FEBS Letters 580 (2006) 4597–4601

Glucuronoyl esterase – Novel carbohydrate esterase produced by *Schizophyllum commune*

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Received 22 June 2006; revised 12 July 2006; accepted 13 July 2006

Available online 21 July 2006

Edited by Ulf-Ingo Flügge

Abstract The cellulolytic system of the wood-rotting fungus *Schizophyllum commune* contains an esterase that hydrolyzes methyl ester of 4-*O*-methyl-D-glucuronic acid. The enzyme, called glucuronoyl esterase, was purified to electrophoretic homogeneity from a cellulose-spent culture fluid. Its substrate specificity was examined on a number of substrates of other carbohydrate esterases such as acetylxylan esterase, feruloyl esterase and pectin methylesterase. The glucuronoyl esterase attacks exclusively the esters of MeGlcA. The methyl ester of free or glycosidically linked MeGlcA was not hydrolysed by other carbohydrate esterases. The results suggest that we have discovered a new type of carbohydrate esterase that might be involved in disruption of ester linkages connecting hemicellulose and lignin in plant cell walls.

Keywords: Glucuronoyl esterase; Methyl ester of 4-*O*-methyl-D-glucuronic acid; Lignin-carbohydrate complex; *Schizophyllum commune*

1. Introduction

The literature contains information on existence of three types of covalent linkages between lignin and hemicelluloses in plant cell walls [1,2]. The first type involves *p*-coumaric or ferulic acid, linked etherically to lignin, and esterically to hemicellulose sugars [3]. This linkage could be theoretically cleaved by feruloyl esterases (EC 3.1.1.73). The second type is represented by ether linkages between OH-groups of saccharides and lignin alcohols [4]. The third type involves ester linkages between 4-*O*-methyl-D-glucuronic acid (MeGlcA) or D-glucuronic acid residues of glucuronoxylans and hydroxyl groups of lignin alcohols [5] (Fig. 1A). This ester linkage has so far been proven only indirectly, and in two ways. One technique uses *in situ* ester reduction with sodium borohydride, leading to conversion of MeGlcA to 4-*O*-methyl-D-glucose [6–9]. 4-*O*-methyl-D-glucose cannot be found in the acid hydrolysate of the tissue before the reductive treatment. The second approach involves oxidative cleavage of fully acetylated uronic acid ester with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, affording free uronic acid and aldehyde or ketone, depending on the character of the alcohol (primary or secondary) involved in the ester linkage [5,10].

Interesting, that the scientific literature does not contain any mention about esterases cleaving the linkages between aromatic alcohols of lignin and the carboxyl group of MeGlcA or D-glucuronic acid residues. This is the first report on existence of a fungal esterase that might fill such a function. The new enzyme, called glucuronoyl esterase, has been identified on the basis of its ability to hydrolyse methyl ester of free or glycosidically linked MeGlcA and its inability to attack substrates of other hemicellulolytic esterases.

2. Materials and methods

2.1. Glucuronoyl esterase substrates

Methyl ester of reduced aldotetraouronic acid [2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-D-xylitol], was an unexpected neutral side product during the preparation of reduced aldotetraouronic acid used as the substrate in the study of the stereochemistry of substrate hydrolysis by α-glucuronidase [11]. The methyl ester was formed during removal of boric acid from a decationized reaction mixture by evaporation with methanol. Its identity was confirmed by its conversion to the corresponding acid after alkaline treatment and also by enzymatic deesterification.

Methyl 4-*O*-methyl-D-glucopyranuronate [12] and 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside [13] (Fig. 1B) were generous gifts from Dr. Ján Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia).

2.2. Auxiliary enzymes and their assays

α-Glucuronidase (EC 3.2.1.139) from *Aspergillus tubigensis* was provided by Drs. Ronald de Vries and Jaap Visser (Agricultural University of Wageningen, The Netherlands). The list of auxiliary enzymes tested for their ability to hydrolyze the substrates of glucuronoyl esterase, is given in Table 1. The glucuronoyl esterase was tested for several hours or overnight for activity on substrates of acetyl esterase [21], acetylxylan esterase [22,23], feruloyl esterase [24,25] and pectin methylesterase. The glucuronoyl esterase was used at a concentration which caused complete deesterification of methyl 4-*O*-methyl-D-glucopyranuronate within a few min. This corresponded to 0.24 mg ml^{-1} of protein and enzyme activity of 1000 U ml^{-1} (definition of U is given below).

