Biochimica et Biophysica Acta 1830 (2013) 5075–5086

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Mode of action of acetylxylan esterases on acetyl glucuronoxylan and acetylated oligosaccharides generated by a GH10 endoxylanase

Peter Biely [a,*], Mária Cziszárová [a], Iveta Uhliariková [a], Jane W. Agger [b], Xin-Liang Li [c], Vincent G.H. Eijsink [b], Bjorge Westereng [b,d]

[a] Institute of Chemistry, Center of Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 84538 Bratislava, Slovakia
[b] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway
[c] Youtell Biotech Inc., Bothell, WA, USA
[d] University of Copenhagen, Faculty of Science, Rolighedsvej 23, 1958 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 15 May 2013
Received in revised form 15 July 2013
Accepted 17 July 2013
Available online 24 July 2013

Keywords:
Acetylxylan esterase
Carbohydrate esterase family
Positional specificity
Acetyl glucuronoxylan
MALDI ToF MS
NMR

ABSTRACT

Background: Substitutions on the xylan main chain are widely accepted to limit plant cell wall degradability and acetylations are considered as one of the most important obstacles. Hence, understanding the modes of action of a range of acetylxylan esterases (AcXEs) is of ample importance not only to increase the understanding of the enzymology of plant decay/bioremediation but also to enable efficient bioconversion of plant biomass.
Methods: In this study, the modes of action of acetylxylan esterases (AcXEs) belonging to carbohydrate esterase (CE) families 1, 4, 5 and 6 on xylooligosaccharides generated from hardwood acetyl glucuronoxylan were compared using MALDI ToF MS. Supporting data were obtained by following enzymatic deacetylation by ^{1}H NMR spectroscopy.
Conclusions: None of the used enzymes were capable of complete deacetylation, except from linear xylooligosaccharides which were completely deacetylated by some of the esterases in the presence of endoxylanase. A clear difference was observed between the performance of the serine-type esterases of CE families 1, 5 and 6, and the aspartate-metalloesterases of family CE4. The difference is mainly due to the inability of CE4 AcXEs to catalyze deacetylation of 2,3-di-*O*-acetylated xylopyranosyl residues. Complete deacetylation of a hardwood acetyl glucuronoxylan requires additional deacetylating enzyme(s).
General significance: The results contribute to the understanding of microbial degradation of plant biomass and outline the way to achieve complete saccharification of plant hemicelluloses which did not undergo alkaline pretreatment.

1. Introduction

Hardwood glucuronoxylan and cereal arabinoxylans are partially acetylated [1–3]. Consequently, all procedures for biorefining of plant biomass that do not involve alkaline pretreatments will generate partially acetylated hemicellulose fractions. Complete hydrolysis of, e.g. hardwood acetyl glucuronoxylan, not only requires glycoside hydrolases (endo-β-1,4-xylanases, α-glucuronidases, β-xylosidases) but also deacetylating enzymes, called acetylxylan esterases (AcXEs) [4]. The role of the deacetylating enzymes is to create new sites for productive binding of glycoside hydrolases, thus enabling complete hydrolysis. Deacetylation is complicated by considerable variation regarding the position of the acetyl group and substitution of the vicinal hydroxyl group (Fig. 1). A major portion of Xyl*p* residues is monoacetylated at position 2 or 3, or 2,3-di-*O*-acetylated. 3-*O*-acetylation frequently accompanies the substitution of Xyl*p* residues with MeGlcA [5–7]. So far, AcXEs have been found in four of the carbohydrate esterase (CE) families defined in the CAZy database [8], namely families 1, 4, 5 and 6 [4].

Which esterases are needed to achieve complete deacetylation of xylan poly- or oligosaccharides still remains to be elucidated and there is a clear need for studying the action of various AcXEs on natural substrates. So far, literature data are scarce. AcXEs of *Chrysosporium lucknowense* belonging to CE families 1 and 5 were found to be capable

