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Biochemical and catalytic properties of two intracellular β-glucosidases from the fungus *Penicillium decumbens* active on flavonoid glucosides

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Abstract

In the presence of rutin as sole carbon source, *Penicillium decumbens* produces two intracellular β-glucosidases named G_I and G_{II}, with molecular masses of 56,000 and 460,000 Da, respectively. The two proteins have been purified to homogeneity. G_I and G_{II} composed of two and four equal sub-units, respectively and displayed optimal activity at pH 7.0 and temperature 65–75 °C. Both β-glucosidases were competitively inhibited by glucose and glucono-δ-lactone. G_I and G_{II} exhibited broad substrate specificity, since they hydrolyzed a range of (1,3)-, (1,4)- and (1,6)-β-glucosides as well as aryl β-glucosides. Determination of k_{cat}/K_m revealed that G_{II} hydrolyzed 3–8 times more efficiently the above-mentioned substrates. The ability of G_I and G_{II} to deglycosylate various flavonoid glycosides was also investigated. Both enzymes were active against flavonoids glycosylated at the 7 position but G_{II} hydrolyzed them 5 times more efficiently than G_I. Of the flavanols tested, both enzymes were incapable of hydrolyzing quercetrin and kaempferol-3-glucoside. The main difference between G_I and G_{II} as far as the hydrolysis of flavanols is concerned, was the ability of G_{II} to hydrolyze the quercetin-3-glucoside.

Keywords: *Penicillium decumbens*; β-Glucosidases; Purification; Kinetics; Deglycosylation; Flavonoids glycosides

1. Introduction

β-Glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) are members of glycosyl hydrolase families 1 and 3 [1,2]. β-Glucosidases hydrolyze *O*-glycosidic bonds at the terminal, nonreducing end of carbohydrates with retention of anomeric configuration. β-Glucosidases demonstrate catalytic activity against a broad range of glycosides including aryl- and alkyl-β-D-glycosides. They have been isolated from members of all three domains of life, i.e. eukarya, bacteria and archaea [3]. These enzymes are widely used in various biotechnological processes, including the production of fuel ethanol from cellulosic materials [4–6] and the synthesis of useful β-glucosides [7,8]. In the flavor industry, β-glucosidases are also studied for its potential to liberate aroma-rich terpenes.

Flavonoids are large class of polyphenolic-plant secondary metabolites providing much of the color and flavor in plant foods. Consequently they occur in many foods and beverages resulting in high human consumption [9]. The flavonoids exist in nature almost exclusively as β-glycosides. The flavanols mainly as the 3-*O*-glycoside, although the 7 and 4′ positions may also be glycosylated in some plants, e.g. onions [10]. Other classes of flavonoids such as the flavones, flavanones and isoflavones, are found mainly glycosylated in the 7 position [11]. The basic structure of the (iso)flavonoids with positions of glycosylation are shown in Fig. 1. Most industrial and domestic food processing procedures do not lead to cleavage of the glycosidic linkage [12–15] and hence flavonoids in foods are generally present as glycosides. Flavonoids have been regarded both as putative vitamins [16] and as potential carcinogens [17], but in the recent years they have attracted considerable interest from both food producers and consumers due to their antioxidative activities [18] and their capacity to inhibit enzymes such as cyclooxygenase and protein kinases involved in cell proliferation and apoptosis [19]. (Iso)flavonoids are potent antioxidants in vitro, and it has become a popular belief that these polyphenols, as part of dietary fruits and vegetables, provide protection against disease by functioning as antioxidants in vivo [20]. The mechanism by which xenobiotics

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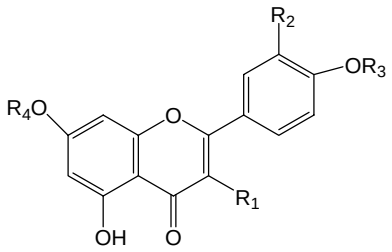
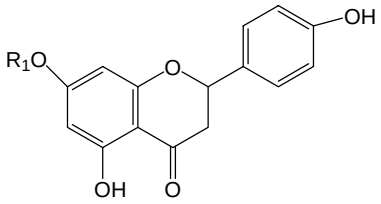
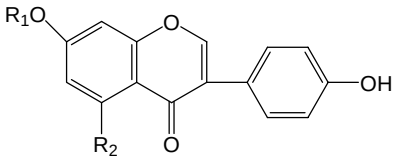


