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Mélanie L'Enfant[a♯], Pricillia Kutudila[b♯], Catherine Rayon[a], Jean-Marc Domon[a], Woong-Hee Shin[c], Daisuke Kihara[c,d], Anne Wadouachi[b], Jérome Pelloux[a], Gwladys Pourceau[b], Corinne Pau-Roblot[a*]

[a] EA3900-BIOPI, Biologie des Plantes et Innovation, SFR Condorcet FR CNRS 3417, Université de Picardie, 33 Rue St Leu, FR-80039 Amiens, France

[b] Laboratoire de Glycochimie, des Antimicrobiens et des Agroressources (LG2A), UMR CNRS 7378 – Institut de Chimie de Picardie FR CNRS 3085, Université de Picardie Jules Verne, FR-80039 Amiens, France.

[c] Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA.

[d] Department of Computer Science, Purdue University, West Lafayette, IN 47907, USA.

[♯]These authors contributed equally to this work.

***Corresponding author:**

Dr. Corinne Pau-Roblot, e-mail: corinne.pau-roblot@u-picardie.fr, Tel: +33 3 22 82 75 36, Fax : + 33 3 22 82 74 36

ABSTRACT

The discovery of molecules that can inhibit the action of phytopathogens is essential to find alternative to current pesticides. Pectin methylesterases (PME), enzymes that fine-tune the degree of methylesterification of plant cell wall pectins, play a key role in the pathogenicity of fungi or bacteria. Here we report the synthesis of new lactoside derivatives and their analysis as potential PME inhibitors using three plants and one fungal PME. Because of its structure, abundance and reduced cost, lactose was chosen as a case study. Lactoside derivatives were obtained by TEMPO-mediated oxidation of methyl lactoside, followed by an esterification procedure. Three derivatives were synthesized: sodium (methyl-lactosid)uronate, methyl (methyl-lactosid)uronate and butyl (methyl-lactosid)uronate. The inhibition of the plant and pathogen enzyme activities by lactoside derivatives was measured *in vitro*, showing the importance of the substitution on lactose: methyl (methyl-lactosid)uronate was more efficient than butyl (methyl-lactosid)uronate. These results were confirmed by docking analysis showing the difference in the interaction between lactoside derivatives and PME proteins. In conclusion, this study identified novel inhibitors of pectin remodeling enzymes.

Abbreviations

PME, Pectin methylesterase; HG, homogalacturonan

Keywords

Pectin methylesterase; chemical inhibitors; lactoside derivatives; docking analysis.

1. Introduction

Considering the deleterious effects of chemical pesticides on the environment, alternatives are needed to reduce the infection of plants by phytopathogens. As an example, a number of strategies were envisaged to limit the infection of *Botrytis cinerea*, one of the most widespread fungus attacking numerous plant species of agronomic interest. This include the use of antifungal protein from *Aspergillus giganteus* [1], essential oil as eugenol [2-3], or oxygenated monoterpenes [4]. Most of bacterial or fungal phytophathogens secrete plant cell wall degrading enzymes that play a central role in the infection. The plant primary cell wall is highly complex, composed of polysaccharides (cellulose, hemicelluloses and pectins) and structural proteins that are linked to form a cohesive but highly dynamic structure outside of the plant cell. Pectins, and in particular homogalacturonan (HG), a homopolymer of α-(1→4)-linked-D-galacturonic acid units, are major components of the plant cell wall and play a central role in regulating the chemistry and mechanics of the cell wall during development and plant pathogen interactions. Pectin remodeling enzymes, which play a key role in determining the phytopathogenicity of the various fungi [5], could thus be considered as a case study to engineer chemical compounds that could inhibit specifically a number of these enzymes. Pectin methylesterases (PME), enzymes that fine-tune the degree of methylesterification of homogalacturonans (HG), are secreted by fungi as *Fusarium asiaticum* or *Rhizoctomia solani* [6-7] during infection, but are as well present in plant cell wall where they can regulate a number of developmental process including primordia emergence at the shoot apical meristem [8-10], adventitious root formation [11], pollen tube elongation [12-13], seed development [14] and dark-grown hypocotyl elongation [15-16]. PMEs from plants and pathogens differ in terms of substrate specificity and sensitivity to inhibitors. For instance, it was reported that proteinaceous inhibitors (PMEIs) are effective against plant PMEs but ineffective against pathogen PMEs [17]. Molecules such as catechins, phenolics

extracts from green tea leaves, as well as aromatics have been identified as inhibitors of plant PME activity, although no inhibition was measured on fungal PME [18-19]. A recent report identified phenylephrine as inhibitor of a number of Arabidopsis pectin methylesterases, with consequent effects on root development [20]. As an alternative of chemical library screen, one of the methods to produce new inhibitors is to synthesize compounds mimicking the structure of the substrates of the enzyme [21]. Due to the recent advances in the chemistry and biochemistry, glycosidase inhibitors have already been synthesized or extracted from natural sources. In particular, some disaccharides were used as glycosidases inhibitors: kojibiose and nigerose were indeed identified as inhibitors of glucosidases and was used as therapeutic agents [22]. α-(1→5)-linked arabinofuranose disaccharides were reported to inhibit arabinosyltransferases of *Mycobacterium tuberculosis* that are induced in tuberculosis disease [23]. The chemical synthesis of oligosaccharides related to plant cell wall were also reported, paving the way for potential modifications [24]. As pectin methylesterase act on methylated homogalacturonan whose monomer unit is GalA methylated at C-6 position, carbohydrate analog with other group than natural COOMe could be envisaged as potential inhibitor of PME activity. Here we report the synthesis of lactose derivatives as bioisoster of PME substrates and the inhibition of plant or fungal pectin methylesterases, produced in vitro or commercially available, by the synthesized lactoside derivatives. Lactose was chosen, firstly because it is an abundant sugar, and secondly, because it includes a galactose unit that is structurally very close to the galacturonic acid present in homogalacturonan, natural substrate of PME enzymes. The results are discussed in the light of docking analysis of the lactosides derivatives with modelled structures of pectin methyesterases, showing the potential of this approach.

