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# Fungal Glucuronoyl Esterases and Substrate Uronic Acid Recognition

Miroslava ĎURANOVÁ, Ján HIRSCH, Katarína KOLENOVÁ, and Peter BIELY†

*Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia*

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**Glucuronoyl esterases are enzymes involved in microbial plant cell-wall degradation. In this study we purified and characterized two recombinant *Phanerochaete chrysosporium* glucuronoyl esterases, *Pc*GE1 and *Pc*GE2. The catalytic activity of these and previously described glucuronoyl esterases was investigated on new synthetic substrates, methyl esters of uronic acids and their glycosides, prepared by esterification with ethereal diazomethane.**

**The data obtained indicate that the enzymes hydrolyzed efficiently not only esters of 4-*O*-methyl-D-glucuronic acid, but also methyl esters of D-glucuronic acid carrying a 4-nitrophenyl aglycon. Moreover, the fact that they did not recognize the 4-epimers of these compounds, the D-galacturonic acid derivatives, supports the hypothesis that these carbohydrate esterases attack ester linkages between 4-*O*-methyl-D-glucuronic acid of glucuronoxylan and lignin alcohols.**

**Key words:** glucuronoyl esterase; *Phanerochaete chrysosporium*; lignin-carbohydrate complex; glucuronoxylan; 4-*O*-methyl-D-glucuronic acid

Glucuronoyl esterases (3.1.1.-., GEs) might be enzymes hydrolyzing ester bonds occurring in plant cell-walls between the 4-*O*-methyl-D-glucuronic acids of hemicelluloses and the hydroxyl alcohols of lignin.[1–3] This hypothesis was suggested by the fact that GEs show activity on (aryl)alkyl esters of 4-*O*-methyl-D-glucuronic acid,[4] substrates that mimic the ester bonds mentioned above. GE activity was detected for first time in the crude cellulolytic system of the fungus *Schizophyllum commune*, and its purification and characterization[3] resulted in the establishment of a new carbohydrate esterase family (CE15, http://www.cazy.org/fam/CE15.html).[5] On the basis of the internal amino acid sequence of *S. commune* glucuronoyl esterase, numerous homologous sequences were found in microbial genomes.[5] The sequences included the *Hypocrea jecorina* Cip2 protein of unknown function over-expressed during growth on cellulose,[6] and the carboxy-terminal domain of the *Ruminococcus flavefaciens* multidomain protein.[7] Now both proteins are considered to be GEs belonging to CE15. The catalytic domain of Cip2 was crystallized by Wood *et al.*,[8] and it awaits solution of its three-dimensional structure. The last GE purified and characterized to date was from the thermophilic fungus *Sporotrichum thermophile*.[9] The *P. chrysosporium* genome also contains two GE genes (*pcge1* and *pcge2*), which have been heterologously expressed.[10] In this study, the corresponding gene products were purified and characterized, and their substrate specificity was investigated on new synthetic substrates.

## Materials and Methods

*Purification of* P. chrysosporium *glucuronoyl esterases.* Recombinant strain *P. cinnabarinus* UU-P001.19 was used in *Pc*GE1 production and *S. commune* UU-S002.2 in *Pc*GE2 production by procedures described previously.[10] The supernatant of a 14-d-old culture was concentrated on 10 kDa cut-off membrane, and the concentrate, containing *Pc*GE1 or *Pc*GE2, was loaded onto a weak anion exchange DEAE Sepharose FF column (2.5 × 30 cm) equilibrated by 50 mM sodium-phosphate buffer, pH 6.0 (buffer A). Elution of proteins was achieved by 400 ml of linear gradient of 0–1 M NaCl in buffer A. The flow rate was set at 4.0 ml/min, and fractions of 8 ml were collected. Fractions containing GE activity were pooled, desalted, and concentrated. After the addition of ammonium sulfate to the resulting 1 M concentration, the sample was loaded onto a Phenyl Sepharose FF column (1.5 × 30 cm) equilibrated by 1 M ammonium sulfate in buffer A. The flow rate was set at 3.0 ml/min, and fractions of 6.0 ml were collected. Bound proteins were eluted by 240 ml of a decreasing gradient of ammonium sulfate, and then by 240 ml of buffer A. The fractions containing GE were pooled and concentrated by ultrafiltration. In the last purification step, two different ion exchange columns were used. The sample containing *Pc*GE1 was diluted in 50 mM sodium acetate, pH 4.0 (buffer B), and loaded onto a weak cation CM HiTRAP FF 5 ml column, and the sample containing *Pc*GE2 was diluted in 50 mM buffer A, and loaded onto a strong anion MonoQ 5/50 GL 1-ml column. Elution of both columns was controlled by an AKTAbasic system (GE Life Science, Piscataway, NJ). Proteins adsorbed on the CM HiTRAP column were eluted by 50 ml of NaCl gradient in buffer B (0–0.5 M NaCl), and proteins adsorbed on MonoQ 5/50 GL by 10 ml of NaCl gradient in buffer A (0–0.4 M NaCl) at a flow rate 2.0 ml/min. Fractions of 1.0 ml were collected. Fractions with GE activity were pooled, desalted and concentrated. The purity of the proteins was determined by SDS–PAGE.