Flavone/Flavonol	R_1	R_2	R_3	R_4
Apigenin 7-glucoside	H	H	H	glucose
Kaempferol 3-glucoside	O-glucose	H	H	H
Quercetin 3-glucoside	O-glucose	OH	H	H
Quercetrin 3-rhamnoside	O-rhamnose	OH	H	H
Quercetin 3-rhamnoglucoside	O-glucose(6→1) α-L-rhamnose	OH	H	H



Flavanone	R_1
Naringenin-7-glucoside	glucose

(a)



Isoflavone	R_1	R_2
Daidzein 7-glucoside	glucose	H

(b)

Fig. 1. Structure of the flavonoid and isoflavonoid glucosides used in the present study (structures compiled from [40]).

are metabolized and adsorbed in humans has received much attention due to the high levels of plant-derived compounds that are ingested orally and bioactive, or which generate potentially toxic or beneficial metabolites [21–24]. Biological activity depends on the presence or absence of the glycosidic residue [21]. The position and nature of the sugar residue may increase the uptake of the compound in the small intestine. However, the aglycone is likely to have a greater biological effect than the glycoside [21], so deglycosylation via a β-glucosidase activity would be an important step in metabolism.

There is considerable interest in altering the form of dietary polyphenols in order to positively affect their bioavailability and/or their biological activities in humans [25,26]. A simple route for altering the form of polyphenols is through deglycosylation with the action of β-glucosidases. In the present work, we describe the purification and kinetic characterization of two intracellular β-glucosidases (viz. G_I and G_{II}) produced by *Penicillium decumbens* which exhibited activity towards flavonoids glycosylated at the 7 position. The most interesting finding was the ability of G_{II} to hydrolyze the quercetin-3-glucoside, while other flavonoids glycosylated at the 3 position were not hydrolyzed by G_I or G_{II}.

2. Experimental

2.1. Microorganism

The microorganism used in this study was *P. decumbens*. It was kindly provided by Prof. Li Ze-Lin, Shichuan Academy of Food and Fermentation Industries, Chengdu, PR China.

2.2. Growth conditions

A three-stage cultivation technique was employed. In the first, the fungus was grown on PDA slants for 4 days at 29 °C. In the second, 25 ml deionized sterile water, was added to a PDA slant and aliquots (5 ml) of the mixture were used to inoculate 3-l Erlenmeyer flasks containing 2.5 g glucose, 3 g $(NH_4)_2HPO_4$ and 500 ml of salts solution containing: KH_2PO_4, 1.0 g l^{-1}; $MgCl_2{\cdot}6H_2O$, 1.0 g l^{-1}; $CuSO_4{\cdot}5H_2O$, 0.014 g l^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.001 g l^{-1}; $FeSO_4{\cdot}7H_2O$, 0.001 g l^{-1}; $MnSO_4{\cdot}H_2O$, 0.001 g l^{-1}. The flasks were incubated at 30 °C for 2 days in an orbital shaker (250 rev min^{-1}) for mycelium production. In the third stage, an inoculum of mycelial suspension (5% v/w) was added to a 20-l bioreactor (MBR, Switzerland). The composition of the mineral medium was as described above and rutin (4 g l^{-1}) and $(NH_4)_2HPO_4$ (9 g l^{-1}) supplemented the medium as carbon and nitrogen source, respectively. The pH of the medium was controlled automatically at 7.0 with the addition of 0.2 M HCl and NaOH solutions. The aeration was adjusted so that the dissolved oxygen was higher than 10% and the agitation was 180 rev min^{-1}. The temperature was controlled automatically at 30 °C. At specific time intervals aliquots were aseptically obtained from the bioreactor in order to monitor biomass production and enzyme activities.

