However, when the highly substituted rye flour arabinoxylan was used as a substrate, exo-β-xylosidase activity changed to endo-xylanase activity, indicating that the enzymatic property of GH43B6 is influenced by the substituted side groups of xylan. For α-L-arabinofuranosidase, arabinose was released from short-chain substrates including *p*-nitrophenyl-α-L-arabinofuranoside and α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*. This study reports the novel trifunctional properties of GH43B6 containing exo- and endo-activity together with xylanolytic debranching enzymatic activity, which increases its potential for application in lignocellulose-based biorefineries.

Keywords: Exo-β-xylosidase/endo-xylanase/α-L-arabinofuranosidase activities; Glycoside hydrolase family 43; Multifunctional enzyme; Paenibacillus curdlanolyticus; Xylanolytic enzyme

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INTRODUCTION

Plant biomass contains a variety of polysaccharides as structural and storage compounds, and the most prominent are cellulose and hemicelluloses (Schwarz 2001). Xylans are the major components of hemicelluloses. Xylan from hardwoods is mainly composed of glucuronoxylan, in which some xylose units within the linear β-(1,4)-D-xylopyranose backbone are decorated with acetyl groups and 4-*O*-methylglucuronic acids (Alén 2000). Meanwhile, arabinoglucuronoxylan was identified in softwoods and grasses. The structure of arabinoglucuronoxylan consists of a linear β-(1,4)-D-xylopyranose backbone decorated with 4-*O*-methyl-α-D-glucopyranosyluronic acid and α-L-arabinofuranosyl linked by α-(1,2) and α-(1,3) glycosidic bonds. Nonetheless, arabinose decoration of xylan from grasses was higher than it was from softwoods (Gírio *et al.* 2010). However, the type and

degree of the substituted side chains on xylan vary with the botanical origin (Biely 1985). Due to the complicated architecture of xylans, the hydrolysis of xylan requires the action of a complex enzyme system of xylanolytic enzymes that act synergistically to depolymerise xylan to its sugar constituents. Xylanolytic enzymes are usually composed of non-debranching enzymes (endo-xylanase and β-xylosidase) and debranching enzymes (α-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase) (Biely 1985).

Glycoside hydrolase family 43 (GH43) is a group of enzymes with broad similarity and a variety of enzymatic functions (Cantarel *et al.* 2009) encompassing monofunctional enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55), β-xylosidase (EC 3.2.1.37), arabinanase (EC 3.2.1.99), xylanase (EC 3.2.1.8), galactan 1,3-β-galactosidase (EC 3.2.1.145), α-1,2-L-arabinofuranosidase (EC 3.2.1.-), exo-α-1,5-L-arabinofuranosidase (EC 3.2.1.-), exo-α-1,5-L-arabinanase (EC 3.2.1.-), and β-1,3-xylosidase (EC 3.2.1-). Moreover, multifunctional enzymes from several origins have been reported in GH43 such as β-xylosidase/α-L-arabinofuranosidase (Kim and Yoon 2 0 1 0) , β-xylosidase/exo-xylanase (GenBank ID: ABD48561.1), and β-1,4-xylosidase/α-1,5-arabinofur(pyr)anosidase/β-1,4-lactase/α-1,6-raffinase/α-1,6-stachyase/β-galactosidase/α-1,4-glucosidase (Ferrer *et al.* 2012). Recently, Viborg *et al.* (2013) classified 92 characterised GH43 enzymes based on sequence similarity and functions to five distinct substrate specificity groups. GH43 enzymes contribute to xylan degradation by releasing substituted side groups from xylan, which allows endo-xylanases to act efficiently for complete hydrolysis (Lagaert *et al.* 2014). This issue is of great interest for biomass utilisation in the biorefinery and bioenergy areas.

We found one open reading frame (ORF) from the *Paenibacillus curdlanolyticus* strain B-6 encoding a protein belonging to GH family 43, which comprises a broad variety of enzyme specificities and similarities. As a result, we speculated that GH43 from strain B-6 (GH43B6) may exhibit enzymatic properties that differ from other GH43 enzymes. *P. curdlanolyticus* strain B-6 is a true cellulolytic/xylanolytic bacterium that produces a unique extracellular xylanolytic-cellulolytic multienzyme complex-like structure capable of degrading insoluble substrates (Pason *et al.* 2006). Therefore, this study was conducted to elucidate the enzymatic properties of GH43 from *P. curdlanolyticus* strain B-6. The results are important in determining how the enzymatic properties of GH43B6 cooperate with endo-xylanases to depolymerise xylans.

EXPERIMENTAL

Strains and Plasmid

P. curdlanolyticus B-6 was isolated from an anaerobic digester, fed with pineapple waste (Pason *et al.* 2006), and deposited in the BIOTEC Culture Collection of the National Center for Genetic Engineering and Biotechnology, Thailand, with the accession number BCC no. 11175. All cloning strategies were performed in *Escherichia coli* DH5α (New England Biolabs, Ipswich, MA, USA). *Escherichia coli* BL21 (DE3) (Novagen, Darmstadt, Germany) was used as the host for the derivative of pET28b(+) (Novagen).