*Glucuronoyl esterase activity assays.* Methyl 4-*O*-methyl-D-glucopyranuronate (Compound I, Fig. 1)[11] was used in TLC monitoring[3] of GE activity during the purification procedures. Specific enzyme activities, kinetic parameters, temperature, and pH optima were determined by HPLC on chromogenic substrate 4-nitrophenyl 2-*O*-(methyl-4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside (Compound VI, Fig. 1),[12] as described previously.[3] One unit of glucuronoyl esterase activity was defined as the amount of enzyme de-esterifying 1 μmol of the substrate in 1 min at 30 °C.

† To whom correspondence should be addressed. Fax: +421-2-5941-0222; E-mail: chempbsa@savba.sk

*Abbreviations*: CBD, cellulose binding domain; GEs, glucuronoyl esterases; *Pc*GE1, *Phanerochaete chrysosporium* glucuronoyl esterase 1; *Pc*GE2, *Phanerochaete chrysosporium* glucuronoyl esterase 2

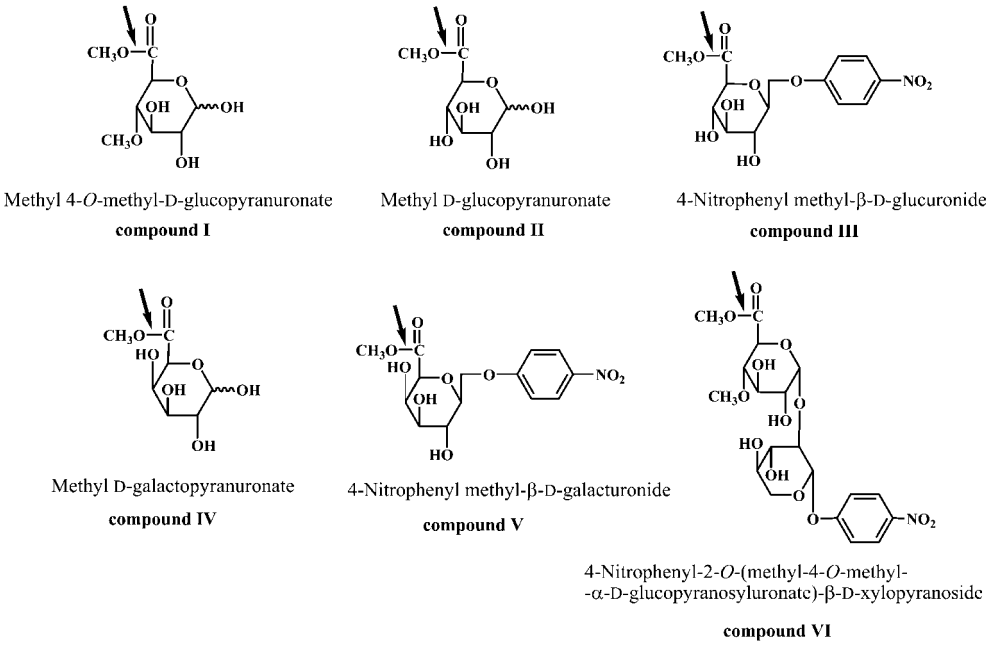


**Fig. 1.** Synthetic Substrates for Determination of GE Activity by TLC (compound I), HPLC (compound VI) and by the Hestrin Procedure (compounds I–V).