2. Materials and methods

2.1. Source of enzymes

Pectin methylesterase (PME) from orange peel (*Citrus sinensis*), referred to as CsPME, was used as a reference (Sigma, P5400).

Escherichia coli strain JM101, carrying the plasmid pREP4 [25] overexpressing PME-*At3g29090,* was used for the expression of the Arabidopsis protein and referred to as AtPME31. To express and produce recombinant AtPME31, recombinant *E. coli* was grown at 30 °C in Lysogeny Broth (LB) medium. 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used as inducer of protein production. Cells were harvested by centrifugation and bacteria were lysed by 4x30 s sonication on ice and the lysate was centrifuged. The clarified culture supernatant was concentrated using a PelliconXL device with a Biomax10 membrane on a Labscale TFF pump (Millipore) and buffer was changed to 0.3 M NaCl, 50 mM sodium phosphate, pH 7.5. The supernatant was applied to an AKTA prime FPLC fitted with a HisTrap FF column at a flow rate of 0.25 $ml.min^{-1}$ and eluted in a linear gradient of 20 to 500 mM imidazole (0.5 $ml.min^{-1}$) over 10 ml.

Transgenic *Nicotiana tabacum* plants (var. PBD6) overexpressing Arabidopsis PME-*At3g14310* was used for the expression of Arabidopsis protein and referred to as AtPME3 [26]. Recombinant PME3 (PME3-6xHis) was purified from *N. tabacum* frozen leaf powder homogenized in 50 mM NaH_2PO_4 containing 2 M NaCl (pH 7). The protein extract was centrifuged and supernatant was diluted 6-fold in a NaH_2PO_4 50 mM buffer (pH 8) containing 40 mM imidazole. The supernatant was loaded onto a Ni-affinity column (HisTrap FF, GE Healthcare, Little Chalfont, United Kingdom) and washed with 10 column volumes of 40 mM imidazole in NaH_2PO_4 50 mM buffer, 500 mM NaCl (pH 8). The recombinant protein was eluted from the affinity resin with 500 mM imidazole in NaH_2PO_4 50 mM buffer, 500 mM

NaCl (pH 8). The purified protein was loaded onto a spin column (Microcon YM-10, Merck Millipore, Tullagreen Carrigtwohill, Ireland) and desalted with 50 mM sodium phosphate buffer (pH 7.5).

Pichia pastoris strain GS115, carrying the plasmid pPIC3.5 expressing *Botrytis cinerea* BcPME1 was referred to as BcPME1 [27]. To express and produce BcPME1, recombinant *P. pastoris* were grown overnight at 30 °C on Yeast Extract Peptone Dextrose (YPD) medium plate. One colony was transferred into 2 ml liquid Buffered Methanol-complex Medium Yeast (BMMY) maintained at 30 °C for 15 h. Methanol was added every 24 h to a final concentration of 0.5% (v/v). After 4 days of expression, yeasts were removed by centrifugation. The supernatant containing proteins was precipitated using ammonium sulfate at 90% for 6 h at 4 °C. Proteins were harvested by centrifugation and dialyzed in GEBAflex tubes (6-8 kDa). Extracts were concentrated using 15 ml amicon tubes (10 kDa) and then purified as follows: the material was applied twice to a DEAE-Sepharose FF column (GE Healthcare, Sweden). Proteins were eluted with sodium-phosphate buffer (50 mM; pH 6.0).

2.2. Lactoside derivatives synthesis

2.2.1. Methyl β-D-lactoside 1 (OH)

The methyl lactoside was synthesized following a four-step sequence as described in literature [28]: a) peracetylation of lactose (quant. yield); b) activation of anomer position via a 2,3,6,2',3',4',6'-hepta-O-acetyl-α-D-lactosyl bromide (quant. yield); c) substitution of lactosyl bromide by a methoxy group (74% yield) and deacetylation (71% yield). Methyl β-D-lactoside **1** (OH) was obtained in 52% overall yield and the characterization (^{1}H, ^{13}C) was in accordance with literature.