2.3. Glucuronoyl esterase assays

Semiquantitative assay to monitor the esterase activity in column effluents was based on TLC analysis of incubation mixtures (30 °C) containing the tested sample and the substrate, 5 mM methyl 4-*O*-methyl-D-glucopyranuronate, in 50 mM sodium phosphate buffer, pH 6.0 (buffer A). Aliquots were chromatographed on thin-layers of Silica gel 60 (Merck, Germany) in 1-butanol:ethanol:water (10:5:2, v/v) and the liberation of MeGlcA (R_f 0.15) from its methyl ester (R_f 0.75) was visualised with *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent [26].

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Abbreviations: MeGlcA, 4-*O*-methyl-D-glucuronic acid; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylene diamine tetraacetic acid

0014-5793/$32.00
doi:10.1016/j.febslet.2006.07.033

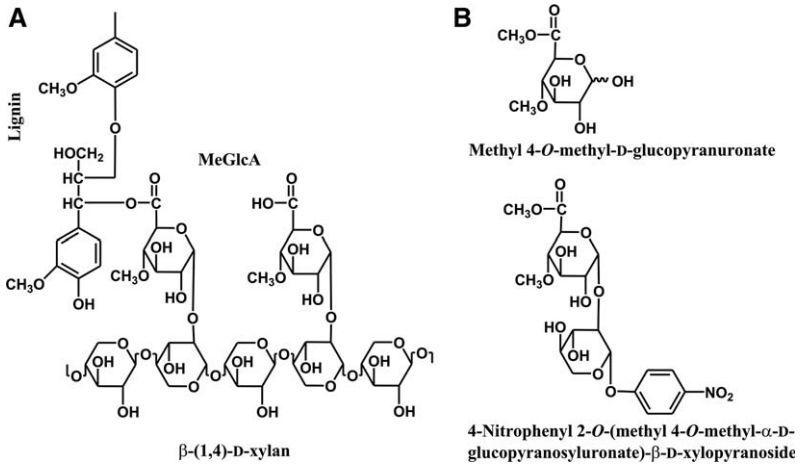

Fig. 1. Part A: Ester linkage between MeGlcA residues of glucuronoxylan and aromatic alcohols of lignin found in plant cell walls. Part B: Formulas of two synthetic compounds used as glucuronoyl esterase substrates.

Table 1
The list of used carbohydrate esterases, pure acetylxylan esterases (EC 3.1.1.72), feruloyl esterases (EC 3.1.1.73) and partially purified fungal and tomato pectin methylesterases (EC 3.1.1.11), and substrates used for the enzyme assays

Esterase	Protein (mg ml^{-1})	Me-MeGlcA (5 mM)	NPhXylMe-MeGlcA (2 mM)	Methyl or ethyl ferulate (2 mM)	NPhAc (2 mM)	Pectin (citrus) (1%)	References to the enzyme source
Glucuronoyl esterase *Schizophyllum commune* [1000 U.mg^{-1}]	0.25	+	+	–	–	–	This paper
FeE *Aspergillus niger*	0.01	–	–	+	+	n.t.	[14]
FeE *Aspergillus oryzae*	0.17	–	–	+	+	n.t.	[15]
FeE *Aureobasidium pullulans*	0.1	–	–	+	+	n.t.	[16]
FeE *Talaromyces stipitatus*	0.08	–	–	+	+	n.t.	[17]
AcXE *Streptomyces lividans*	0.2	–	–	–	–	n.t.	[18]
AcXE *Trichoderma reesei*	0.15	–	–	–	+	n.t.	[19]
AcXE *Schizophyllum commune*	0.17	–	–	–	+	n.t.	[20]
Pectin methylesterase from tomato extract	16.5	–	–	–	+	+	Markovič, O. (this Institute)
Pectinex®Ultra SP-L	6.35	–	–	+	+	+	Novozymes, Denmark

Abbreviations used: Me-MeGlcA, methyl 4-*O*-methyl-D-glucopyranuronate; NPhXylMe-MeGlcA, 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside; NPhAc, 4-nitrophenyl acetate; FeE, feruloyl esterase; AcXE, acetylxylan esterase; +, positive test; –, negative test; n.t., not tested.

