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β-Glucuronidase-coupled assays of glucuronoyl esterases

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ABSTRACT

Glucuronoyl esterases (GEs) are microbial enzymes with potential to cleave the ester bonds between lignin alcohols and xylan-bound 4-*O*-methyl-D-glucuronic acid in plant cell walls. This activity renders GEs attractive research targets for biotechnological applications. One of the factors impeding the progress in GE research is the lack of suitable substrates. In this work, we report a facile preparation of methyl esters of chromogenic 4-nitrophenyl and 5-bromo-4-chloro-3-indolyl β-D-glucuronides for qualitative and quantitative GE assay coupled with β-glucuronidase as the auxiliary enzyme. The indolyl derivative affording a blue indigo-type product is suitable for rapid and sensitive assay of GE in commercial preparations as well as for high throughput screening of microorganisms and genomic and metagenomic libraries.

1. Introduction

Plant cell walls are composed of cellulose fibers embedded in a complex network of hemicellulose and pectin or lignin [1]. Lignin, which is a group of highly branched phenylpropanoid polymers derived from sinapyl, *p*-coumaryl and coniferyl alcohols, is covalently bound to hemicellulose in the form of lignin-carbohydrate complexes (LCCs) [2–4]. This linkage is preserved in plant biomass pretreated by non-alkaline procedures such as steam explosion or hot water or mild acid treatment [5–7]. Enzymatic separation of lignin from carbohydrates could reduce recalcitrance of plant biomass, improve extraction of hemicelluloses and suppress the inhibitory effect of lignin on enzymatic saccharification [7–10].

Several lines of evidence suggest that the ester bond between lignin alcohols and 4-*O*-methyl-D-glucuronic acid (MeGlcA) could be the target of glucuronoyl esterase (GEs; EC 3.1.1.-), the carbohydrate esterase discovered ten years ago in the cellulolytic system secreted by wood-rotting fungus *Schizophyllum commune* [11]. The enzyme was found to be homologous to *Trichoderma reesei* Cip2 protein of unknown function, inducible by cellulose and sophorose [12]. Orthologues of *S. commune* GE were identified in a number of microbial genomes [13], that served as the basis for introduction of new carbohydrate esterase family CE15. So far eight CE15 GEs have been purified and biochemically characterized (http://www.cazy.org/CE15_characterized.html). 3-D structures of two of them, *Trichoderma reesei* GE (Cip2) [14] and *Myceliophthora thermophila* GE2 [15], have been solved by X-ray crystallography. The catalytic triad of both esterases, Ser-His-Glu, are located on the surface of the structures, indicating ability to attack ester linkage between high molecular mass components. In fact, the enzymes tolerate both bulky carbohydrate moieties carrying MeGlcA and bulky aromatic alcohols [11,16–19] resembling lignin fragments [20].

The GE activity can be determined on alkyl as well as arylalkyl esters of D-glucuronic acid and MeGlcA [11,20] and also on the corresponding aryl α- and β-glycosides [17] by chromatographic methods (TLC, HPLC). Commercially available benzyl glucuronate can be used for the spectrophotometric determination of GE activity employing quantification of liberated glucuronic acid using the glucuronic acid dehydrogenase kit from Megazyme [21]. There is no report on activity of GEs on natural substrates such as plant cell walls or isolated LCCs, however. The evidence for positive effect of GEs on saccharification process has so far been only indirect. Addition of GEs to cellulolytic systems considerably increased the yield of saccharification of heat-pretreated corn fiber [22]. Interesting changes were observed in plants expressing fungal GEs. Genetically modified *Arabidopsis* and poplar showed altered cell

Abbreviations: CE15 family, carbohydrate esterase family 15; GE, glucuronoyl esterase; GlcA-*p*NP, 4-nitrophenyl β-D-glucuronide; MeGlcA, 4-*O*-methyl-D-glucuronic acid; Me-GlcA-*p*NP, methyl (4-nitrophenyl β-D-glucopyranosid)uronate; Me-GlcA-X, methyl (5-bromo-4-chloro-3-indolyl β-D-glucopyranosid)uronate; Me-MeGlcA, methyl 4-*O*-methylglucopyranuronate.