2.2.2. Sodium (methyl β-D-lactosid)uronate 2 (Na)

To a solution of 2.39 g of methyl β-D-lactoside **1** (6.8 mmol, 1 eq) in 25 ml of acetonitrile and 20 ml of phosphate buffer (0.67M, pH 6.7) were added 544 mg of TEMPO (3.6 mmol, 0.5 eq), 34 ml of 2 M aqueous $NaClO_2$ solution (67.9 mmol, 10 eq) and 323 µl of 2.1M aqueous NaOCl solution (0.679 mmol, 0.1 eq) portion wise. The reaction mixture was heated at 35 °C and monitored by TLC. After completion, the reaction mixture was evaporated to dryness, and then water and dichloromethane were added. Aqueous phase was extracted three times with dichloromethane (3 x 25 ml) and concentrated. The crude residue was diluted in water and dialyzed against water during 24h. After freeze-drying, sodium (methyl β-D-lactosid)uronate **2** (Na) was obtained as a white solid (1.15 g, 2.67 mmol, 40%)

^{1}H NMR (300 MHz, D_2O) : δ (ppm) 4.45 (d, $J_{H\text{-}1',H\text{-}2'}$= 7.8 Hz, 1H, H-1'), 4.43 (d, $J_{H\text{-}1,H\text{-}2}$= 8.1 Hz, 1H, H-1), 4.22 (dd, $J_{H\text{-}4',H\text{-}3'}$= 3.5 Hz, $J_{H\text{-}4',H\text{-}5'}$=1.3 Hz, 1H, H-4'), 4.10 (d, $J_{H\text{-}5',H\text{-}4'}$= 1.3 Hz, 1H, H-5'), 3.91 – 3.86 (m, 1H, H-4), 3.77 – 3.65 (m, 3H, H-3', H-3, H-5), 3.61 – 3.53 (m, 4H, H-2', OCH_3), 3.43 – 3.33 (m, 1H, H-2).

^{13}C NMR (75 MHz, D_2O): δ (ppm) 175.2 (C-6'), 174.6 (C-6), 103.0 (C-1), 102.8 (C-1'), 81.1 (C-5 or C-3 or C-3'), 75.7 (C-4), 75.5 (C-5'), 74.6 (C-5 or C-3 or C-3'), 72.6 (2s, C-2 & C-5 or C-3 or C-3'), 70.6 (C-2'), 70.1 (C-4'), 57.3 (OCH_3).

2.2.3. Methyl (methyl β-D-lactosid)uronate 3 (Me)

To a solution of 204 mg of sodium (methyl β-D-lactosid)uronate **2** (0.47 mmol, 1 eq) in 16 ml of MeOH were added 1.6 ml of 2,2-dimethoxypropane (0.013 mol, 28 eq) and a catalytic quantity of an aqueous 37% HCl solution. The reaction mixture was irradiated by microwaves at 63 °C during 40 min then neutralized by addition of Et_3N and evaporated to dryness. The crude product was then purified by flash chromatography (EtOAc/MeOH) to obtain methyl (methyl β-D-lactosid)uronate **3** (Me) as white powder (36.9 mg, 0.087 mmol, 19%).

^{1}H NMR (300 MHz, D_2O): δ (ppm) 4.52 (d, $J_{H\text{-}1',H\text{-}2'}$ = 8.1 Hz, 1H, H-1'), 4.52 (d, $J_{H\text{-}1,H\text{-}2}$= 1.5 Hz, 1H, H-1) 4.43 (d, $J_{H\text{-}4,H\text{-}5}$= 7.8 Hz, 1H, H-5), 4.30 (dd, $J_{H\text{-}2,H\text{-}3}$= 3.4 Hz, $J_{H\text{-}2,H\text{-}1}$=1.3 Hz, 1H, H-2), 4.27 (d, $J_{H\text{-}4',H\text{-}5'}$= 9.7 Hz, 1H, H-5'), 3.92 – 3.85 (m, 1H, H-4'), 3.85 (s, 6H, $COOCH_3$), 3.76 (t, $J_{H\text{-}3',H'2}$=$J_{H\text{-}3',H\text{-}4'}$=9.0 Hz, 1H, H-3'), 3.75 (dd, $J_{H\text{-}3,H\text{-}4}$=10.2 Hz, $J_{H\text{-}3,H\text{-}2}$=3.3 Hz, H-3) 3.59 (s, 3H, OCH_3), 3.54 (dd, $J_{H\text{-}4,H\text{-}3}$ = 10.0 Hz, $J_{H\text{-}4,H\text{-}5}$=7.8 Hz, 1H, H-4), 3.41 (dd, $J_{H\text{-}2',H\text{-}3'}$ = 9.3 Hz, $J_{H\text{-}2',H\text{-}1'}$=8.0 Hz, 1H, H-2').

^{13}C NMR (75 MHz, D_2O): δ (ppm) 170.2-170.1 (2s, C-6, C-6'), 103.4 (C-1'), 102.8 (C-5), 80.6 (C-4'), 74.1 (C-1), 73.9 (C-3), 73.1 (C-5'), 72.4 (C-2'), 71.7 (C-3'), 69.9 (C-4), 69.3 (C-2), 57.6 (OCH_3), 53.3-53.0 (2s, $COOCH_3$)

2.2.4. Butyl (methyl β-D-lactosid)uronate 4 (Bu)

To a solution of 99 mg of sodium (methyl β-D-lactosid)uronate **2** (0.23 mmol, 1 eq) in 3 ml of butanol were added 500 mg of Na_2SO_4 (32 mmol, 139 eq) and a catalytic quantity of an aqueous 37% HCl solution. The reaction mixture was irradiated by micro-waves at 70 °C during 40 min then neutralized by addition of Et_3N, filtrated to remove Na_2SO_4 and evaporated to dryness. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH) to obtain butyl (methyl β-D-lactosid)uronate **4** (Bu) as white powder (109,7 mg, 0.22 mmol, 95%).