Gene Manipulation

Genomic DNA was extracted from *P. curdlanolyticus* B-6 by using a chromosomal DNA extraction kit (Viogene, New Taipei City, Taiwan) and subjected to *GH43B6* amplification. The oligonucleotide primers were forward primer 5' CGGGATCCCGATGGA CAACAAACCGGTAAA 3' and reverse primer 5' CCGCTCGAGCGGTTTCAATTTGCTA

TAATCGAGC 3'; they were designed based on a genomic library of strain B-6 and constructed using a CopyControl Fosmid Library Production Kit (Epicenter, Madison, WI), which has been described previously (Sudo *et al*. 2010). The suitable *Bam*HI and *Xho*I recognition sites for cloning were added to the forward and reverse primers, respectively (underlined sequences). The PCR product was introduced into pET28b(+) at the same restriction sites to yield p*GH43B6*.

Expression, Purification, and Optimum Conditions of GH43B6

Plasmid p*GH43B6* was transformed into *E. coli* BL21 (DE3). A transformant harbouring p*GH43B6* was grown on Luria Bertani medium, supplemented with kanamycin (30 µg/mL final concentration). The culture was incubated at 37 °C and 200 rpm until the OD_{600} reached 0.6. Protein expression was induced with a 1 mM final concentration of isopropyl β-D-1-thiogalactopyranoside, and the culture was further incubated at 16 °C for 16 h. The cells were harvested and disrupted. Cell-free extracts were applied to HisTrap™ FF columns (GE healthcare, Little Chalfont, UK) for affinity purification. The purity of purified proteins was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). α-L-Arabinofuranosidase was used for monitoring the target protein. The optimum pH of GH43B6 for hydrolysis was determined at a pH ranging from 3.0 to 10.0 (pH 3.0 to 6.0, acetate buffer; pH 6.0 to 8.0, phosphate buffer; pH 8.0 to 9.0, Tris-HCl buffer; pH 9.0 to 10.0, carbonate buffer) for 30 min at 50 °C. The optimum temperature was determined in the range of 30 to 90 °C for 30 min at the optimum pH.

Enzyme Assays and Protein Determination

The following substrates—*p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA), *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), *p*-nitrophenyl acetate, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-D-galactopyranoside (*p*NPGal), and *p*-nitrophenyl-β-D-cellobioside (all from Sigma-Aldrich, St. Louis, MO, USA)—were used for assaying α-L-arabinofuranosidase, β-xylosidase, acetyl esterase, β-glucosidase, galactan 1,3-β-galactosidase, and cellobiohydrolase activities, respectively. Enzyme aliquots were incubated with 100 µL of 0.9 mM substrate in 50 mM sodium phosphate buffer (SPB) (pH 7.0) at 50 °C for 30 min, and the reaction was stopped by adding 1 mL of 1 M Na_2CO_3. Liberation of *p*-nitrophenol was measured by absorbance at 410 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required for liberating 1 µmol of *p*-nitrophenol per min. Xylanase activity was assayed on 1% xylan from birchwood (Sigma-Aldrich) in 50 mM SPB (pH 7.0) at 50 °C for 10 min. Liberated reducing sugars were analysed by the Nelson-Somogyi method (Nelson 1944). One unit (U) of xylanase activity was defined as the amount of enzyme used for liberating 1 µmol of xylose per minute. Protein concentration was determined as described by Lowry *et al*. (1951).

Determination of the Hydrolysis Action of GH43B6

The hydrolysis action of purified GH43B6 was elucidated on various types of substrates. To assess β-xylosidase action, 0.025% (w/v) of xylohexaose and xylobiose (Megazyme International, Wicklow, Ireland), were used as substrates. The action of xylanase was determined on 1% (w/v) xylans from birchwood, oat spelt (Sigma-Aldrich), wheat flour arabinoxylan (Megazyme), and rye flour arabinoxylan (Megazyme). The α-L-arabinofuranosidase action was determined on 1% (w/v) rye flour arabinoxylan, oat spelt xylan, and 0.025% (w/v) α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p* (A-X-A). Aliquots of 0.1 U of α-L-arabinofuranosidase, β-xylosidase, or xylanase from GH43B6 were mixed with

their substrate in 50 mM SPB buffer pH 7.0 and incubated at 50 °C. The hydrolysis reaction was stopped by boiling for 15 min.

Preparation of A-X-A

Rye flour arabinoxylan (1%) in 50 mM SPB pH 7.0 was hydrolysed by 0.1 U of xylanase Xyn10C from *P. curdlanolyticus* strain B-6 (Imjongjairak *et al.* in press) at 50 °C for 16 h. The hydrolysis products were separated by thin layer chromatography (TLC) as described by Sornyotha *et al*. (2007). The A-X-A was cut out from the TLC plate at the same position as the standard A-X-A that had been prepared in the laboratory as described by Imjongjairak *et al.* (in press); it was verified by electrospray ionization mass spectrometry and enzymatic analysis using *Bacillus licheniformis* α-L-arabinofuranosidase Axh43A (Sakka *et al*. 2012), suspended in deionised water, and then sonicated. Silica was removed by centrifugation at 9,200 x *g* for 1 min. The supernatant was collected and dried in a speed vacuum (Univapro 100 ECH, UniEquip, Planegg, Germany).

Analysis of Hydrolysis Products

Hydrolysis samples from the xylans were centrifuged at 9,200 x *g* for 1 min, and the supernatants were collected. The analyses of the hydrolysis products from xylohexaose and xylans were carried out by TLC. The xylobiose hydrolysis products were analysed by high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) with a reflective index detector (Shimadzu RID-10A) on BP-100 Pb^{++} carbohydrate columns (Benson polymeric, Sparks, NV, USA) operated at 85 °C with deionised water at a flow rate of 0.6 mL/min.

