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A novel family of hemicellulolytic α-glucuronidase

Olena Ryabova [a], Mária Vršanská [a], Satoshi Kaneko [b], Willem H. van Zyl [c], Peter Biely [a,*]

[a] Institute of Chemistry, Slovak Academy of Sciences, Department of Carbohydrate Enzymology, Dúbravská cesta 9, 845 38 Bratislava 45, Slovakia
[b] National Food Research Institute, 2-1-12 Kannondai, Tsukuba 305-8642, Ibaraki, Japan
[c] Department of Microbiology, Stellenbosch University, Stellenbosch 7600, South Africa

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ABSTRACT

Investigation of the xylanolytic enzyme system of the xylose-fermenting yeast *Pichia stipitis* resulted in the discovery of an extracellular α-glucuronidase efficiently debranching hardwood glucuronoxylan. This activity is not exhibited by more extensively investigated α-glucuronidases of glycoside hydrolase (GH) family 67, operating on substrates in which the uronic acid is linked to the non-reducing xylopyranosyl residues of main chain fragments. The N-terminus of the purified enzyme corresponded exactly to the *P. stipitis* gene ABN67901 coding for a protein of unknown function. BLAST search revealed the presence of similar genes in genomes of other microorganisms. These results lead to the emergence of a new family of α-glucuronidases.

1. Introduction

Efficient hydrolysis of plant xylans using microbial enzymes represents a part of our effort to develop environmentally friendly processes for plant biomass conversion. The enzymes hydrolyzing xylan can be divided into two categories: (i) enzymes degrading the polysaccharide main chain, which is endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) and (ii) enzymes that liberate side chains, the main chain substituents, so called accessory xylanolytic enzymes, that include α-glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72) and feruloyl esterase (3.1.1.73) [1].

There are several types of enzymes hydrolyzing β-D-xylosidic linkages in the main chain. Both endoxylanases and β-xylosidases are found in several glycoside hydrolase (GH) families, which differ in the protein folding and in substrate structure requirements [2]. Some endo-xylanases of GH5, for instance, recognize 4-O-methyl-D-glucuronic acid (MeGlcA) or GlcA xylan side chains as specificity determinants [3,4]. Some of the accessory enzymes also show great diversity in terms of the architecture and substrate specificities [1,5]. α-L-Arabinofuranosidases can be found in five (GH3, 43, 51, 54, 62) GH families. The greatest diversity occurs particularly among acetylxylan esterases classified in seven of 16 carbohydrate esterase families [6].

There is also a great diversity among feruloyl esterases [7], however, their classification is awaiting more data from biochemical, physiological and structural studies. In contrast to the above quoted accessory enzymes, only one GH family, GH67, harbours α-glucuronidases [2]. These α-glucuronidases do not operate on polymeric substrates, glucuronoxylans [8–10]. They liberate MeGlcA or GlcA only from those fragments of glucuronoxylan (aldouronic acids), in which the uronic acid is linked to the non-reducing terminal xylopyranosyl residue (Fig. 1) [8–11]. One such aldouronic acid, Xyl(MeGlcA)–Xyl–Xyl* is generated from glucuronoxylan by family 10 endoxylanases [12]. The GH67 α-glucuronidases also do not attack aryl glycosides of GlcA or MeGlcA [1]. An α-glucuronidase that hydrolyzes aryl α-D-glucuronidase can be found in GH4, however, this enzyme does not recognize as substrates glucuronoxylan or its fragments, and therefore it cannot be considered to be a hemicellulolytic glycosidase [13,14].

The only α-glucuronidase described so far that is capable of liberating MeGlcA side chains from hardwood glucuronoxylan is the enzyme present in the cellulolytic system of the wood rotting fungus *Schizophyllum commune* [15]. The N-terminal sequence of this enzyme has been reported [15], however, it does not match any gene sequences in available databases. The partial sequence also does not match the sequences of a new family of α-glucuronidase that is described in this paper.

Here, we report isolation of an extracellular α-glucuronidase from the xylanolytic system of the xylose-fermenting xylanolytic yeast *Pichia stipitis* CBS 6054 [16] of which the genome sequence became recently available [17]. The *P. stipitis* enzyme liberates

Abbreviations: MeGlcA, 4-O-methyl-D-glucuronic acid; GH, glycoside hydrolase

* Corresponding author. Fax: +421 2 59410 222.
E-mail address: chempbsa@savba.sk (P. Biely).

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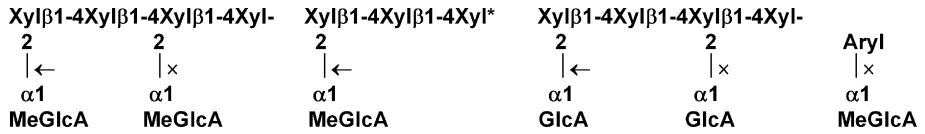

Fig. 1. Glycosidic linkages in fragments of glucuronoxylan attacked (←) and not attacked (×) by GH67 α-glucuronidases. The enzymes do not hydrolyze aryl α-glucuronides, which serve as substrates of non-hemicellulolytic family 4 α-glucuronidases. The asterisk marks the reducing end.

MeGlcA residues linked to terminal or internal xylopyranosyl residues of glucuronoxylan and aldouronic acids generated from the polysaccharide on the action of endoxylanases. The *P. stipitis* CBS 6054 α-glucuronidase was purified, partially sequenced and the sequence used to establish a new family of α-glucuronidase. The α-glucuronidases with such catalytic properties might be of great importance in processes of enzymatic biomass conversion as well as tools for altering rheological properties of glucuronoxylans.