^{1}H NMR (600 MHz, CD_3OD): δ (ppm) 4.36 (d, $J_{H\text{-}4',H\text{-}5'}$ = 1.4 Hz, 1H, H-5'), 4.33 (d, $J_{H\text{-}1',H\text{-}2'}$= 7.4 Hz, 1H, H-1') 4.30 (d, $J_{H\text{-}1,H\text{-}2}$= 7.8 Hz, 1H, H-1), 4.26 – 4.12 (m, 5H, OCH_2, H-4'), 4.02 (d, $J_{H\text{-}4,H\text{-}5}$= 9.7 Hz, 1H, H-5), 3.77 (dd, $J_{H\text{-}3,H\text{-}4}$=8.8 Hz, $J_{H\text{-}4,H\text{-}5}$= 9.6 Hz, H-4), 3.60 (t, $J_{H\text{-}2,H\text{-}3}$=$J_{H\text{-}3,H\text{-}4}$=8.9 Hz, 1H, H-3), 3.55 (dd, $J_{H\text{-}3',H\text{-}4'}$= 9.8 Hz, $J_{H\text{-}2',H\text{-}3'}$=3.2 Hz, H-3') 3.51-3.50 (m, 4H, OCH_3, H-2'), 3.28 (dd, $J_{H\text{-}1,H\text{-}2}$ = 7.8 Hz, $J_{H\text{-}2,H\text{-}3}$= 9.1 Hz, 1H, H-2), 1.76 – 1.57 (m, 4H, OCH_2CH_2-), 1.50 – 1.36 (m, 4H, $OCH_2CH_2CH_2$), 0.96 (2t, J=7.4Hz, 6H, CH_2CH_3)

^{13}C NMR (150 MHz, CD_3OD): δ (ppm) 170.0 (C-6), 169.7 (C-6'), 105.6 (C-1), 104.9 (C-1'), 82.7 (C-4), 75.8 (C-3), 75.5 (C-5'), 75.2 (C-5), 74.5 (C-2), 74.0 (C-3'), 71.6 (C-2'), 71.2 (C-4'), 66.8 –

66.3 (OCH_2) 57.5 (OCH_3), 31.7 – 31.5 (2s, OCH_2CH_2), 20.1 (2s, $OCH_2CH_2CH_2$), 14.0 (2s, CH_2CH_3).

2.3. Quantitative assay of PME activity and inhibition

Inhibition of CsPME, AtPME3 and AtPME31 activity was quantified using a colorimetric microassay adapted from Klavons and Bennett [29] on *Citrus* pectin >85% methylesterified (Sigma P9561) at 20 $mg.ml^{-1}$ 10 µM to 1500 µM of lactoside derivatives. The mixture was incubated at 30 °C for 30 min. Results were expressed as nmol $MeOH.min^{-1}.\mu g^{-1}$ of protein using the methanol standard curve.

Considering the optimal pH activity of BcPME1 (pH 6) and the limitations of the alcohol oxidase assay with regards to pH, the inhibition of BcPME1 activity was measured using the Anthon and Barrett method [30] on *Citrus* pectin >85% methylesterified (Sigma aldrich P9561) at 20 $mg.ml^{-1}$ and with 10 µM to 1500 µM of lactoside. PME activity was calculated as the difference in absorbance between saponified and non-saponified samples and expressed, using the standard curve, as nmol $MeOH.min^{-1}.\mu g^{-1}$ of proteins.

2.4. Protein-lactosides derivatives docking

The structural homology modeling of AtPME3 and at AtPME31 was established by Dedeurwaerder et al [25] and Sénéchal et al [26] respectively.

Binding site loop modeling for AtPME31 was modeled by GalaxyWeb server [31].

Interactions between the proteins and lactosides derivatives were predicted by AutoDock4 [32]. For a regular ligand-protein docking analysis, Autodock4 (considering flexibility of the ligands) would be suitable because the method is developed and tuned specifically for ligand-protein interactions and practically has a good balance between accuracy and computational speed.

3. Results

3.1. Lactosides derivatives synthesis

Three lactosides derivatives, modified at C-6 position by replacing primary alcohol (**1**) (OH) with carboxylate (**2**) (Na), carboxymethyl (**3**) (Me) or carboxybutyl (**4**) (Bu) groups, were synthesized as bioisostere substrates (Scheme 1).

These derivatives were obtained following two-steps procedure starting from methyl β-D-lactoside **1** (OH), obtained as described in literature [28] using TEMPO-mediated oxidation followed by acid-catalyzed esterification [33]. Sodium (methyl β-D-lactosid)uronate **2** (Na), methyl (methyl β-D-lactosid)uronate **3** (Me) and butyl (methyl β-D-lactosid)uronate **4** (Bu) were obtained with moderate to high yields (19-95%).

3.2. Inhibition of PME by lactosides derivatives

First, four PMEs previously identified for their various substrates specificities [18] were used to test the inhibitory potential of lactosides derivatives. Commercial PME from orange peel (*Citrus sinensis:* CsPME), two PMEs from *Arabidopsis thaliana* (AtPME3 and AtPME31) previously reported to differ in their structure [26] and in their inhibition by PMEI and one fungal PME from *Botrytis cinerea.* The two PMEs from *Arabidopsis thaliana* were expressed in heterologous system (*N. tabacum* leaves or *E. Coli*) and purified by affinity binding on a His-Trap FF column [18;26]. The last PME, from *B. cinerea*, was produced in *P. pastoris* [27] and purified using a DEAE-Sepharose FF column.