*Cellulose-binding studies.* *Pc*GE1 (2 µg) was added to 100 µl of 5% w/v suspension of Avicel PH101 in 50 mM sodium phosphate buffer (pH 6.0), and mixture was incubated for 4 h at 4 °C. Control was run in parallel in the absence of cellulose. Samples were centrifuged at 10,000 × *g* for 10 min. The activity of GE in the supernatant and the control enzyme solution was determined by HPLC.

*New substrates of glucuronoyl esterases and their de-esterification.* Methyl esters of D-glucuronic acid (compound II, Fig. 1), 4-nitrophenyl *β*-D-glucuronide (compound III, Fig. 1), D-galacturonic acid (compound IV, Fig. 1), and 4-nitrophenyl *β*-D-galacturonide (compound V, Fig. 1) were prepared by esterification (Sigma-Aldrich, St. Louis, MO) with ethereal diazomethane.

*Preparation of methyl esters of D-uronic acids.* Dried uronic acid or its glycoside (1 g) was dissolved in 15 ml of absolute methanol in a two-necked flask equipped with a dropping funnel, a stirrer, and a $CaCl_2$ drying tube. The solution was cooled to −10 °C, and throughout esterification the temperature of the solution was kept below 0 °C. Then the freshly prepared solution of ethereal diazomethane was added dropwise. When the color of the reaction mixture remained slightly yellow permanently, esterification was complete. Usually 3 g of *N*-nitrosomethyl urea is required for the preparation of diazomethane for complete esterification of 1 g of D-saccharide acid. The ether and methanol were removed under reduced pressure to obtain the uronic acid methyl ester. Ethereal diazomethane was prepared by the procedure described by Müller *et al.*[13]

*Characterization of the new esters by NMR and FTIR.* $^1$H and $^{13}$C-NMR spectra were recorded in $D_2O$ or $CD_3OD$ at 25 °C on a Varian 400MR spectrometer operating at 400 MHz and 100.6 MHz respectively. COSY and HSQC pulse programs were also used. IR spectra were measured with an FTIR spectrometer Nicolet 6700, and the Diamond ATR technique (Smart Orbit accessory, Thermo Fisher Scientific Inc., Waltham, MA) was applied for measuring in a solid state.

*Methyl D-glucopyranuronate* (II) (a mixture of *α* and *β* anomers) $^1$H-NMR ($D_2O$, 400 MHz) *δ*: 5.16 (1H, d, H1*α*), 4.58 (1H, d, H1*β*), 4.27 (1H, m, H5*α*), 3.96 (1H, d, H5*β*), 3.70 (3H, s, $OCH_3$), 3.71–3.36 (5H, m, H2*α*, H3*α*, H3*β*, H4*α*, H4*β*), 3.20 (1H, m, H2*β*). $^{13}$C-NMR ($D_2O$, 100.6 MHz) *δ*: 174.5 (*C*OOCH$_3$ *α*), 173.5 (*C*OOCH$_3$ *β*), 98.7 (C1*β*), 94.9 (C1*α*), 77.7, 77.1, 76.2, 74.8, 74.0, 73.8, 73.5, 73.2 (C2*α*, C2*β*, C3*α*, C3*β*, C4*α*, C4*β*, C5*α*, C5*β*), 55.6 ($OCH_3$).

*4-Nitrophenyl methyl-β-D-glucuronide* (III) $^1$H-NMR ($CD_3OD$, 400 MHz) *δ*: 8.21 (2H, d, Ar), 7.21 (2H, d, Ar), 5.18 (1H, d, H1), 4.13 (1H, d, H5), 3.77 (3H, s, $OCH_3$), 3.63 (1H, m, H2), 3.53 (2H, m, H3, H4). $^{13}$C-NMR ($CD_3OD$, 100.6 MHz) *δ*: 170.8 (*C*OOCH$_3$), 163.5, 144.1, 126.7 (×2), 117.7 (×2) (6 × Ar), 101.5 (C1), 77.0, 76.7, 74.4, 72.8 (C2, C3, C4, C5), 53.0 ($OCH_3$).