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wall composition facilitating saccharification [23,24]. These effects stimulate general interest in GEs.

One of the factors impeding the progress in GE research is the lack of suitable substrates. In this work, we report a new way of detection and determination of GE activity on methyl esterified 4-nitrophenyl β-D-glucuronide and 5-bromo-4-chloro-3-indolyl β-D-glucuronide as prochromogenic substrates using β-glucuronidase as the coupling enzyme. The latter substrate affording the blue indigo-type chromophore is particularly convenient for high-throughput screening for GE activity in commercial preparations, culture media, and genomic and metagenomic libraries.

2. Materials and methods

2.1. Enzymes

Ruminococcus flavefaciens, *Trichoderma reesei* and *Schizophyllum commune* GEs were prepared as reported previously [19,11]. Commercial β-glucuronidase IX-A from *Escherichia coli* was purchased from Sigma-Aldrich (St. Louis, MO, USA), recombinant *E. coli* β-glucuronidase (catalogue No. E-BGLAEC) from Megazyme (Bray, Ireland) and β-glucuronidase from the digestive tract of the snail *Helix pomatia* was obtained from Boehringer (Mannheim, Germany). Protein concentration was determined by a dye-binding method [25] using bovine serum albumin as a standard.

2.2. Commercial preparations tested for GE activity

GE-positive preparations: Rohament clicellulase (cellulolytic preparation) and Depol™ (endo-carbohydrase preparation) were products of AB Enzymes (Darmstadt, Germany) and Biocatalysts (Cefn Coed, UK), respectively. Albazyme 40S-4X and Celluclast® 1.5L were purchased from Novo Nordisk (Bagsvaerd, Denmark). Celluclast® 1.5 L (*Trichoderma reesei* cellulase preparation), Dextrozyme® GA 1.5X (glucoamylase preparation), Fungamyl® (α-amylase preparation), Novozym® 735 (lipase preparation), Resinase® HT (lipase preparation), Spirizyme® Ultra (glucoamylase preparation), Suberase®, Ultraflo™ L (*Humicola insolens* β-glucanase/xylanase preparation) and Ultrazym® AFP were obtained from Novozymes (Bagsvaerd, Denmark). Cartazyme HS 10 (xylanase preparation) was supplied by Sandoz (Holzkirchen, Germany).

GE-negative preparations: Papain (Corolase® L10), Rohavin® Color, Rohavin® LX, Rohavin® MX, Veron® 191 and Veron® Special were purchased from AB Enzymes (Darmstadt, Germany). Rapidase AR2000® (*Aspergillus niger* polygalacturonase) was obtained from DSM (Heerlen, the Netherlands). Enzeco® (*Aspergillus niger* lipase concentrate) was a product of Enzyme Development Corporation (New York, NY, USA). Lallzyme Cuvée Blanc (pectinase preparation) and Lallzyme Beta (pectinase and β-glucosidase preparation) were products from Lallemand (Blagnac, France). Amylyve® A30 (*Bacillus subtilis* α-amylase), Lipolyve® AN (*Aspergillus niger* lipase) and Peclyve® LVG were obtained from Lyven (Colombelles, France). Gammanase® 1,5L, Pulpzyme® HA and Pulpzyme® HB were commercial products of Novo Nordisk (Bagsvaerd, Denmark). AMG 300 L (*Aspergillus niger* exo-α-1,4-D-glucosidase), Carezyme® 4500L (cellulase enzyme preparation), Dextrozyme DX 1.5X (*Aspergillus niger* glucoamylase), Lactozym 3000 L HP-G (*Kluyveromyces fragilis* β-galactosidase), Liguanase® 2.5 L, Maltogenase (*Bacillus subtilis* maltogenic amylase), Novozym® 188 (cellobiase preparation), Novozym® 435 (*Candida antarctica* lipase), Pectinex Ultra SP-L (*Aspergillus aculeatus* pectinase), Pectinex® SMASH XXL (pectin lyase preparation), Pectinex® UF (*Aspergillus aculeatus* polygalacturonase), Pectinex® ULTRA MASH (*Aspergillus aculeatus* and *Aspergillus niger* pectin lyase), Pectinex® Yieldmash Extra (*Aspergillus niger* pectin methyl esterase), Pentopan 500 BG (multi-component xylanase), Savinase® 16 L Type EX (protease preparation) and Viscozyme® (*Aspergillus aculeatus* β-glucanase) were purchased from Novozymes (Bagsvaerd, Denmark).