We investigated the inhibition of enzyme activities from plant and fungi PMEs by lactoside derivatives using increasing concentrations of molecules (0 to 1500 µM). Results are presented in Fig. 1. Inhibition of CsPME was effective at a concentration of lactoside of 500 µM. The most effective derivative was the methyl (methyl β-D-lactosid)uronate **3** (Me) which induced a maximum decrease in CsPME activity of about 50%. Methyl β-D-lactoside **1** (OH)

had also an effect on CsPME with a maximum reduction of 40% of the enzymatic activity at 1500 µM. In contrast, the other two lactoside derivatives had a weak effect on PME activity. The same effect was observed for another plant PME, AtPME3: the methyl (methyl β-D-lactosid)uronate **3** (Me) inhibited most strongly the enzyme with a maximal decrease of about 80% of the activity at a concentration of 1500 µM. This lactoside derivative had a significant effect from a concentration of 100 µM. Methyl β-D-lactoside **1** (OH) inhibited AtPME3 with a maximum decrease in the activity of 40% compared to the control. Similarly to what observed for CsPME, the other two lactoside derivatives had only a slight effect on PME activity. In contrast, the third plant PME, AtPME31, was only very weakly inhibited by any of the lactoside derivatives (maximum decrease of about 15% compared to the control), and whatever the concentration of the molecules. Lactoside derivatives showed also a weak inhibition of the fungal BcPME1.

3.3. Docking analysis revealed difference in the affinity of lactoside derivatives for PME

In order to understand the differences in the inhibiting capacity of the molecules on distinct PMEs, we used functional and catalytic actives sites prediction, as well as and docking analysis [34]. Only AtPME3 and AtPME31 were chosen for this study because of their plant origin and a large contrast in their response to lactoside derivatives. The 3D structure of AtPME3 was modeled in a previous study [26]. AtPME31 was modeled using AtPME3 coordinates as template. As observed in Fig. 2B and 2D, homology modeling showed the presence of a supplementary loop of amino acids in AtPME31, as compared to AtPME3 conformation (Fig. 2A and 2C).

Results of docking of the lactoside derivatives into the active site of AtPME3 and AtPME31 are presented in Fig. 3. If all the lactoside derivatives were included in the active sites for AtPME3, this was not observed for AtPME31.

The binding affinity, number of hydrogen bonds and the binding residues calculated by docking for AtPME3 and AtPME31 are presented in table 1. The negative values observed for AtPME3 revealed the high interaction between the enzyme and the inhibitor. The docking analyses showed that H-bonds were formed between lactoside derivatives with catalytic sites residues Arg, Asp and Gln. Methyl β-D-lactoside **1** (OH) and methyl (methyl β-D-lactosid)uronate **3** (Me) presented the more H-bonds than the over derivatives. These analyses indicated that the inhibition could occur via the interaction of lactoside derivatives with the substrate-binding site of AtPME3. On the over hand, the predicted binding affinity were very positive for AtPME31 and no H-bonds were predicted to be present. Based on these elements, no interaction between lactoside derivatives and binding site can be achieved with AtPME31. This could notably be explained by the presence of a supplementary loop of amino acids in AtPME31, not present in AtPME3, which narrows binding site (Fig. 2), impairing the binding of ligand to the catalytic site.

4. Discussion

Pectin methylesterases from plants and pathogens play a central role in regulating plant development and pathogenicity, respectively [8]. The discovery of a novel molecules that could inhibit PMEs is thus of a broad interest to either control i) plant growth and/or ii) plant-pathogen interactions. Over the recent years, a number of proteinaceous (PMEI) or chemical inhibitors, were shown to be effective on PME activity [20]. However, no generic PME inhibitor could be identified as, for instance, PMEI are ineffective on bacterial and fungal enzymes and chemical inhibitors remain largely untested on non-plant enzymes [17-19]. In order to effectively inhibit PMEs, we designed a strategy based on the engineering of novel chemical inhibitors. Considering previous studies showing that the use of bioisoster substrates can effectively inhibit enzymes is one of the solutions [35], we synthesized molecules derived

from lactose, and exhibiting oxidized groups such as carboxylate, carboxymethyl group (as the native substrate of the enzyme) and carboxybutyl group. These compounds were obtained from lactose following a multi-step procedure. Methyl β-D-lactoside **1** (OH) was obtained, as described in literature [28] by first peracetylation of lactose, then activation of the C1 position using bromide group, substitution of the anomer bromide by a methoxy group and final deacetylation. The primary hydroxyl groups (at C6 and C6' positions) of this compound were then oxidized using TEMPO-assisted procedure to lead to sodium (methyl β-D-lactosid)uronate **2** (Na) which was then esterified in acidic conditions using two different alcohols: MeOH (leading to methyl (methyl β-D-lactosid)uronate **3** (Me)) or BuOH (leading to butyl (methyl β-D-lactosid)uronate **4** (Bu)). TEMPO-catalyzed oxidation of carbohydrates is a well-known and powerful strategy when using protected carbohydrates. When free carbohydrates were used, the procedure can be very slow and tricky. Indeed, about 1 week was necessary to oxidize the methyl β-D-lactoside, with portion additions of TEMPO and co-oxidants. Furthermore, esterification of uronic acids is challenging since, under acidic conditions, (trans)glycosylation and esterification were competitive, leading to degradation products such as shorter saccharides. In the procedure, a careful control of the conditions was necessary to avoid by-product formation.