*Methyl D-galactopyranuronate* (IV) (a mixture of *α* and *β* anomers) $^1$H-NMR ($D_2O$, 400 MHz) *δ*: 5.20 (1H, d, H1*α*), 4.64 (1H, m, H5*α*), 4.49 (1H, d, H1*β*), 4.34 (1H, m, H5*β*), 4.21 (1H, m, H4*α*), 4.15 (1H, dd, H4*β*), 3.82 (1H, dd, H3*α*), 3.70 (4H, m, $OCH_3$, H2*α*), 3.60 (1H, dd, H3*β*), 3.38 (1H, m, H2*β*). $^{13}$C-NMR ($D_2O$, 100.6 MHz) *δ*: 171.4 (*C*OOCH$_3$ *α*), 170.5 (*C*OOCH$_3$ *β*), 96.2 (C1*β*), 92.3 (C1*α*), 74.1, 72.1, 71.1, 70.4, 70.0, 69.6, 68.4, 67.7 (C2*α*, C2*β*, C3*α*, C3*β*, C4*α*, C4*β*, C5*α*, C5*β*), 52.8 ($OCH_3$).

*4-Nitrophenyl methyl-β-D-galacturonide* (V) $^1$H-NMR ($CD_3OD$, 400 MHz) *δ*: 8.21 (2H, d, Ar), 7.25 (2H, d, Ar), 5.08 (1H, d, H1), 4.56 (1H, s, H5), 4.22 (1H, s, H4), 3.85 (1H, t, H2), 3.77 (3H, s, $OCH_3$), 3.69 (1H, dd, H3). $^{13}$C-NMR ($CD_3OD$, 100.6 MHz) *δ*: 170.2 (*C*OOCH$_3$), 163.9, 144.1, 126.6 (×2), 117.8 (×2) (6 × Ar), 101.8 (C1), 75.8 (C5), 74.1 (C3), 71.4 (C2), 71.2 (C4), 52.8 ($OCH_3$).

The ester character of compounds II–IV was also confirmed by FTIR. Absorption peaks characteristic of esters were found at 1,732 cm$^{-1}$, 1,730 cm$^{-1}$, 1,735 cm$^{-1}$, and 1,713 cm$^{-1}$ for II, III, IV, and V, respectively.

*Action of GEs on methyl esters of uronic acids and their glycosides.* The ability of the GEs to hydrolyze compounds II-V and also compound I (Fig. 1) was followed by determination of the remaining esters following Hestrin.[14] The low sensitivity of this method does not allow determination of kinetic constants, and hence only specific activities were determined. The reaction mixture, containing 20 mM substrate and 3 µg of enzyme in 50 mM sodium phosphate buffer (pH 6.0), was incubated at 30 °C for 15 min (compounds I–III) or 20 h (compounds IV–V). The remaining esters were converted to hydroxamic acid by a reaction with 2 volumes of solution containing 2 M $NH_2OH{\cdot}HCl$ and 3.5 M NaOH (1:1, v/v). One volume of HCl:$H_2O$ solution (1:2, v/v) was added to the mixture after 60 s, and subsequently, after the addition of 1 volume of $FeCl_3{\cdot}6H_2O$ in 0.1 M HCl, the hydroxamic acid was complexed with ferric ions, resulting in a measurable chromophore (540 nm). The performance of the GEs from *P. chrysosporium* were compared with the action of GEs from *S. commune*[3] and *H. jecorina*.[5]

## Results

*Purification of* P. chrysosporium *glucuronoyl esterases*

The *P. chrysosporium* GEs produced by recombinant strains were purified by a three-step procedure that included ion-exchange chromatography and hydropho-

**Table 1.** Summary of Recombinant *P. chrysosporium* GE1 (A) and GE2 (B) Purification

A

| Purification step | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Culture fluid | 681 | 166 | 369 | 2.23 | 100 | 1 |
| Ultrafiltration | 40 | 43.84 | 261 | 5.95 | 70.7 | 2.7 |
| DEAE Sepharose FF | 6 | 31.60 | 219 | 6.93 | 59.3 | 3.1 |
| Phenyl Sepharose FF | 0.80 | 2.54 | 70 | 27.56 | 19 | 12.4 |
| HiTrap CM FF | 0.17 | 0.84 | 33 | 38.81 | 8.9 | 17.4 |