Quantitative glucuronoyl esterase assay was based on the measurement of the decrease of 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside concentration due to deesterification. The ester (2 mM) was incubated with glucuronoyl esterase-containing sample in buffer A at 30 °C and its concentration was followed in time by HPLC on a Separon SGX C18, 7 μm column (250 × 4 mm) (Watrex, Czech Republic) eluted with acetonitrile:water (2:1, v/v) at a flow rate 0.7 ml min^{-1} using a UV-detector DVW-10 (D-Star Instruments, Manassas VA, USA) operating at 308 nm. One unit of glucuronoyl esterase activity is defined as the amount of the enzyme deesterifying 1 μmol of 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside in 1 min at 30 °C.

2.4. Purification of glucuronoyl esterase from Schizophyllum commune

The enzyme was purified from 11 day cellulose-spent culture fluid of the wood-rotting fungus *Schizophyllum commune* ATCC 38548 [27], concentrated on a 10 kDa cut-off membrane. The concentrate was

applied to DEAE Sepharose fast flow column (2.5 × 30 cm) (Pharmacia, Uppsala, Sweden), equilibrated with buffer A and eluted with increasing gradient of NaCl (0–0.5 M) in buffer A. Presence of glucuronoyl esterase in fractions was established by TLC assay described above.

Pooled and desalted glucuronoyl esterase active fractions were applied to Phenyl Sepharose column (0.9 × 9 cm) (Pharmacia, Uppsala, Sweden) equilibrated with 1.2 M $(NH_4)_2SO_4$ in buffer A. Adsorbed proteins were liberated from the carrier with decreasing gradient of $(NH_4)_2SO_4$ (1.2–0.0 M) in buffer A. The chromatographic step was repeated and the glucuronoyl esterase active fractions were pooled, concentrated and desalted by ultrafiltration.

2.5. Activity stain on IEF gels

IEF gel with separated proteins (pH range 3–10) was washed twice with 5 mM sodium phosphate buffer, pH 5.5, for 10 min. The gel was then brought into contact with 2.5% agar (DIFCO) detection gel, prepared in 2 mM sodium phosphate buffer, pH 5.5, containing 7.5 mM methyl 4-*O*-methyl-D-glucopyranuronate and 0.06% (w/v) bromcresol green as acid–base indicator. Liberation of MeGlcA at the location of glucuronoyl esterase lowers the pH, which results in a colour change of the indicator from blue to yellow.

3. Results and discussion

3.1. Discovery of glucuronoyl esterase

The novel carbohydrate esterase was discovered during the studies of the stereochemistry of the hydrolysis of glycosidic linkage by α-glucuronidase [11]. The methyl ester of 2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-D-xylitol served as a substrate of purified α-glucuronidase only after alkaline deesterification. This was in consonance with our earlier finding that compounds with 4-*O*-methyl-α-D-glucuronosyl residues esterified with methanol did not serve as α-glucuronidase substrates [28]. However, in contrast to the resistance of the methyl ester to pure α-glucuronidase, the MeGlcA was liberated from the ester by a crude cellulolytic system of the wood-rotting fungus *Schizophyllum commune* containing α-glucuronidase, which suggested that the fungal system contains an enzyme that catalyses deesterification of the 4-*O*-methyl glucuronosyl residue. Our anticipation proved to be correct. Glucuronoyl esterase activity after 11 day growth on cellulose reached about 10 U ml^{-1}. The enzyme is inducible on cellulose since its level in glucose medium was negligible.