RESULTS AND DISCUSSION

The gene encoding glycoside hydrolase family 43 from *P. curdlanolyticus* B6 (*GH43B6*) was cloned in this study. *GH43B6* (accession No. KM272280) was annotated to encode an enzyme belonging to GH43 based on amino acid sequence similarity. It contains an ORF of 990 nucleotides corresponding to 330 amino acids with a predicted molecular mass of 37.5 kDa. GH43B6 amino acid sequence positions 11 through 321 were confidently predicted by SMART software (Letunic *et al.* 2014) to be a glycoside hydrolase family 43 catalytic domain, while two unknown regions were also identified at positions 1 through 10 and 322 through 330, respectively. Signal peptide prediction by the SignalP 4.0 server (Petersen *et al.* 2011) suggested the absence of a signal peptide in GH43B6. BlastP analysis of GH43B6 revealed high similarity to uncharacterised GH43 mono- and bi-functional enzymes such as α-L-arabinofuranosidase, β-xylosidase, and bifunctional β-xylosidase/α-L-arabinofuranosidase, with the highest identity (84%) to α-L-arabinofuranosidases from *Paenibacillus* sp. J14 (GenBank ID: WP_028537372.1) and *Paenibacillus* sp. oral taxon 786 (WP_009224277.1). Comparison of GH43B6 with GH43 α-L-arabinofuranosidases from *P. lactis* (GenBank ID: WP_007127515.1), *P. vortex* (WP_006211951.1), and *P. curdlanolyticus* (WP_006040336.1) showed sequence identities of 81%, 77%, and 72%, respectively. Moreover, comparisons with GH43 xylosidase/arabinofuranosidase from *Clostridium saccharoperbutylacetonicum* N1-4(HMT) (YP_007457878.1) and β-xylosidases from *Thermobacillus composti* KWC4 (YP_007212002.1) and *Aspergillus terreus* (ADF63137.1) showed sequence identities of 77% 76%, and 64%, respectively. Subsequently, almost all the catalytic domain of GH34B6 was subjected to multiple sequence alignment with other GH43 members (Fig. 1).

Multiple conserved regions were identified, especially aspartic acids (positions 69, 81, 145, 151, 153, 186, 201, 211, and 285) and glutamic acids (positions 196, 202, 218, and 302). A phylogenetic tree of GH43B6 with characterised GH43 representatives was constructed based on categories described by Viborg *et al.* (2013). Representatives were chosen by the criteria of enzymatic function and sequence similarity compared with GH43B6. The results indicated that GH43B6 belongs to group III, which consists of α-L-arabinofuranosidase, β-xylosidase, endo-xylanase, and β-xylosidase/α-L-arabinofuranosidase (Fig. 2). However, AAD30363.1 bifunctional endo-xylanase/α-L-arabinofuranosidase from *Caldicellulosiruptor* sp. Tok7B.1, which has been classified to group III by Viborg *et al.* (2013), was individually separated due to low sequence similarity with GH43B6 (22.54%).

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GH43B6                          HGEVLHMKD----VPWVSKQMWAPDCAFK-NNTYYLYFPARDKDGIFRIGVATSSRPEGP 115
C. saccharoperbutylacetonicum   HGEALHLKD----IPWASKQLWAPDAVYK-NGTYYLFFPARDKDEIFRIGVATSSNPAGP 57
C. papyrosolvens                NGEALHMKD----VPWVSKQMWAPDAAFK-NNTYYLYFPARDKDGIFRIGVASSSSPAGP 65
P. lactis                       HGEVLHVKD----VPWAQKQMWAPDAAFK-NDTYYLYFPARDHEGIFRIGVATSSSPSGP 115
P. vortex                       HGQALHVKD----VPWAKKQMWAPDAAYK-NDTYYLYFPARDHNDIFRLGVATSSSPSGP 57
P. curdlanolyticus              HGQALHVKD----VPWASKQMWAPDAAFK-NDTYYLFFPARDHDDIFRIGVATSPSPAGP 57
T. composti                     HGEALHVRD----VPWASKQMWAPDAAFK-NGKYYLYFPARDLEGNFRIGVAVSDKPAGP 111
C. japonicus                    CGVALHVKD----VPWAERQMWAPDAITK-DGKYYLYFPARARDGLFKIGVAIGDQPEGP 58
                                          *               *

GH43B6                          FKAEPNYIEGSYSIDPAVFVDEDNRAYMYFGGLWGGQLEKWQTGEFLGDVTEGPAADAPA 175
C. saccharoperbutylacetonicum   FKAEENYIPGSFSIDPAVLMDDDNRSYVYFGGLWGGQLEKWQTGTFKADA-EGPAVTAPA 116
C. papyrosolvens                FTAQKEPIPGSFSIDPAVLVDDDNRAYIYFGGLWGGQLEKWQTGSFSPDA-EGPDVSAPA 124
P. lactis                       FTPEPNYIPGSFSIDPAVFVDDDNRAYMYFGGLWGGQLEKWQTGTFVPDA-EGPAADAPA 174
P. vortex                       FTPEPDYIPGSYSIDPAVFVDDDNRAYIYFGGLWGGQLEQWQTGSHIPDG-EGPAADAPA 116
P. curdlanolyticus              FEPQPEYIPGSFSIDPAVFVDEDDRAYMYFGGLWGGQLEKWQTDAYVAEP-AEIEPDQPA 116
T. composti                     FKPEPNYIPGSFSIDPAVLVDDDGEAYMYFGGLWGGQLEKWQTGTFNPEG-KEPAPDAPA 170
C. japonicus                    FVAEPEPIAGSYSIDPAVFGDDDGEFYLYFGGIWGGQLQKYRDNTYSEIH-EEPTADQPA 117
                                          *     * *