2.3. Substrates

4-Nitrophenyl β-D-glucuronide (GlcA-*p*NP) was purchased from Sigma. 5-Bromo-4-chloro-3-indolyl β-D-glucuronide was obtained from Carbosynth (Compton, United Kingdom) in the form of sodium salt. Methyl 4-*O*-methyl-D-glucopyranuronate (Me-MeGlcA) [11] was a generous gift from Dr. Ján Hirsch (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia). Prochromogenic substrates for GE, i.e. methyl (4-nitrophenyl β-D-glucopyranosid) uronate (Me-GlcA-*p*NP) and methyl (5-bromo-4-chloro-3-indolyl β-D-glucopyranosid)uronate (Me-GlcA-X) were prepared from the corresponding glycosides (Fig. 1). 200 mg of GlcA-*p*NP or 5-bromo-4-chloro-3-indolyl β-D-glucopyranosiduronate were dissolved in 15 ml of freshly distilled methanol and 2 g of a strong cation exchanger Dowex 50WX8 (Acros, Geel, Belgium; H^+ form, extensively washed with freshly distilled methanol and air-dried) were added. The suspension was stirred at 400 rpm and room temperature in the dark. The resin-catalyzed esterification was followed by TLC. After 24 h, when the esterification was completed, the suspension was filtered and the filtrate evaporated to give the desired products.

2.4. β-Glucuronidase assay

β-Glucuronidase activity was determined at 35 °C in 50 mM sodium phosphate buffer, pH 6.0, using 1 mM GlcA-*p*NP as the substrate in a total volume of 0.125 ml. The reaction was started by addition of the enzyme and terminated by addition of 0.750 ml of a saturated solution of $Na_2B_4O_7$, typically after 10 min. Released 4-nitrophenol was determined spectrophotometrically at 405 nm using 1-cm light path cuvettes. One unit of β-glucuronidase activity is defined as the amount of enzyme producing 1 μmol of 4-nitrophenol in 1 min.

2.5. TLC assay of GE

Qualitative detection of GE activity on methyl 4-*O*-methyl-D-glucopyranuronate was conducted by TLC according to Špániková and Biely [11].

2.6. Novel glucuronoyl esterase assays

Standard spectrophotometric GE assay was performed similarly to β-glucuronidase assay using 4 mM prochromogenic substrate, Me-GlcA-*p*NP, in the presence of 200 mU β-glucuronidase from Megazyme. One unit of GE activity was defined as the enzyme amount producing 1 μmol of 4-nitrophenol in 1 min under these conditions.

Cup-plate agarose assay of GE was done as follows. A thin layer of low-melting-point agarose gel (1%, w/v) containing 1 mM Me-GlcA-X in 0.05 M sodium phosphate buffer, pH 6.0, and β-glucuronidase (4 U/ml) in Petri dishes was used in this assay. The agarose was dissolved at 90–95 °C and Megazyme β-glucuronidase and the substrate were added after cooling the agarose solution to 50 °C. After gel solidification the wells were dwelled with the aid of cork borer. Upon thermal equilibration to 35 °C, 10 μl of tested enzyme preparations were added and the plate was incubated at the same temperature in a wet chamber to prevent desiccation. Alternatively, the agarose gel contained the substrate only and the Megazyme β-glucuronidase (20 mU) was pipetted into the wells along with the enzyme preparations tested.