2.3. Enzyme assays

α-Rhamnosidase and quercetinase were determined as described previously [27]. β-Glucosidase activity was determined photometrically with *p*-nitrophenyl-β-D-glucopyranoside (*p*-NP-β-D-glu) (Sigma Chemical Co., St. Louis, MO) as substrate. Fifty microliters of properly diluted sample, were added in 220 μl of 1 mM substrate solution in 100 mM MES–KOH buffer (pH 6.0). The rate of increase in absorbance at 410 nm was measured in a microplate reader (Molecular Devices Corporation, Sunnuvale, USA). The rate

of the reaction was determined with reference to a standard curve, prepared by adding various amounts of *p*-nitrophenol to the corresponding assay buffer. One unit of enzyme activity is defined as the amount of the enzyme liberating 1 μmol *p*-nitrophenol per minute.

2.4. Crude enzyme preparation

Growth of the microorganism was terminated at the 6th day of culture, a time when all three enzyme activities involved in rutin degradation had reached their maximum values [27]. The mycelial biomass was collected on a sieve (125 μm) and washed several times with 50 mM phosphate buffer (pH 7.0). The washed biomass (40 g of total dry weight) was suspended in 800 ml of the above buffer, pre-cooled at 4 °C and homogenized in a household blender (3 min at maximum speed).

Disruption of the mycelia was completed using a laboratory sonicator (Model VC 600, Sonics and Materials, Inc., USA) in 200-ml batches under continuous cooling. At 1-min intervals an aliquot (∼0.3 ml) was pooled from the sonication vessel and filtered (0.45-μm filter cartridges, Millipore, Billerica, USA). β-Glucosidase activity and total protein content was determined in the filtrate, in order to monitor the release of the intracellular material. Based on the above results, a total sonication time of 10 min was selected for maximum release of all three rutin degrading enzyme activities (data not shown).

Following sonication, the crude extract was collected by centrifugation (25 min, 12,500 rpm, 10 °C) and concentrated in an Amicon ultrafiltration apparatus (Amicon chamber 8400 with membrane Diaflo PM30, exclusion size 30 kDa) (Millipore, Billerica, USA), to a final volume of 355 ml. The above concentrated and desalted preparation was used as the final crude enzyme source.

2.5. Protein determination

The protein concentration was determined by the Micro BCA™ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, USA) using bovine serum albumin as a standard. A_{280} was used to monitor protein in column effluents.

2.6. Enzyme purification

The crude enzyme sample was further concentrated with ammonium sulfate precipitation (30–80% saturation levels) prior to chromatographic separations. The first chromatographic step involved anion exchange chromatography on a Q-Sepharose column (Sigma) (2.5 cm i.d., 30 cm length). The column was initially equilibrated with 50 mM MES–KOH buffer at pH 6.0. Following introduction of the crude enzyme sample, the column was eluted with 300 ml of the equilibration buffer at a flow rate of 240 ml h^{-1}. A 1200-ml linear NaCl gradient (0–0.5 M) in equilibration buffer was subsequently applied at the same flow rate. Two peaks with apparent β-glucosidase activity were eluted at 0.22 and 0.34 M NaCl and designated as G_I and G_{II}, respectively. The corresponding fractions were pooled and concentrated in an Amicon ultrafiltration apparatus (Amicon chamber 8400 with membrane Diaflo PM10, exclusion size 10 kDa) (Millipore, Billerica, USA).

Each one of the two β-glucosidases was further separately purified, by applying two identical successive chromatographic steps. The first step involved anion exchange chromatography on a DEAE-Toyopearl 650-M column (Sigma) (2.5 cm i.d., 30 cm length) at pH 5.5. Following application of the sample, the column was washed with 400 ml of 20 mM histidine–HCl buffer at a flow rate of 300 ml h^{-1} and eluted with 800 ml of a linear NaCl gradient from 0 to 0.35 M at the same buffer and flow rate. The second step involved hydrophobic interaction chromatography on a *t*-butyl column (Bio-Rad Laboratories, USA) (2 cm i.d., 25 cm length) at pH 6.8 (100 mM sodium phosphate buffer at a flow rate of 300 ml h^{-1}). After sample application, the column was first eluted with 125 ml of a 2 M $(NH_4)_2SO_4$ solution at the equilibration buffer, followed by a 600 ml linear gradient from 2 to 0 M $(NH_4)_2SO_4$.