GH43B6                          IGPRVAELSDDMLSIKGEVKEISIVDENGNPIVAGDEDRRYFEGPWMHKYNGYYYLSYST 235

C. saccharoperbutylacetonicum   LGPRVAELNEDMLTFKESPEEISIVDEDGNPLLAGDEDRRYFEGPWVHKYNGNYYLSYST 176
C. papyrosolvens                IGPRVAELSDDMLTFKEAPEEISIVDEEGNPILAGDEDRRYFEGPWMHKYNGNYYLSYST 184
P. lactis                       LGPRVAELSDDMLTFKAAPQEISIVDENGKPIPAGDEDRRYFEGPWMHKYNGTYYLSYST 234
P. vortex                       IGPRVAELSDDMLTFKDTPQEISIIDENGSPITAGDEERRYFEGPWVHKYNGTYYLSYST 176
P. curdlanolyticus              LGPRVAELSEDMLTFRERPVEIAIVDEQGNALLAGDEERRYFEGPWMHKYKGKYYLSYST 176
T. composti                     LGPRVARLSGDMLTFAETPREVQILDENGEPIKAGDEDRRYFEGPWMHKYNGKYYLSYST 230
C. japonicus                    LGARVARLSADMKSFVEASREVVILDEQGQPLLAGDNSRRYFEGPWMHKYQGKYYLSYST 177
                                          *         *    **        *      *

GH43B6                          GSTHKLVYAMSKNPEGPFVFKGTILTP---------------VIGWTTHHSIVEFQGKWY 280
C. saccharoperbutylacetonicum   GSTHYIVYAMSKNPKGPYTFKGKILDP---------------VIGWTTHHSIVQFQDKWY 221
C. papyrosolvens                GTTHTIVYAVGNNPKGPFVFKGKILTP---------------VVGWTTHHSIVQYQDKWY 229
P. lactis                       GSTHKIVYATSQSPTGPFEYKGTILTP---------------VLGWTTHHSIVEFKNKWY 279
P. vortex                       GSTHQIVYGTSQSPTGPFEFKGTILTP---------------VIGWTTHHSIVQFQDKWY 221
P. curdlanolyticus              GSTHQIVYGTSQSPTGPFEFKGTILTP---------------VIGWTTHHSIVQFQDKWY 221
T. composti                     GTTHKLVYAIGDNPYGPFTYKGVILTP---------------VIGWTTHHSIVEFRGKWY 275
C. japonicus                    GDTHFLCYATSDNPYGPFTYQGQILTP---------------VVGWTTHHSICEFEGKWY 222

GH43B6                          LFYHDSSLSGGVNHKRCVKYTEIKYNEDGTI 311
C. saccharoperbutylacetonicum   LFYHDSSLSGGSDNKRCVKFTELKYNEDGTI 252
C. papyrosolvens                LFYHDSSLSGGRDNKRCVKFTELKYNEDGTI 260
P. lactis                       LFYHDSSLSGGADNKRSVKFTELKYNEDGTI 310
P. vortex                       LFYHDSSLSGGADNKRSVKFTELKYNADGTI 252
P. curdlanolyticus              LFYHDSSLSGGADNKRSVKFTELEYNEDGTI 252
T. composti                     LFYHDASLSGGVNHLRCVKYTELHYNPDGTI 306
C. japonicus                    LFYHDSVLSEGVTHLRSVKVTELHYEADGKI 253
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Fig. 1. Amino acid sequence alignment of the catalytic domain of GH43B6 compared with uncharacterised β-xylosidase/α-L-arabinofuranosidase from *Clostridium saccharoperbutylacetonicum* (YP_007457878.1) and *Cellvibrio japonicus* Ueda107 (YP_00198 1807.1), α-L-arabinofuranosidases from *C. papyrosolvens* (EPR10382.1), *Paenibacillus lactis* (WP_007 127515.1), *P. vortex* (WP_006211951.1) and *P. curdlanolyticus* (WP_006040336.1), and β-xylosidase from *Thermobacillus composti* KWC4 (YP_007212002.1). Conserved regions are highlighted; conserved aspartic acid (D) and glutamic acid (E) are marked with asterisks.