Purified proteins, either from plant or fungal sources, were used to test the inhibition of their activity by lactoside derivatives. The inhibition of CsPME and AtPME3 activities by lactoside derivatives was dependent upon substitution: the most efficient being the methyl (methyl β-D-lactosid)uronate **3** (Me) with an inhibition of the activity of CsPME and AtPME3 of 50 and 80%, respectively. The other derivatives only decreased the activity by 20%. In contrast, the inhibition of AtPME31 and BcPME1 was not more that 20% whatever the molecules tested. The methyl (methyl β-D-lactosid)uronate **3** (Me) was the substrate the most similar to the natural substrate : it has composed of two uronate derivatives esterified by a methyl group

while the substrate of the PME is homogalacturonan methyl esterified in C-6 [36]. The methyl group could probably interact with amino acid residues involved in the catalytic site. The butyl (methyl β-D-lactosid)uronate **4** showed no inhibitor activity probably due to the length of the ester group which was so not cleavable by the enzyme. We thus managed to design novel chemical inhibitors of PME, that had distinct effects depending on the PMEs considered (plant vs fungi). Early report showed that plant PMEs can be inhibited by green tea catechins such as polyphenon-60 and epigallocatechin-3-gallate [19]. This last compound was as well effective in inhibiting a PME from *Aspergillus flavus* when it was used at very high concentrations (20 mg ml^{-1}) [37]. We previously reported the inhibition of another fungal PME, BcPME1 using EGCG concentrations above 1 mg ml^{-1} [18]. Although this appears in accordance with the above-mentioned report, the use of such high concentrations of chemicals questions the affinity of fungal PMEs towards EGCG. Application of this latter chemical on plant seedlings had drastic effects on root growth, showing the importance of PME-mediated tuning of pectins in the control of plant development [38]. Similarly, a chemical library screen recently identified phenylephrine, an amine, as an inhibitor of plant PME activity, with consequent effect on root development; Application of phenyledrine indeed induced a strong decrease in root length [20]. We previously showed that the screen of the LATCA chemical library allowed the identification of a number of compounds that differed in their inhibition potential of plant and fungal PMEs [18], suggesting that enzymes of distinct structures do not have the same binding affinity towards chemical compounds. This is similar to what was shown in our current work where the synthetized lactoside derivatives did not have the same impact on plant and fungal PME activities. To understand what could drive the interaction between the various lactoside derivatives and PME, we used structural homology modeling and docking analyses. These approaches were proven to be powerful tools to assess the potential binding of ligands to proteins [34]. Considering the fact that AtPME3 and AtPME31

are plant PMEs but differ in their inhibition by lactoside derivatives, we used the two proteins as case-studies. The 3D structure of AtPME3 was modeled in a previous study [26]. The 3D structure of AtPME31 were investigated and showed that the overall structure of plant PMEs was well conserved, but a supplementary loop was present in the structure. Docking analysis was carried out using AtPME3 and AtPME31 and the different lactoside derivatives. This strongly suggests that inhibition of AtPME3 activity, as what previously shown for PMEI [39-40] occurs through the interaction of lactoside derivatives with the ligand-binding cleft structure within AtPME3. This interaction is dependent on the structure of the lactoside derivatives and the best interaction occurred with the bioisosteres with structure close to natural substrate of the enzyme. Docking interaction between AtPME3 and lactoside derivatives predicted number of stable hydrogen bonds, but no hydrogen bonds could be predicted for the interaction between AtPME31 and lactoside derivatives. This result was probably due to the presence of a supplementary loop above the ligand binding site.

5. Concluding remarks

In conclusion, we used purified proteins from plants and fungi to test some newly synthetized potential inhibitors of PME activity. Lactoside derivatives of distinct structure were shown to inhibit differentially plant and fungal PMEs. The difference in the inhibiting capacity of lactoside derivatives towards two plant PMEs of different structures could be modeled using docking analyses, showing good correlation with experimental data. Our study paves the way for the chemical synthesis of novel specific inhibitors of PME activity that could be used to target either plant or pathogen enzymes;

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Figures captions:

Scheme 1. Synthesis of sodium (methyl-lactosid)uronate **2** (Na), methyl (methyl-lactosid)uronate **3** (Me) and butyl (methyl-lactosid)uronate **4** (Bu) starting from methyl-lactoside **1** (OH)