B

| Purification step | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Culture fluid | 825 | 112.8 | 402 | 3.56 | 100 | 1 |
| Ultrafiltration | 150 | 38 | 311 | 8.18 | 77.3 | 2.3 |
| DEAE Sepharose FF | 15 | 3.3 | 89 | 26.96 | 22.1 | 7.6 |
| Phenyl Sepharose FF | 0.75 | 1.3 | 59 | 45.38 | 14.7 | 12.7 |
| MonoQ 5/50 GL | 0.21 | 0.6 | 32 | 53.33 | 8 | 15 |

bic interaction chromatography. A summary of *Pc*GE1 purification is presented in Table 1A, and of *Pc*GE2 in Table 1B. Concentrated filtrate of *P. cinnabarinus* growth medium containing *Pc*GE1 was loaded onto a DEAE Sepharose column at pH 6.0 for the purpose of pre-purification. GE activity was found in the protein fractions unadsorbed by the resin. During subsequent chromatography on a Phenyl Sepharose column, *Pc*GE1 together with other proteins remained trapped on the column. Most of the proteins were eluted with a decreasing gradient of ammonium sulfate, but *Pc*GE1 was not eluted until we washed the column with plain buffer. Several other proteins still present in the sample were removed by chromatography on a weak cation exchange HiTRAP CM FF column. *Pc*GE1 adsorbed to the column at pH 4.0, but the other proteins did not. Protein *Pc*GE1 was eluted by 0.1 M NaCl, and showed homogeneity on SDS–PAGE (Fig. 2A, lane 2). The same purification procedure was applied to *Pc*GE2. In contrast to *Pc*GE1, this protein was adsorbed to a DEAE Sepharose at pH 6.0 and was eluted by 0.05 M NaCl. Subsequent chromatography on a Phenyl Sepharose column disclosed that the protein had hydrophobicity similar to that of *Pc*GE1. In contrast to most of the proteins eluted during the decreasing ammonium sulfate gradient, *Pc*GE2 was released from the column during elution with a buffer free of ammonium sulfate. The protein was further purified by chromatography on a strong anion exchange Mono Q resin. *Pc*GE2 did not adsorb to the resin at pH 6.0. The isolated protein showed homogeneity on SDS–PAGE (Fig. 2B, lane 2).

*Properties of* P. chrysosporium *glucuronoyl esterases*

The molecular masses of the *P. chrysosporium* GEs were determined by SDS–PAGE (Fig. 2). The molecular mass appeared to be 47 kDa (calculated at 47.2 kDa) for *Pc*GE1, and 42 kDa (calculated at 42.3 kDa) for *Pc*GE2. These masses were similar to the calculated values, although we expected higher masses considering the presence of putative glycosylation sites (one for *Pc*GE1 and seven for *Pc*GE2).[10] pH and temperature optima were very similar for both enzymes: pH optimum 5.0–6.0 and temperature optimum 45–55 °C. The isoelectric point for *Pc*GE1 was found to be 6.5 *vs.* calculated 5.5 and 4.7 *vs.* calculated 4.8 for *Pc*GE2. The presence of CBD in the protein *Pc*GE1 was experimentally confirmed. Only 20% of the enzyme activity remained in the supernatant after 4 h of incubation of the enzyme with a 5% suspension of cellulose (Avicel PH101, Sigma-Aldrich, St. Louis, MO).