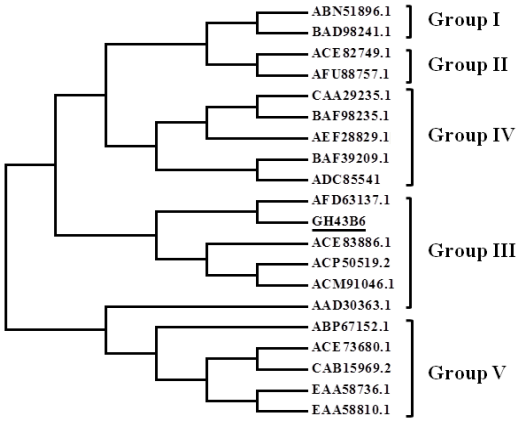

Fig. 2. Phylogenetic tree constructed based on Viborg *et al.* (2013) category by the Neighbor-Joining method of GH43B6 and representatives of the GH family 43. Five distinct groups represent a different specificity: group I consists of galactan 1,3-β-galactosidase; group II consists of α-L-arabinofuranosidase; group III was divided into a cluster of α-L-arabinofuranosidase, β-xylosidase, endo-xylanase, and β-xylosidase/α-L-arabinofuranosidase and another individual branch of endo-xylanase/α-L-arabinofuranosidase; group IV consists of α-L-arabinofuranosidase, β-xylosidase, endo-xylanase, β-xylosidase/α-L-arabinofuranosidase, and β-1,3-xylosidase; and group V consists exclusively of endo-arabinanase. Sequences were designed with the GenBank protein accession number.

Recombinant expression of GH43B6 yielded 71.77% purified protein and a 16.72-fold purification. The purified protein showed a single band of approximately 39 kDa by SDS-PAGE (Fig. 3). The histidyl tag from the pET28b(+) system contributed an extra mass to that which was predicted for the recombinant protein. The GH43B6, which fused with histidyl tag at the C-terminus, retained the enzymatic function indicated that histidyl tag did not interfere the function of GH43B6. The presence of the histidyl tag had no significant effect on protein structure (Carson *et al.* 2007). Thus, biochemical characterization of GH43B6 could be conducted without cleaving of histidyl tag. Determination of the enzymatic activities of GH43B6 showed α-L-arabinofuranosidase, β-xylosidase, and xylanase (Table 1). Furthermore, xylanase activity of GH43B6 on various structural types of xylan exhibited the highest xylanase activity of 2.86 U/mg on xylan from birchwood, and 1.46 U/mg on xylan from oat spelt. Meanwhile, low xylanase activity was observed (0.03 and 0.005 U/mg, respectively) on the highly substituted wheat flour arabinoxylan and rye flour arabinoxylan. The reduced xylanase activity on oat spelt xylan, wheat flour arabinoxylan, and rye flour arabinoxylan may be due to xylooligosaccharides, which exhibit less reducing power than xylose according to the Nelson-Somogyi method. Determination of the optimum pH and temperature for enzyme activities revealed that all enzymatic activities of GH43B6 showed the same optimum hydrolysis condition at pH 6.0 and 50 °C.

Table 1. Substrate Specificity of the Purified GH43B6

Substrate	Specific activity (U/mg protein)
p-Nitrophenyl-α-L-arabinofuranoside	0.30 ± 0.01
p-Nitrophenyl-β-D-xylopyranoside	0.30 ± 0.02
p-Nitrophenyl acetate	ND
p-Nitrophenyl-β-D-glucopyranoside	ND
p-Nitrophenyl-β-D-galactopyranoside	ND
p-Nitrophenyl-β-D-cellobioside	ND
Birchwood xylan	2.86 ± 0.14
Oat spelt xylan	1.46 ± 0.06
Wheat flour arabinoxylan	0.030 ± 0.001
Rye flour arabinoxylan	0.0050 ± 0.0001

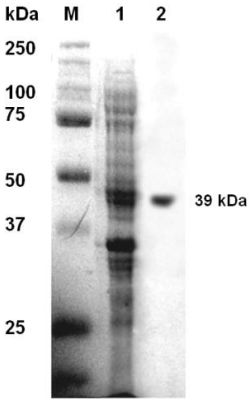

Fig. 3. SDS-PAGE of GH43B6 purified via Ni-NTA columns. Lanes M, 1, and 2 represent a molecular mass marker, a cell free extract, and purified GH43B6, respectively.

Elucidation of GH43B6 hydrolysis action was performed on xylohexaose (X_6), xylobiose (X_2), and xylans. GH43B6 predominantly produced xylose as a hydrolysis product from X_6 (Fig. 4a). The GH43B6 immediately converted X_6 to xylopentaose (X_5), and X_5 was suddenly reduced to xylotetraose (X_4). Successive reduction of X_6 to X_4 revealed the continuous accumulation of xylose. However, trace amounts of xylotriose (X_3) and X_2 were observed, while X_6, X_5, and X_4 still remained after the reaction had been processed for 1 h (Fig. 4a, lane 11). Although reduction of X_2 to xylose occurred slowly, xylose was obviously detected after a 16-h hydrolysis, while small amounts of X_2 remained (Fig. 4b). Predominant and continuous increasing of xylose from X_6 hydrolysis indicated the exo-acting activity of β-xylosidase. GH43B6 cleaved one xylose from the end of a long chain substrate, such as X_6 and X_5, faster than from a short chain substrate (X_2). Generally, true β-xylosidases prefer xylobiose (Wong *et al.* 1988) and the affinity of the enzyme toward xylooligosaccharides decreases with increasing degrees of polymerisation (Bajpai 1997). In contrast to common β-xylosidases, exo-β-xylosidase of GH43B6 showed weak activity against xylobiose and preferably hydrolysed xylooligomers with a degree of polymerisation higher than four. However, the existence of GH43 with a broad xylooligosaccharide specificity toward X_2-X_6 has been reported (Lagaert *et al.* 2011).