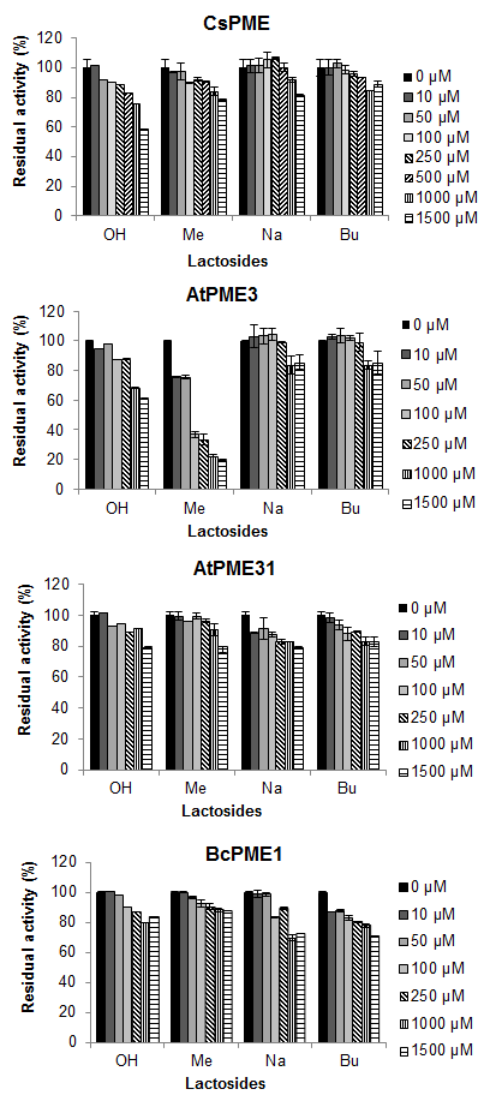
Fig.1: Relative activity (%) of CsPME, AtPME3, AtPME31 and BcPME1 in presence of various amounts of lactosides. Results are means ± SD of three replicates.

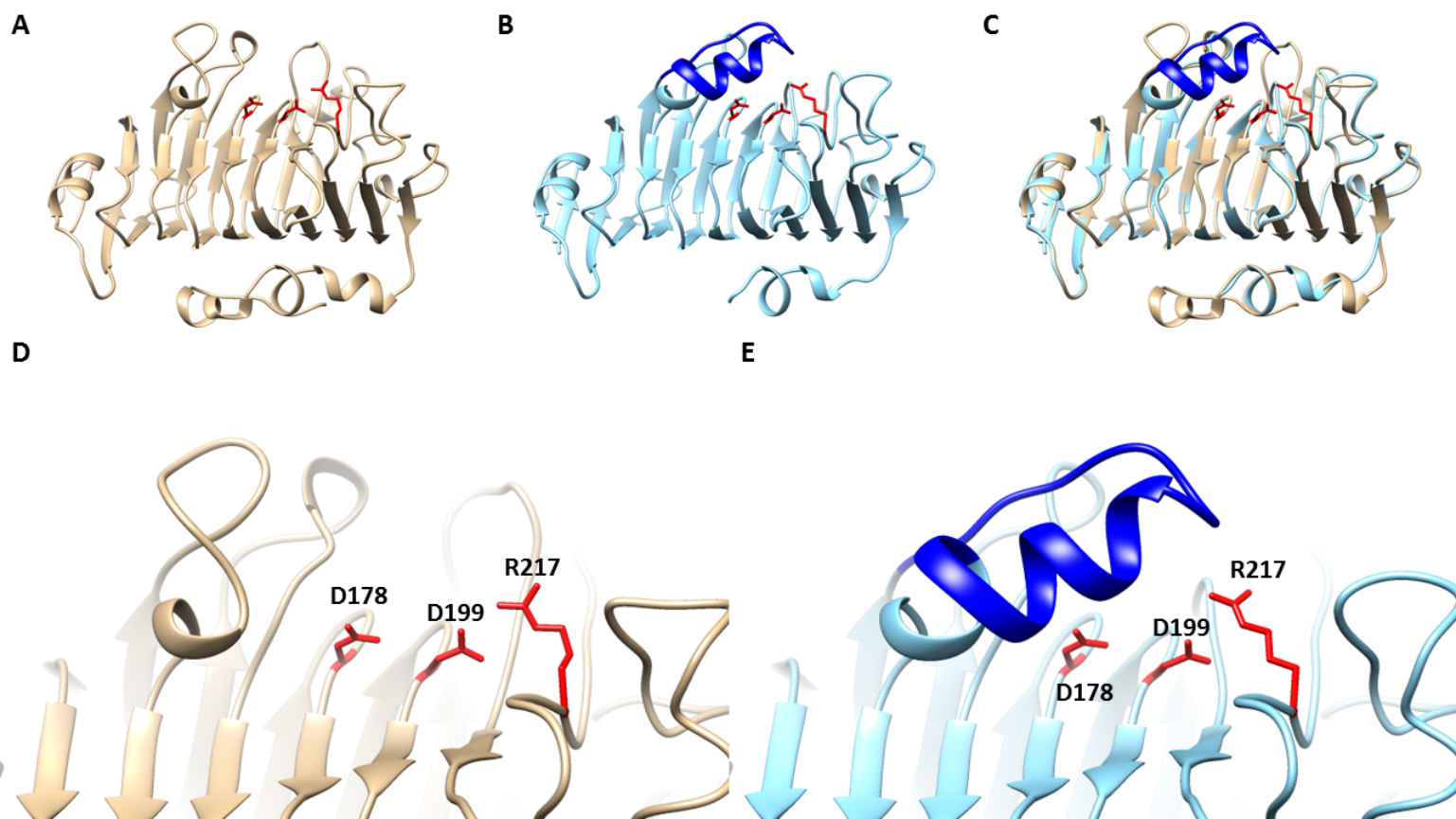
Fig. 2. Predicted 3D structures of AtPME3 and AtPME31. Overall structures of A) AtPME3 (Gold), B) AtPME31 (sky blue), and C) their superposed structures; Binding sites of D) AtPME3 and E) AtPME31. Supplementary loop structure of AtPME31 is colored in blue, and three catalytic residues, D178, D199, and R267, are colored in red.