3.2. Purification of glucuronoyl esterase

Concentrated cellulose-spent culture fluid was chromatographed first on a column of DEAE Sepharose. Glucuronoyl esterase was found among the adsorbed proteins and was eluted after application of a linear NaCl gradient in a peak with some other proteins at 0.25–0.3 M salt concentration (Fig. 2A). The active fractions, eluted mainly after the two peaks of feruloyl esterase activity (elution volume 1100–1400 ml), were pooled, desalted, concentrated and subjected to two subsequent hydrophobic interaction chromatographies on a Phenyl Sepharose column. Glucuronoyl esterase, applied in a solution of 1.2 M $(NH_4)_2SO_4$, was trapped on the column and liberated with decreasing $(NH_4)_2SO_4$ gradient as a single protein peak at 20 mM $(NH_4)_2SO_4$ concentration (Fig. 2B). In the three steps the glucuronoyl esterase of *S. commune* was purified 110 times in a 2.3% overall yield (Table 2).

3.3. Some properties of glucuronoyl esterase

The purified glucuronoyl esterase was found to be homogenous on SDS–PAGE (Fig. 3A). Its molecular mass was

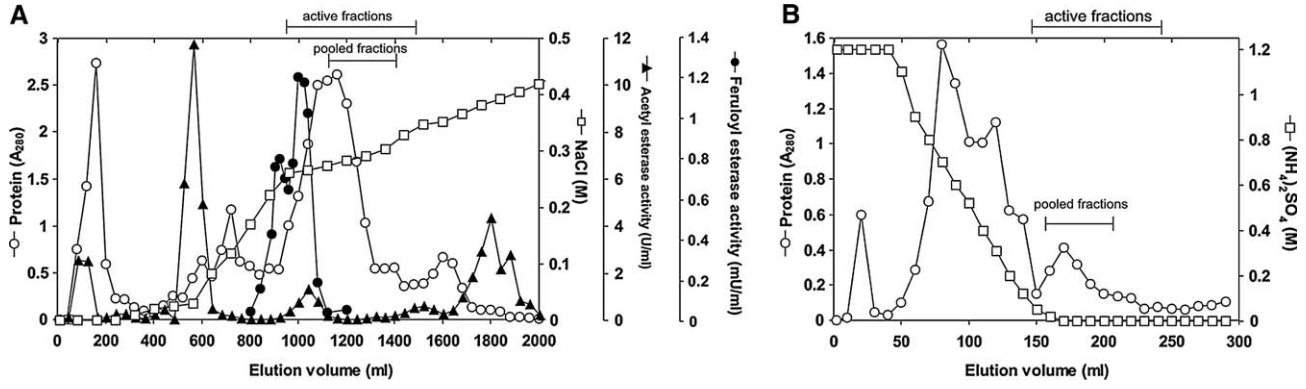

Fig. 2. Part A: Anion exchange chromatography of concentrated cellulose-spent culture medium of *Schizophyllum commune* on a DEAE Sepharose column. Part B: The hydrophobic interaction chromatography on a Phenyl Sepharose column of glucuronoyl esterase active fractions pooled after DEAE Sepharose chromatography. Fractions were tested for protein (○), NaCl or $(NH_4)_2SO_4$ concentration (□), acetyl esterase (▲), feruloyl esterase (●) and glucuronoyl esterase activity using the TLC assay (active and pooled fractions are marked with the bars).

Table 2
Summary of purification of *Schizophyllum commune* glucuronoyl esterase

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Degree of purification
Culture fluid	1000	1120	10 177.9	9.1	100	1
DEAE Sepharose chromatography	8.5	583.7	7 040.3	12.1	69.2	1.3
1st Phenyl Sepharose chromatography	5.3	26.0	2 971.5	114.2	29.2	12.6
2nd Phenyl Sepharose chromatography	1.0	0.24	239.0	1000.4	2.3	110.5