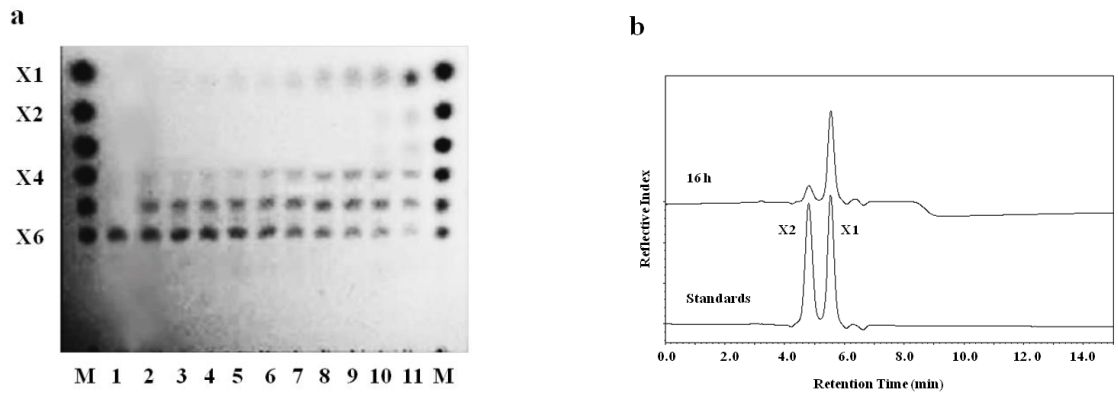

Fig. 4. Chromatograms of the hydrolysis products of xylohexaose (a) and xylobiose (b) by GH43B6. Standard xylose and xylooligosaccharides, X2-X6 are marked by M; lanes 1 through 6 represent hydrolysis products from 0 to 5 min; lanes 7 through 10 represent hydrolysis products from 10, 15, 20, and 30 min; lane 11 represents hydrolysis products at 1 h. Hydrolysis products of xylobiose were analysed via HPLC.

The hydrolysis action of GH43B6 was further elucidated when low branching xylan from birchwood (Fig. 5a) showed that xylose was detected in the early stage of hydrolysis and continuously accumulated, which also indicated the action of exo-β-xylosidase. After 10 min of hydrolysis, xylooligosaccharides were observed between X_4 and X_5 (Fig. 5a, lanes 5 through 7), which implied the presence of an endo-acting mode of GH43B6. Based on xylan structure, xylan from birchwood had the simplest structure, containing trace numbers of substitution (Adams *et al.* 2004). This may suggest that exo-β-xylosidase continuously cleaved one xylose from the end of the xylan chain, and its function was changed to endo-xylanase when GH43B6 was obstructed by the substituted side chain. Based on this evidence, the substituted side groups of xylan may have influenced the enzymatic functions of GH43B6. In addition, 16-h hydrolyses of various structural types of xylans showed that birchwood xylan yielded predominantly xylose and xylooligosaccharides larger than X_5 (Fig. 5b, lane 1), while hydrolysis products from oat spelt xylan resulted in xylose and xylooligosaccharides of sizes between those of X_2 to X_5 (Fig. 5b, lane 2).

Detection of xylooligosaccharides and xylose from oat spelt xylan hydrolysis indicated that GH43B6 exhibited endo-xylanase and β-xylosidase activities. This may result from the structure of oat spelt xylan, which contains more substitution than xylan from birchwood (Puls *et al.* 1987); this makes it difficult for GH43B6 to attack near the substituted end and facilitates its move to the inner chain. Thus, the dominant function of GH43B6 was endo-xylanase activity. Moreover, predominant hydrolysis products resulting from the highly substituted xylan, rye flour arabinoxylan that contains both α-1,3 and α-1,2 monosubstitutions and O2, O3 disubstituted α-L-arabinofuranosides were xylooligosaccharides larger than X_5 (Pitkänen *et al.* 2009) (Fig. 5b, lane 3) and trace amounts of xylose. This also reinforced the mode of action of GH43B6 as mainly that of an endo-xylanase. The arabinose/xylose ratio of rye flour arabinoxylan is 38/62 (Megazyme). As a result of a high degree of side chain decoration, a substituted side group of xylan could limit the exo-β-xylosidase activity of GH43B6, thereby allowing for its endo-xylanase function. These results revealed that the type and frequency of substituted side chains on xylan might influence GH43B6 function.