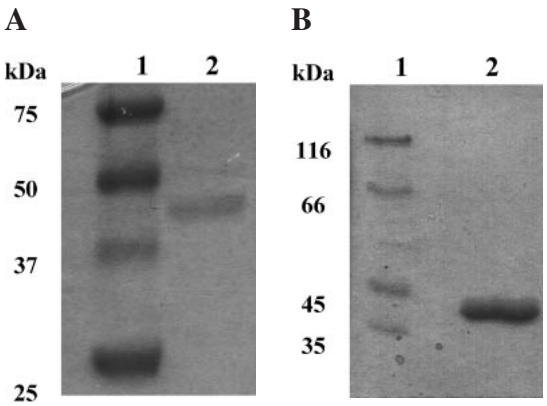


**Fig. 2.** SDS–PAGE of Recombinant *P. chrysosporium* GEs.
A, Lane 1, molecular weight markers (Biorad, Hercules, CA); lane 2, purified *Pc*GE1 (2 μg). B, Lane 1, molecular mass standards (Fermentas, Ontario); lane 2, purified *Pc*GE2 (3 μg).

The kinetic parameters of the *P. chrysosporium* GEs were determined on 4-nitrophenyl 2-*O*-(methyl-4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside (compound VI, Fig. 1) (Table 2). *Pc*GE1 showed 2 times higher catalytic affinity ($K_m$) than *Pc*GE2, but the maximum velocity ($V_{max}$) of substrate hydrolysis by *Pc*GE2 was 6 times higher that *Pc*GE1. The catalytic efficiency parameter ($k_{cat}/K_m$) was 2.5 times higher for *Pc*GE2 than for *Pc*GE1. The results obtained were compared with previously reported kinetic parameters for *H. jecorina*, *S. commune*, and *S. thermophile* GEs for the same substrate. *P. chrysosporium* GEs showed lower catalytic affinity for the substrate, but a considerably higher maximum velocity of hydrolysis than the other GEs (Table 2).

*New substrates for glucuronoyl esterases*

The de-esterification rates of methyl esters of uronic acids and their glycosides (Fig. 1) by GEs from strains

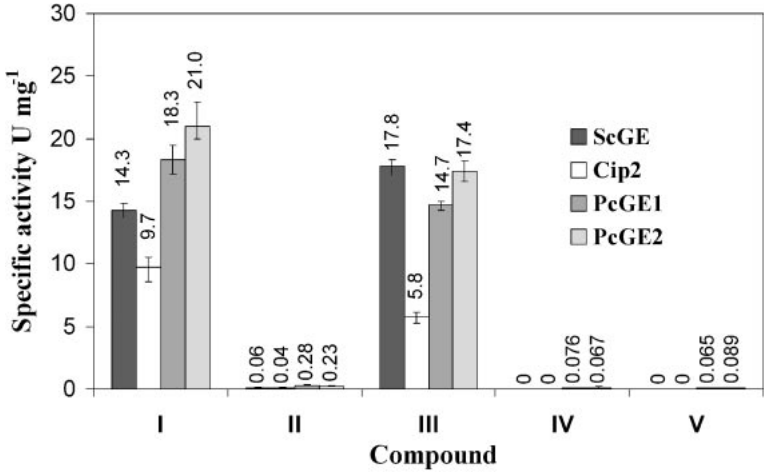


**Fig. 3.** Specific Activities (U/mg) of GEs on Compounds I to V (see Fig. 1) Determined at a Substrate Concentration of 20 mM. The error bars represent standard deviations of three independent measurements.

**Table 2.** Kinetic Parameters of *P. chrysosporium* GEs Determined on 4-Nitrophenyl 2-*O*-(methyl-4-*O*-methyl-α-D-glucopyranosyluronate)-β-D-xylopyranoside and a Comparison with Other Characterized GEs

| Enzyme | $K_m$ (mM) | $V_{max}$ μmol/min/mg | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ (mM/s) | Ref. |
|---|---|---|---|---|---|
| *Pc*GE1 | 0.83 | 14.2 | 11.2 | 13.5 | — |
| *Pc*GE2 | 1.82 | 88.4 | 62.3 | 34.2 | — |
| *Sc*GE | 0.31 | 4.4 | 3.2 | 10.3 | 3) |
| Cip2 | 0.50 | 5.5 | 4.5 | 9.0 | 5) |
| *St*GE | 1.3 | — | 0.8 | 0.6 | 9) |