Abbreviations: AcXE, acetylxylan esterase; CE, carbohydrate esterase; Xyl*p*, D-xylopyranose or D-xylopyranosyl; Xyl_2–Xyl_7, β-1,4-xylobiose–β-1,4-xyloheptaose; MeGlcA, 4-*O*-methyl-D-glucuronic acid or 4-*O*-methyl-D-glucuronosyl; $MeGlcA^3Xyl_3$, 4-*O*-methyl-D-glucuronosyl–α-1,2-D-xylopyranosyl–β-1,4-xylopyranosyl–β-1,4-xylopyranose (the upper index indicates the number of the xylosyl residues from the reducing end substituted with MeGlcA); Xyl_xAc_y, acetylated β-1,4-xylooligosaccharide containing x xylose residues and y acetyl groups; $MeGlcAXyl_xAc_y$, acetylated aldouronic acid containing one MeGlcA, x xylose residues and y acetyl groups; $HexXyl_xAc_y$, oligosaccharide containing one hexopyranose residue of unknown nature, x xylose residues and y acetyl groups; $UXyl_xAc_y$, oligosaccharide containing an unknown component U, x xylose residues and y acetyl groups

* Corresponding author. Tel.: +421 2 59410275; fax: +421 2 59410222.
E-mail address: chempbsa@savba.sk (P. Biely).

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.07.018

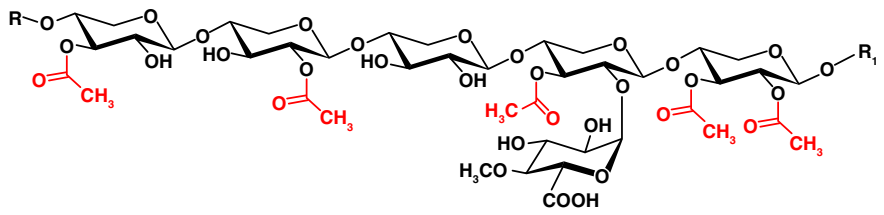

Fig. 1. Four types of acetylation of Xyl*p* residues in hardwood acetyl glucuronoxylans. From the left: 3-*O*-acetyl-, 2-*O*-acetyl-, 3-*O*-acetyl-2-*O*-MeGlcA- and 2,3-di-*O*-acetyl-Xyl*p*.

of removing acetyl groups from both positions 2 and 3 of linear xylooligosaccharides [9]. This conclusion was based on monitoring the deacetylation of linear oligosaccharides by MALDI ToF MS in combination with capillary electrophoresis of derivatized products [9]. The mode of action of *Streptomyces lividans* CE4 and *Orpinomyces* sp. CE6 AcXEs on birchwood acetyl glucuronoxylan has been studied by ^{1}H NMR spectroscopy [10]. The release of individual acetyl groups was monitored by measuring two sets of signals, one in the anomeric region of the spectrum, and the other with the signals of the acetyl methyl groups [5–7,10]. The targets of both enzymes were 2- and 3-*O*-monoacetylated Xyl*p* residues. The *Orpinomyces* sp. esterase also attacked the 2,3-di-*O*-acetylated Xyl*p* residues [10]. Both enzymes ignored the 3-*O*-acetyl group on Xyl*p* residues that were α-1,2-substituted with MeGlcA. It is interesting that AcXEs, in general, showed dual positional specificity on acetylated methyl glycosides [11–13], but strong selectivity for position 2 on monoacetyl derivatives of 4-nitrophenyl glycosides [4,14].

Taking together the current status of knowledge and the increasing need for controlled and efficient conversion of hemicelluloses, the following pertinent questions arise: i) How do the various AcXEs, belonging to different CE families, perform on acetyl glucuronoxylan in the presence or absence of endo-β-1,4-xylanases?, ii) Will endoxylanase cleavage of the xylan main chain make all acetyl groups accessible to esterases?, iii) What is the mechanism allowing some of the enzymes to deacetylate two different positions on Xyl*p* residues? and iv) Is the apparent positional flexibility of some of the enzymes due to migration of the acetyl group between positions 2 and 3?