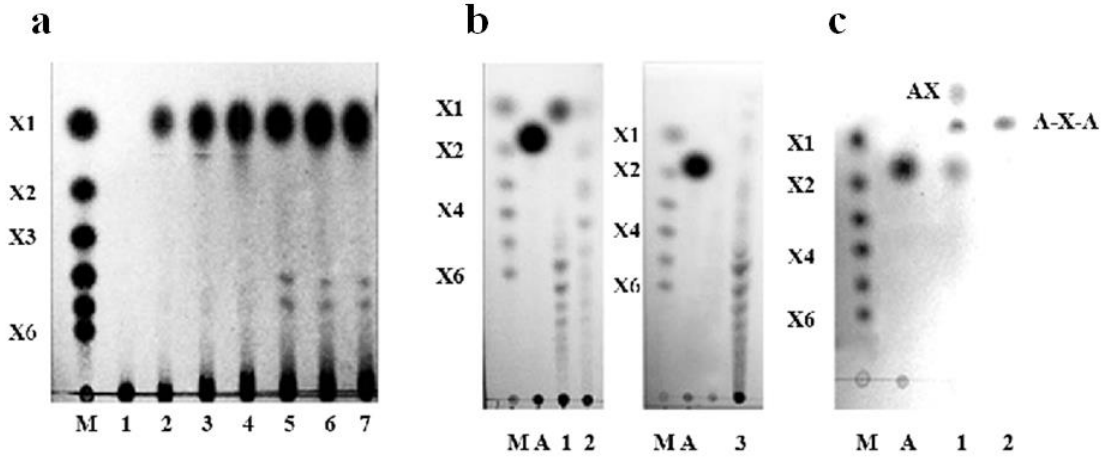

Fig. 5. Chromatograms of xylans and A-X-A hydrolysed by GH43B6. Monitoring of xylan from birchwood hydrolysis products are shown in panel (a). Standard xylooligosaccharides, X1 to X6 are marked by M; lanes 1 through 4 represent hydrolysis products at 0, 1, 2, and 4 min, respectively; lanes 5 through 6 represent hydrolysis products at 10 and 15 min; and lane 7 represents hydrolysis products at 1 h. The 16-h hydrolysis products from xylans are shown in panel (b), birchwood (lane 1), oat spelt (lane 2), and rye flour arabinoxylan (lane 3). α-L-Arabinofuranosidase was determined (c), A-X-A hydrolysis products at 16 h (lane 1) were compared with those of standard A-X-A (lane 2). Standard arabinose is marked by A.

Determination of α-L-arabinofuranosidase activity of GH43B6 showed that there was no arabinose released from the hydrolysis of arabinoxylans from oat spelt and rye flour. Conversely after a 16-h hydrolysis, GH43B6 could partially hydrolyse A-X-A to arabinose and AX (Fig. 5C, lane 1), whereas GH43B6 could release arabinose from aryl-arabinoside, *p*NPA, within 30 min. This suggested that arabinose liberation depends on the chain length of the substrate. GH43B6 preferred only short-chain substrates, which differed from the long chains preferred by GH43 α-L-arabinofuranosidase from *Bacillus licheniformis* SVD1 (Sakka *et al.* 2012) and the broad chain lengths preferred by GH43 α-L-arabinofuranosidase from a compost starter mixture (Wagschal *et al.* 2009). A comparison between A-X-A and aryl-arabinoside revealed that GH43B6 could release arabinose from aryl-arabinoside faster than from A-X-A. This result suggested that the orientation and/or types of backbones forming between arabinosyl and an aromatic ring or xylose might have different effects on GH43B6 arabinofuranosidase activity. In addition, aryl-arabinoside containing an α-1,4 glycosidic linked arabinoside on the aromatic ring might be more easily hydrolysed than the α-1,2 and 1,3 disubstitution on xylose of A-X-A. However, the specificity of the arabinosyl position on AX was not examined. Arabinofuranosidases are categorised by the specificity of the arabinosyl positions, which are specific on α-1,2 and 1,3 monosubstitution (Bourgois *et al.* 2007; Lagaert *et al.* 2010), and only on α-1,3 of diarabinosyl substitution (van den Broek *et al.* 2005, Sørensen *et al.* 2007).

Even though multiple sequence alignment indicated a low similarity between the amino acid sequence of GH43B6 and other GH43 enzymes, conserved residues (aspartate and glutamate in particular) were identified. The catalytic action of GH43 proceeds *via* an inverting mechanism with two aspartates playing a role as the general base and pK_a modulator, respectively, and one glutamate playing a role as a general acid (Pitson *et al.* 1996). Nurizzo *et al.* (2002) established that aspartates 38 and 158, and glutamate 221 are involved in the catalytic mechanism of GH43 α-L-arabinofuranosidase from *Cellvibrio japonicus*. Furthermore, Shallom *et al.* (2005) found that aspartate 14 and glutamate 187 were general bases and general acid catalytic residues of GH43 β-D-xylosidase from *Geobacillus stearothermophilus* T-6, respectively. Moreover, both catalytic actions of bifunctional β-xylosidase/α-L-arabinofuranosidase from *Cel. japonicus* Ueda 107 were also driven by aspartates and glutamate. (Cartmell *et al.* 2011) These results revealed that the monofunctional and bifunctional activities of GH43 are driven by the same catalytic residues. Hence, analysis of the sequence homology with known structures GH43 was further conducted. GH43B6 was compared in terms of the amino acid sequence homology via SWISS-MODEL automated protein structure homology-modelling server (Bordoli *et al.* 2008). GH43B6 showed the highest identity at 53.48% homologue with β-xylosidase RS223 from an uncultured organism (PDB code: 4mlg) and 40% identity homologue with bifunctional β-xylosidase/α-L-arabinofuranosidase from *Cel. japonicus* Ueda107 (CJA_3018) (PDB code: 3qed) (DeBoy *et al.* 2008). Nevertheless, GH43B6 exhibits multi-enzymatic functions; therefore, we selected β-xylosidase/α-L-arabinofuranosidase CJA_3018 (*Cj*Abf43A) to study the comparative analysis. The study of CJA_3018 indicated that Asp41, Asp168, and Glu215 exhibit a significant role in catalytic residues. Moreover, Trp103, Ile167, His267, and Arg295 were shown to play a role in substrate binding sites (Cartmell *et al.* 2011). The comparative analysis of amino acid sequences of GH43B6 and CJA_3018 revealed that although GH43B6 showed low sequence homology with CJA_3018, all key catalytic residues (Asp41, Asp168, and Glu215) and amino acids in substrate binding sites (Trp103, Ile167, His267, and Arg295) were conserved (Fig. 6). Moreover, GH43B6 contains only one GH43 catalytic domain and the optimal conditions of all enzymatic activities of GH43B6 were the same. Additionally, Ferrer *et al*. (2012) suggested that although the