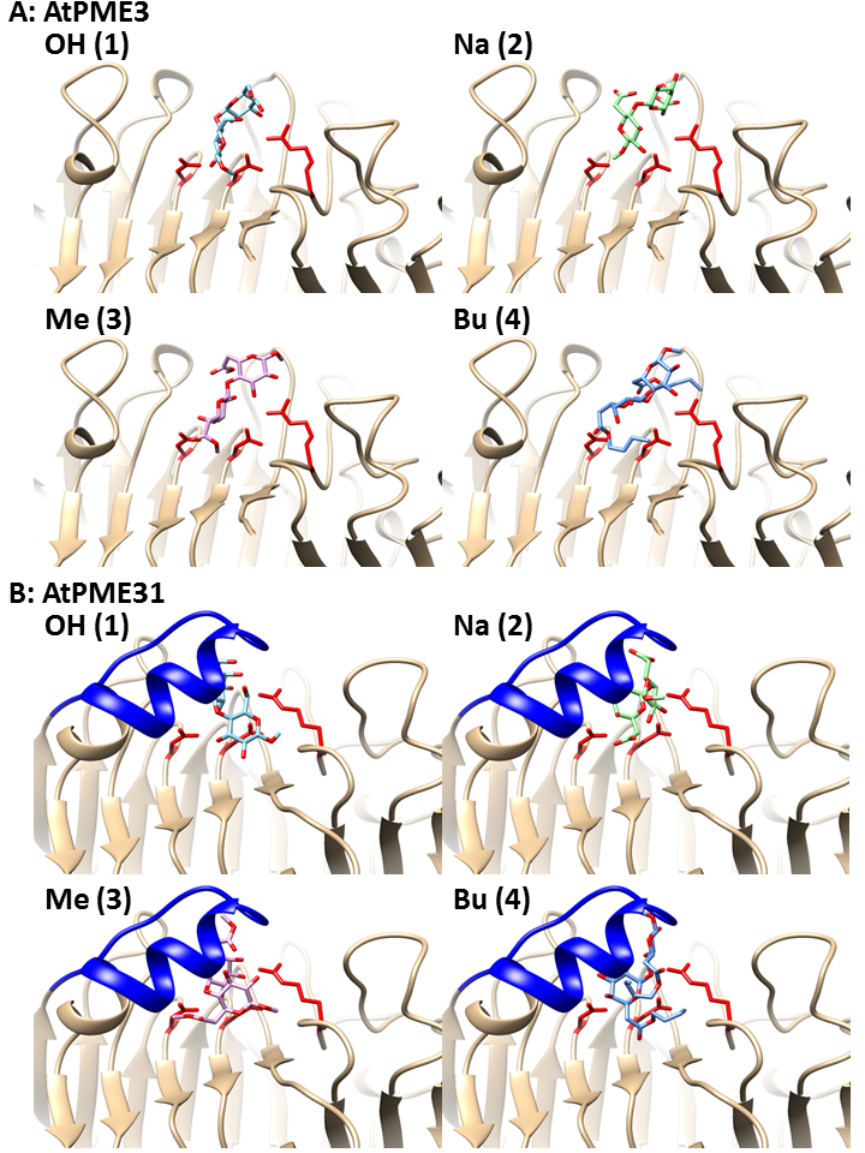
Fig. 3. Comparison of A) AtPME3 with B) AtPME31 in interaction with lactoside derivatives, OH: methyl-lactoside **1**; Na: sodium (methyl-lactosid)uronate **2**; Me: methyl (methyl-lactosid)uronate **3**; Bu: butyl (methyl-lactosid)uronate **4**. Supplementary loop structure of AtPME31 and catalytic residues for both proteins are colored in blue and red, respectively.

Table 1: Binding affinity, number of hydrogen bonds and the binding residues calculated by docking for AtPME3 and AtPME31

	Lactoside derivatives	OH	Me	Na	Bu
AtPME3	Predicted binding affinity (kcal/mol)	-9.08	-5.16	-4.59	-0.32
	N° of H-bonds	5	4	1	0
	Residues	Arg (2), Asp (2), Gln (1)	Asp (2), Gln (2)	Gln	-
AtPME31	Predicted binding affinity (kcal/mol)	24.25	70.30	42.75	213.35
	N° of H-bonds	0	0	0	0
	Positions residues	-	-	-	-


CsPME
Residual activity (%)
120
100
80
60
40
20
0
OH
Me
Na
Bu
Lactosides
0 µM
10 µM
50 µM
100 µM
250 µM
500 µM
1000 µM
1500 µM
AtPME3
Residual activity (%)
OH
Me
Na
Bu
Lactosides
0 µM
10 µM
50 µM
100 µM
250 µM
1000 µM
1500 µM
AtPME31
Residual activity (%)
OH
Me
Na
Bu
Lactosides
0 µM
10 µM
50 µM
100 µM
250 µM
1000 µM
1500 µM
BcPME1
Residual activity (%)
OH
Me
Na
Bu
Lactosides
0 µM
10 µM
50 µM
100 µM
250 µM
1000 µM
1500 µM


A
B
C
D
E
D178
D199
R217
D178
D199
R217


A: AtPME3
OH (1)
Na (2)
Me (3)
Bu (4)
B: AtPME31
OH (1)
Na (2)
Me (3)
Bu (4)