The pooled β-glucosidase fractions from the two final hydrophobic columns (G_I and G_{II}), were concentrated and desalted in an Amicon ultrafiltration apparatus (Amicon chamber 8400 with membrane Diaflo PM10, exclusion size 10 kDa) (Millipore, Billerica, USA) using a 20 mM MES–KOH buffer at pH 6.0. These two purified enzyme samples, were used in all subsequent experiments.

2.7. Electrophoresis

Native and SDS–PAGE, on the purified samples was performed in a PhastSystem electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) using appropriate PhastGels according to the manufacturer's recommendations. The gels were silver-stained according to the method of Blum et al. [28].

2.8. Determination of optimum pH and temperature

The response of the purified β-glucosidases (G_I and G_{II}) to pH was determined using the standard assay described above except that appropriate buffers were included in the standard assay. The following buffer systems (100 mM each) were used: citric acid-*di*-sodium hydrogen phosphate (pH 3.5–7.5), MES–KOH (pH 5.5–6.5) and Tris–HCl (pH 7.5–9.0). Temperature effects on activity were measured using the standard assay described above at 30–80 °C.

2.9. Inhibition by glucose and glucono-δ-lactone

The inhibition pattern from glucose and glucono-δ-lactone was determined by the addition of different concentrations of these two compounds in the *p*-NP-G assay system at the optimum pH and temperature for each β-glucosidase. The

inhibition constants were determined from the corresponding Lineweaver–Burk plots using standard linear regression techniques.

2.10. Activity on aryl-glucosides and saccharides

Activity of the purified β-glucosidases (G_I and G_{II}) on aryl-glucosides, all purchased from Sigma, were determined under the following conditions: 1 mM aryl-glucoside in 100 mM MES–KOH buffer (pH 6.5) at 40 °C. The reaction rate was followed from the increase in absorbance at 410 nm in a microplate reader (Molecular Devices Corporation, Sunnuvale, USA). Kinetic constants (K_m, k_{cat}, k_{cat}/K_m), were determined by measuring the activity at varying concentrations of aryl-glucosides (0.05–10 mM).

The activity of the purified β-glucosidases (G_I and G_{II}) against natural substrates (saccharides), all purchased from Sigma was determined as follows: 25 μl from each purified enzyme sample (equal number of units), were added into 225 μl of substrate solution in MES–KOH buffer (pH 6.5). The reaction mixtures were incubated at 40 °C for 60 min and the product (glucose) was estimated by a glucose oxidase/peroxidase assay kit. The kinetic constants (K_m, k_{cat}, k_{cat}/K_m), were determined by measuring the activity at varying concentrations (0.1–1.0 mM) of those saccharides where significant activity was detected.

2.11. Deglycosylation of flavonoids

The following flavonoids were used: quercitrin (quercetin-3-rhamnoside) (Sigma), quercetin-3-glucoside, kaempferol-3-glucoside, daidzein-7-glucoside, apigenin-7-glucoside and naringenin-7-glucoside (Apin Chemicals, Abingdon, Oxon, UK). All flavonoids were dissolved in DMSO and served as 100-mM stock solutions stored at 4 °C. A specific volume from the stock solution was properly diluted in 100 mM MES–KOH buffer at pH 6.5 to yield the desired concentration in each reaction mixture.

Deglycosylation reactions were carried out by adding 100 μl of each purified enzyme sample (equal number of units), into 900 μl of flavonoids solutions of various concentrations. Reactions were carried out at 40°C and stopped by the addition of methanol containing 1 mM ascorbic acid. The samples were centrifuged and the reaction products were detected both through glucose determination (glucose oxidase/peroxidase kit) and HPLC. The analytical HPLC method described by Day et al. [21] was used.