catalytic action of GH43 from a fiber-adherent microbial community from a dairy cow rumen was achieved *via* the same key residues as other GH43, promiscuous enzymatic activities occurred by the atypical folding of the active site. Atypical folding of the active site enhanced the flexibility of the active site for binding with various types of substrates such as, *p*NPA, *p*-NP-α-arabinopyranoside, *p*NPX, *p*NPGal, *p*NPG, *p*-NP-α-D-maltoside, 1,4-β-xylooligosaccharides (X_1-X_6), 1,5-α-L-arabinooligosaccharides (A_2-A_4), maltooligosaccharides (M_2-M_7), lactose, raffinose, and stachyose. These evidences lead to the logical conclusion that multifunctional properties of GH43B6 were driven by one active site. The significant difference in amino acid sequences contributes to the promiscuous characteristics of GH43B6, which may be caused by atypical folding of the binding site within the active site, compared to other GH43 enzymes.

```
CJA_3018        MTTSLNSRRLWLHRLCALLLGTGSALVQAENPIFTDVFTADPAALVHKGRVYLYAGRDEA 60
GH43B6          ----------------------MDNKPVKPNQPLVTHIYTADPSAHVFEGKIYIYPSHDID 39
                                      ..  *:.::*:.*.::****:* *.:*::*:*..:*

CJA_3018        P-----DNTTFFVMNEWLVYSSDDMAN-WEAHGPGLRAKDFTWAKGDAWASQVIERNGKF 114
GH43B6          HDGPDNDNGDQYKMEDYHVLSLDDFNSPCVDHGEVLHMKDVPWVSKQMWAPDCAFKNNTY 99
                      **   : *::: * * **: .    **  *: **..*.. : **.:   :*..:

CJA_3018        YWYVTVRHDDTKPGFAIGVAVGDSPIGPFKDALGKALITNDMTTDTPIDWDDIDPSVFID 174
GH43B6          YLYFPARDKD--GIFRIGVATSSRPEGPFKAEP--NYIEGSYS---------IDPAVFVD 146
                * *...*..*    * ****... * ****       * .. :         ***:**:*

CJA_3018        DDGQAYLFWG----------------------------NTRPRYAKLKKNMVELDGPIRA 206
GH43B6          EDNRAYMYFGGLWGGQLEKWQTGEFLGDVTEGPAADAPAIGPRVAELSDDMLSIKGEVKE 206
                :*.:**:::*                                ** *:*..:*:.:.* ::

CJA_3018        IEGLP-------------EFTEAIWVHKYQDNYYLSYAMGFPEKIGYAMGKSIKGPWVYK 253
GH43B6          ISIVDENGNPIVAGDEDRRYFEGPWMHKYNGYYYLSYSTGSTHKLVYAMSKNPEGPFVFK 266
                *. :              .: *. *:***:. *****: * ..*: ***.*. :**:*:*

CJA_3018        G-ILNEVAGNTPTNHQAIIEFNNKHYFIYHTGAGRPDGGQYRRSVSIDELFYNPDGTIKR 312
GH43B6          GTILTPVIG--WTTHHSIVEFQGKWYLFYHDSS-LSGGVNHKRCVKYTEIKYNEDGTIQM 323
                * **. * *   *.*::*:**:.* *::** .:  ..* :::*.*.  *: ** ****:

CJA_3018        IVMTTEGVAPNKSPERVKKAAK 334
GH43B6          LDYDKLK--------------- 330
                :   .
```

Fig. 6. Comparative analysis of amino acid sequence of GH43B6 with the bifunctional β-xylosidase/α-L-arabinofuranosidase from *Cel. japonicus* Ueda107 (CJA_3018, GenBank ID ACE83886.1) accomplished with ClustalW2 (Larkin *et al.* 2007). The conserve catalytic residues were indicated in box and amino acids in substrate binding sites were shaded in gray.

In summary, this study is the first report of GH43 exhibiting a new combination of exo- and endo-activity together with the side chain removing activity of exo-β-xylosidase/endo-xylanase/α-L-arabinofuranosidase. The different mode of action of GH43B6 from the endo-xylanases suggests that GH43B6 may be a good accessory enzyme in xylanolytic enzyme cocktails to boost the hydrolysis efficiency for the bioconversion of biomass.

CONCLUSIONS

1. Glycoside hydrolase family 43 from *P. curdlanolyticus* strain B-6 (GH43B6) exhibited multifunctional properties including a combination of exo- and endo-acting enzymatic activity together with xylanolytic debranching enzymes.
2. GH43B6 showed an exo-acting function toward xylooligosaccharides and low-substituted xylan.
3. Substituted side chains of xylan influenced the change in the mode from exo- to endo-action of GH43B6.
4. α-L-Arabinofuranosidase of GH43B6 could release arabinose from short-chain substrates, α-L-ara*f*-(1→2)-[α-L-ara*f*-(1→3)]-β-D-xyl*p* and *p*-nitrophenyl-α-L-arabinofuranoside.

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