To answer these questions and to provide a view on AcXE variability, we have studied the actions of enzymes belonging to CE families 1, 4, 5 and 6 on acetylated xylooligosaccharides generated from aspen acetyl glucuronoxylan by a GH10 endoxylanase. We found that the NMR technique introduced for studying AcXE mode of action on a polymeric substrate [10] cannot be applied to a mixture of acetylated xylooligosaccharides due to excessively complex spectra. Therefore, MALDI ToF MS was adopted for product analysis. After resolving the acetylated xylooligosaccharides in the starting material, they were treated with different AcXEs in the absence or presence of an endo-xylanase and changes in the degree of acetylation were monitored. The NMR approach was used for monitoring the rates of specific deacetylation processes for those enzymes that had not previously been studied in this manner. Apart from illustrating differences between the various AcXE types, the results confirmed the interplay between xylanases and esterases. Maximum deacetylation required the presence of an active endoxylanase further degrading the partially deacetylated xylooligosaccharides. Complete deacetylation of all oligosaccharides could not be achieved. The resistant acetyl groups have been tentatively identified based on knowledge of: i) the architecture of GH10 xylanases, ii) the known catalytic properties of AcXEs on artificial substrates such as acetylated glycosides, and iii) the positional specificity of the esterases on polymeric substrates as demonstrated by ^{1}H NMR spectroscopy. It appears that xylooligosaccharides contain additional resistant acetyl groups which are not attacked by the typical AcXEs. The combination of existing and newly generated data discussed below shows that complete removal of the acetyl groups from acetyl glucuronoxylan requires deacetylases with specificities that have not yet been detected.

2. Materials and methods

2.1. Polysaccharides

Birchwood acetyl glucuronoxylan prepared by steam explosion of birchwood saw dust was supplied by Dr. Henry Schneider (NRCC, Ottawa, Canada). Aspen acetyl glucuronoxylan with an approximate mass distribution from 500 Da to 2500 Da (roughly estimated from MALDI-TOF MS analyses) was prepared by steam explosion (at 200 °C for 10 min) of milled aspen wood (milled on a Retsch mill with a 500 μm sieve) using the experimental setup described by Horn et al. (2011) [15]. The resulting soluble material was purified and concentrated by ultrafiltration on a tangential flow ultrafiltration unit (Pellicon, Millipore) collecting the permeate in the first filtration step (10 kDa cut-off) and the retentate in a subsequent filtration with a 1 kDa cut-off membrane. The xylan containing retentate was collected and lyophilized.

2.2. Enzymes

Endo-β-1,4-xylanase of GH10 family from *Clostridium thermocellum* (Xyn Z or *Ct*GH10) was a recombinant enzyme prepared as described below. A DNA sequence coding for residues 511 to 837 of *Ct*GH10 [16] was synthesized. To facilitate cloning of the sequence into pET-24b(−) (Invitrogen Corp., Carlsbad, CA), BamHI and XhoI sites were added on the 5′ and 3′ ends, respectively, leading to a total fragment size of 991 bp, which was synthesized by GenScript Corp. (Piscataway, NJ). After cloning of the gene into pET-24b(−) and verification of the sequence by sequencing with T7 promoter and terminator oligonucleotides as primers, *Escherichia coli* HMS 174 (DE3) competent cells were transformed with the plasmid and four primary clones were analyzed for xylanase production after induction in liquid culture and disruption of the cells. The sonicated supernatant of all the four cultures displayed activity against birchwood xylan. One liter culture of the highest xylanase-producing clone was prepared, and a clarified cell-free supernatant was subject to purification using a prepacked nickel affinity column (GE Life Science USA, Piscataway, NJ, USA) controlled by an AKTAbasic System (GE). The 35-kDa His-tagged xylanase was further purified by anion exchange chromatography with a MonoQ HR 16/10 column (GE) using 50 mM Tris/HCl, pH 7.5 for binding; proteins were eluted by a gradient of 0–0.5 M NaCl in 50 mM Tris/HCl, pH 7.5. The flow rate was 3.0 ml/min and fractions of 1.5 ml each were collected. Fractions containing a high level of xylanase activity were pooled and concentrated using a Centricon Plus tube (Millipore Co., Billerica, MA, USA).

The CE1 AcXE from *Schizophyllum commune* (*Sc*CE1) was purified as described [11]. Purified CE4 AcXE from *S. lividans* (*Sl*CE4) [17] was kindly supplied by Drs. Claude Dupont and Dieter Kluepfel (Institute of Armand Frappier, Laval, Canada). A similar enzyme from *C. thermocellum* (*Ct*CE4) [18] was provided by Profs. Carlos M.G.A. Fontes (Universidade Técnica de Lisboa, Portugal) and Gideon J. Davies (University of York, UK). A purified CE5 AcXE from *Trichoderma reesei* (*Tr*CE5) [19] was kindly provided by Prof. Maija Tenkanen (University of Helsinki, Finland). A recombinant CE6 AcXE from *Orpinomyces* sp. (*O*CE6) was from Megazyme Int. (Ireland).