3. Results and discussion

3.1. Purification of the two β-glucosidases

Dinstinct β-glucosidase, α-rhamnosidase and quercetinase activities were detected when *P. decumbens* was grown in a 20-l bioreactor using rutin as a sole carbon and energy source. Rutin, was chosen since, apart from being an inducer for quercetinase, it has been also shown to induce β-glucosidic activity in *Aspergillus* species [29,30]. Most of the β-glucosidic activity was detected intracellularly and as a result, it was decided to purify the enzyme(s) from the washed mycelial extract, which was (were) collected after 6 days of cultivation (maximum observed β-glucosidic activity) [27].

Two intracellular β-glucosidase were purified using a procedure, which involved, ammonium sulfate fractionation, two ion-exchange and a hydrophobic interaction chromatographies. The summary of the purification is presented in Table 1.

3.2. Enzyme characterization

Both β-glucosidases (G_I and G_{II}), as eluted from the final chromatographic step, appeared to be electrophoretically pure as shown in Figs. 2 and 3, respectively. The two enzymes seem to differ very much in their structure, since, significant differences both in the molecular weight as well as in the tertiary structure have been identified. More specifically, G_I revealed a dimeric structure with a sub-unit MW of

Table 1
Summary of the purification procedure for the G_I and G_{II} β-glucosidases of *Penicillium decumbens*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification factor (fold)
Crude enzyme	45.1	119.9	0.38	100	1.00
Ammonium sulfate	26.0	39.6	0.66	57.6	1.74
Q-Sepharose					
G_I	13.9	11.3	1.23	30.8	3.24
G_{II}	11.5	4.5	2.56	25.5	6.74
DEAE-Toyopearl					
G_I	12.3	7.1	1.73	27.3	4.55
G_{II}	11.3	3.12	3.62	25.1	9.53
t-Butyl					
G_I	4.95	2.55	1.94	11.0	5.11
G_{II}	4.22	1.34	3.15	9.36	8.23

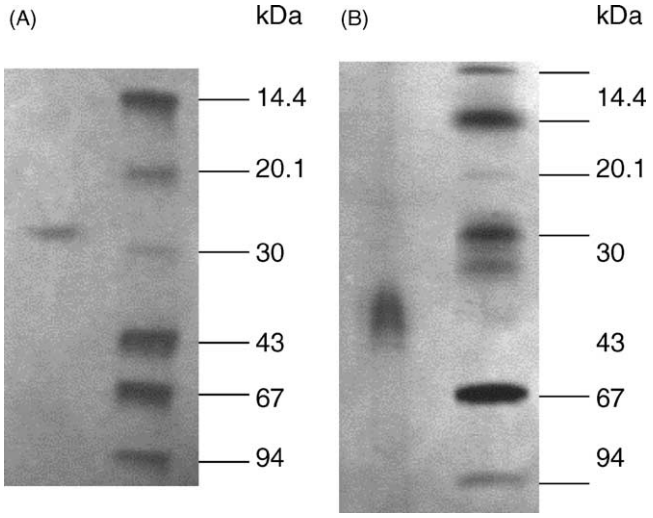


Fig. 2. SDS–PAGE (A) and Native-PAGE (B) of the low-molecular weight β-glucosidase (G_I) of *P. decumbens* on 8–25% gradient and 20% homogenous PhastGels, respectively.

28 kDa, while G_{II} is, apparently, a tetramer with a sub-unit MW of 115 kDa.

Most fungal β-glucosidases reported are either momomers or dimers of a reduced molecular weight between 43 and 131 kDa [29–36], but in the case of G_I the sub-unit size of 28 kDa is, to our knowledge, the smallest reported so far for fungal enzymes. On the contrary, G_{II} has one of the highest molecular mass values for β-glucosidases found in literature. It apparently comprises of four sub-units of 115 kDa each, resulting in a total molecular mass of 460 kDa. β-Glucosidases with high molecular mass reported in the literature are those of *Botrytis cinerea* [37] *Monilia* sp. [38], *Aureobasidium pullulans* [39] and *Trichoderma longibrachiatum* [40] which have values of 350, 480, 340 and 350 kDa, respectively.