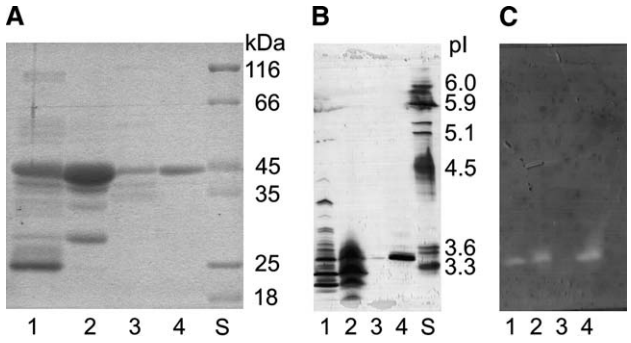

Fig. 3. SDS–PAGE (Part A) and IEF (Part B and C) monitoring of the purification of *Schizophyllum commune* glucuronoyl esterase. Lane 1, concentrated culture filtrate, 30 μg of protein; lane 2, glucuronoyl esterase active fractions obtained after DEAE Sepharose chromatography, 35 μg of protein; lane 3, glucuronoyl esterase active fractions obtained after the first Phenyl Sepharose chromatography, 3 μg of protein; lane 4, purified glucuronoyl esterase obtained after the second Phenyl Sepharose, 5 μg of protein; lane S, standards with molecular masses indicated (Part A) or standards with indicated pI values (Part B). Proteins were detected with Coomassie Brilliant Blue R-250. Activity stain (Part C) based on the liberation of MeGlcA from its methyl ester at the location of glucuronoyl esterase, which results in a colour change of the acid–base indicator.

estimated to be 44 kDa. On IEF the enzyme gave a single protein band of pI value 3.5 that coincided with the enzyme activity (Fig. 3B and C). On 4-nitrophenyl 2-*O*-(methyl 4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside the enzyme showed pH optimum 7 and temperature optimum 50 °C. At pH 6, selected for the enzyme assay to avoid spontaneous deesterification of the substrates at higher pH, the enzyme exhibited 75% of the optimum activity and had K_m 0.31 mM, V_{max} 4.4 μmol min^{-1} mg^{-1} and k_{cat} 3.2 s^{-1}.

As shown in the Table 1, the enzyme did not attack substrates of other carbohydrate esterases. On other hand, none of the tested acetylxylan esterases, feruloyl esterases and pectin methylesterases hydrolyzed the substrates of glucuronoyl esterase (Table 1). In the case of pectin methylesterases it is known that they hydrolyze neither methyl ester of D-galactopyranuronic acid nor the mono- and di-methyl esters of its dimer, but readily attack methyl esters of higher oligogalacturonides and D-galacturonans [29–31]. These data are in accord with our finding that pectin methylesterases were not active on the glucuronoyl esterase substrates, even if they would not discriminate galacturonic acid from MeGlcA.

Glucuronoyl esterase was not inhibited by 1 mM PMSF suggesting that it does not belong to serine-type esterases. The enzyme did not loose activity on addition of 1 mM EDTA as an evidence that it is not a metal-dependent enzyme. At pH 6.0 the enzyme did not bind to cellulose indicating the absence of a cellulose binding module.

At this stage of work we do not have direct experimental evidence for catalytic ability of the enzyme to hydrolyse ester linkages between uronic acids of hemicellulose and hydroxyl groups of lignin-forming alcohols *in situ*. Larger amounts of the enzyme will be required to investigate its biotechnological potential. We do not have sufficiently sensitive assays to test the enzyme action on plant materials. It is highly possible that the action of the enzyme will be apparent only in the presence of other plant cell wall hydrolysing enzymes. However, we shall report soon that the enzyme hydrolyzes other synthetic substrates, D-glucopyranuronates and 4-*O*-methyl-D-glucopyranuronates of phenylalkyl alcohols, supporting the view that the enzyme recognizes the uronic acid residue (Biely et al. in preparation). We do not report the N-terminal amino acid sequence of the isolated enzyme because it was found blocked. Therefore, our current effort is directed toward isolation of larger amount of glucuronoyl esterase and determination of its internal amino acid sequence(s) after trypsin digestion.

Acknowledgements: The authors thank Dr. Ján Hirsch (this Institute) for the glucuronoyl esterase substrates and all colleagues who supplied pure enzymes. This work was supported by a Grant 2/6130/26 from the Slovak Grant Agency VEGA, a Grant from the Slovak Academy of Sciences to the Center of Excellence GLYCOBIOS, and by a Grant from the Slovak Research and Development Agency under the Contract No. APVV-51-003805.

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