*S. commune*, *H. jecorina*, and *P. chrysosporium* were followed by determination of residual esters by the Hestrin procedure[14] (Fig. 3). All of the enzymes showed specific activity between 9.7–21.0 U/mg on methyl 4-*O*-methyl-D-glucuronic acid (compound I), whereas the activity on methyl ester of D-glucuronic acid (compound II) was negligible, 65–242 times lower. These surprising results were obtained with the methyl ester of 4-nitrophenyl β-D-glucuronide (compound III), on which all the GEs showed specific activities between 5.8–17.8 U/mg despite the lack of the methyl group at position 4. These values are comparable to the specific activities obtained for compound I possessing the 4-*O*-methyl group. Finally, the substrates in which D-glucuronic acid was replaced by D-galacturonic acid were also tested (compounds IV and V). As can be seen in Fig. 3, the specific activities of the GEs on the galacto-derivates were insignificant.

## Discussion

To date, GEs from *S. commune*,[3] *H. jecorina*,[5] and *S. thermophile*[9] have been characterized. Two genes (*pcge1* and *pcge2*) coding for this recently discovered carbohydrate esterase have been identified in the genome of the white-rot basidiomycete *P. chrysosporium*.[5] While the gene *pcge2* codes for an enzyme that has only catalytic domain, *pcge1* codes for an enzyme that also has a family 1 CBD. Because the purification of both *P. chrysosporium* GEs from sugar-beet pulp containing growth media of the fungus proved to be very difficult, the enzymes were successfully produced as recombinant proteins by two different hosts, *P. cinnabarinus* and *S. commune*.[10] Selection of the regulatory element located in the front of the reading frames made possible their production and purification from glucose media. The enzyme containing CBD showed binding affinity to crystalline cellulose and also a higher molecular mass than the enzyme without CBD. In spite of the fact that the *Pc*GE1 and *Pc*GE2 sequences contain putative glycosylation sites, their estimated molecular masses correspond to the calculated ones. It seems that the CBD influences the p*I* values of the enzymes, which reflects their different affinities towards the ion-exchangers used in enzyme purification. Consequently, identical purification steps might not be applied to the two GEs. The differences in physical properties between two *P. chrysosporium* GEs are similar to those observed between the CBD-free *S. commune* GE[3] and the CBD-containing GEs from *H. jecorina*[5] and *S. thermophile*.[9]

Since there is no experimental evidence that GEs hydrolyze the ester linkages between uronic acids in hemicellulose and lignin alcohols,[15–20] the physiological role of these carbohydrate esterases in microbial plant cell-wall degradation remains unclarified. In this study, we tested and compared the catalytic properties of the two *P. chrysosporium* GEs and previously isolated GEs on new synthetic substrates prepared by esterification of commercially available uronic acids and their derivatives using ethereal diazomethane. These compounds mimic the ester linkages in lignin-carbohydrate complexes. Examination of the GE activity on the methyl esters of different uronic acids and their derivatives indicates the important role of the 4-*O*-methyl group in substrate recognition. The methyl ester of 4-*O*-methyl-D-glucuronic acid was hydrolyzed by all four GEs at least 65 times faster than the methyl ester of D-glucuronic acid. Previous study has also shown that *S. commune* GE hydrolyzes esters of 4-*O*-methyl-D-glucuronic acid with much higher efficiency than those of D-glucuronic acid. Here we found that the change of the methyl ester of free glucuronic acid into its 4-nitrophenyl β-glycoside considerably increased the rate of hydrolysis by GEs. The introduction of a hydrophobic aglycon increased substrate affinity for the enzyme catalytic site. In

addition, this result serves as evidence that GEs accept esterified D-glucuronic acid as their substrate also in the form of its $\beta$-anomer. The de-esterification of 4-nitrophenyl methyl-$\beta$-D-glucuronide (compound III) by all four tested enzymes points to the possibility of elaborating a simple enzyme coupled assay of GEs using $\beta$-glucuronidase as the auxiliary enzyme.

The above remarks do not apply to the methyl ester of free D-galacturonic acid and its 4-nitrophenyl $\beta$-glycoside. These two compounds did not serve practically as substrates for any of the tested GEs. Based on these observations, we conclude that the gluco-configuration on C-4 is decisive for enzyme activity. These results underline the uniqueness of GE substrate specificity in confrontation with pectin methyl esterases and the need for a further search for GE native substrates.

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