2. Materials and methods

2.1. P. stipitis strain and its cultivation

P. stipitis CBS 6054 was grown in flasks on a medium containing YNB (Difco, 6.7 g/l), L-asparagine (2 g/l), KH_2PO_4 (5 g/l) and carbon source (glucose or beechwood glucuronoxylan, 10 g/l) at a temperature 30 °C and agitation of 180 rpm. Exponential-grown cells were harvested at cell density 1.0–1.5 mg/ml (dry weight).

2.2. α-Glucuronidase substrates and products

Deacetylated glucuronoxylan was extracted from beech sawdust [18]. Aldotetraouronic acid Xyl(MeGlcA)–Xyl–Xyl, the shortest acidic product of glucuronoxylan hydrolysis by family 10 endoxylanases, and aldopentaouronic acid Xyl–Xyl(MeGlcA)–Xyl–Xyl, the shortest acidic product of glucuronoxylan hydrolysis by family 11 endoxylanases were prepared as described earlier [12]. MeGlcA was prepared by de-esterification of its methyl ester by *S. commune* glucuronoyl esterase [19].

2.3. P. stipitis α-glucuronidase production in the absence of xylan

The α-glucuronidase enzyme, prior to purification, was produced in induction experiments which were done as follows. Exponential-phase cells grown in a 1% glucose YNB medium were collected by centrifugation and washed twice with basal YNB medium (without carbon source) and then suspended in the same medium supplied with 0.5 mg/ml of xylooligosaccharides mixture (XYLO-OLIGO 70, Suntory Limited, Japan) and 0.33 mg/ml of methyl-β-xylopyranoside. The cell concentration was 5.0 mg/ml dry weight (105 °C). After 24 h incubation on a shaker (180 rpm) at 30 °C for 24 h the mixture was centrifuged and the clear supernatant was used for purification of extracellular α-glucuronidase which was co-induced with endo-β-1,4-xylanase.

2.4. Purification of P. stipitis α-glucuronidase

The clear induction supernatant (600 ml) was 300-fold concentrated on Amicon 10 kDa cut-off membranes. In the first step, secreted proteins were fractionated by anion-exchange chromatography on a HiTrap DEAE-FF (GE Healthcare, Sweden) column using elution with NaCl gradient (0–1.0 M) in 50 mM sodium-phosphate buffer (pH 7.0). Fractions containing α-glucuronidase, eluted as a peak between 0.2 and 0.25 M NaCl, were pooled, and after concentration and desalting, equilibrated in 50 mM acetate buffer (pH 4.0) containing 2 M $(NH_4)_2SO_4$ and subjected to hydrophobic interaction chromatography on a Butyl-FF column (5 ml) (GE Healthcare) eluted with a decreasing gradient of $(NH_4)_2SO_4$ in the same buffer. α-Glucuronidase was eluted between 1.1 and 0.61 M concentration of $(NH_4)_2SO_4$. The active fractions were pooled, desalted, concentrated and subjected to two additional anion-exchange chromatography steps on a Tricorn MonoQ 5/50GL (GE Healthcare) column (polystyrene/divinylbenzene). In the first step, the column was equilibrated with 50 mM sodium acetate buffer (pH 4.0) and eluted with increasing gradient of NaCl (0–1.0 M). In the second step the acetate buffer was replaced by 50 mM sodium-phosphate buffer (pH 7.0). Active fractions, eluted between 0.25 and 0.3 M NaCl, were desalted and concentrated by membrane filtration on Microcon (10 kDa cut-off, Millipore Co., USA).

2.5. Sequence analysis of purified protein

Purified α-glucuronidase was separated by SDS–PAGE in 10% acrylamide gels and electro-blotted onto a polyvinylidene difluoride membrane (Milipore Corp., USA). The sequence of 15 N-terminal amino acids was determined on an HP G105A protein sequencer (Hewlett–Packard, Palo Alto, CA, USA).

2.6. α-Glucuronidase assay

α-Glucuronidase activity was qualitatively followed by TLC on silicagel (Merck Silica gel 60 on aluminum plates) in ethyl acetate:acetic acid:1-propanol:formic acid:water (25:10:5:1:15, by vol.) on the basis of appearance of free MeGlcA from aldouronic acids (10 mg/ml) or glucuronoxylan (2%) dissolved in 0.05 M sodium acetate buffer (pH 4.4). Enzyme was usually used at concentration 50 μg protein/ml. The substrate and products were detected with N-(naphtylethylenediamine)-dihydrochloride reagent [20]. The reagent gives a brown color with MeGlcA in contrast to a purple color with xylose-containing compounds. Quantitative assay was based on determination of free MeGlcA liberated from aldopentaouronic acid Xyl–Xyl(MeGlcA)–Xyl–Xyl, 10 mg/ml) or beechwood glucuronoxylan (2%) by the method of Milner and Avigad [21]. Protein samples (1–10 μg, depending on purity) were incubated for 10–60 min in 0.1 ml of reaction mixture containing the substrate in 50 mM acetate buffer (pH 4.4). The reaction was stopped by addition of 0.3 ml of the copper reagent and boiling for 10 min at 100 °C, followed by addition of 0.2 ml of the Nelson reagent and 0.4 ml of water. Absorbance was measured at 600 nm using calibration with GlcA. One unit of α-glucuronidase is defined as the amount of enzyme producing 1 μmol of uronic acid in 1 min from aldopentauronic acid Xyl–Xyl(MeGlcA)–Xyl–Xyl.

2.7. Other procedures

Protein was determined according to Bradford [22] using bovine serum albumin as standard. Protein molecular weight was determined by SDS–PAGE [23] using unstained protein molecular weight markers (FERMENTAS, Canada) and dyed protein markers (SERVA, GmbH). IEF was performed on Multiphor II system (GE Healthcare, Sweden) using SERVALYT PRECOTES 3–6 precast gels and IEF markers 3–10 (SERVA, GmbH).