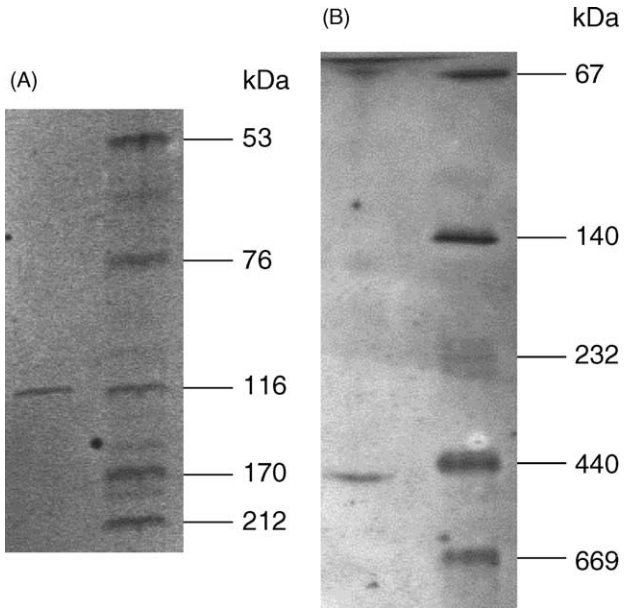


Fig. 3. SDS–PAGE (A) and Native-PAGE (B) of the high-molecular weight β-glucosidase (G_{II}) of *P. decumbens* on 12.5% homogenous and 4–15% gradient PhastGels, respectively.

The pH for optimal enzyme activity of both enzymes (pH 7.0) was higher than that reported for other fungal β-glucosidases [29–36,39] and similar to *B. cinerea* β-glucosidase [37] which is characterized by molecular weight and tetrameric structure similar to G_{II}.

The optimum temperature for G_I and G_{II} was found to be 65 and 75 °C, respectively. In general, optimum temperature for fungal β-glucosidases ranged from 50 to 65 °C [29,30,32,34–37]. None of the enzymes though showed any exceptional thermal stability since both completely lost their activity after 1 h of incubation at 70 °C.

3.3. Inhibition by glucose and glucono-δ-lactone

For both enzymes, glucono-δ-lactone, proved to be the most potent inhibitor, since the corresponding reaction rates were decreased more than 90% in the presence of 5.6 mM of the compound. Similar inhibition levels required glucose concentrations higher than 110.9 mM.

Comparison among the two enzymes, revealed that G_I was more strongly affected by both inhibitors tested. Initial reaction rate experiments at various inhibitor concentrations, within a range of substrate concentrations, revealed that both β-glucosidases were competitively inhibited by glucose and glucono-δ-lactone (data not shown). The corresponding dissociation constant (K_i) values determined from the Lineweaver–Burk plots where 243 and 0.531 μM for G_I and 297 and 0.578 μl for G_{II}, glucose and glucono-δ-lactone, respectively. Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases although there are exceptions like β-glucosidases produced by several *Aspergillus* species [29,30,36]. Most microbial β-glucosidases have glucose inhibition constants (K_i) ranging from 0.35 mM to no more than 100 mM [29]. The glucose inhibition constants for G_I and G_{II} are the smallest ones reported for fungal β-glucosidases [32,33,37,39] comparable only to those for β-glucosidase from *Acremonium prersicinum* [31].

Glucono-δ-lactone, which is known to have structural similarity with an intermediary compound of the reaction, was by far the most potent inhibitor for both G_I and G_{II}, a characteristic common to other fungal β-glucosidases. K_i values reported for glucono-δ-lactone are in the range of 4–20 μM [31–33,37] with exception of β-glucosidases from *Aspergillus oryzae* and *Sclerotium glucanicum* which exhibited K_i values of 12.5 and 12.79 mM, respectively [29,41].

The comparison of K_i values for each enzyme indicated that glucono-δ-lactone inhibited both enzymes 2×10^3 times more strongly than glucose. Although the above values are quite close for both enzymes, they indicate a possible higher resistance for G_{II} against both inhibitors.

Table 2
Relative initial rate of hydrolysis of various aryl-glycosides and saccharides by purified β-glucosidases from *Penicillium decumbens* (conditions are described in Section 2)

Substrates[a]	Linkage of glycosyl group	Relative initial rate of hydrolysis (%)[b]	
		G_I	G_{II}
Aryl-glycosides			
p-NP-β-D-glucopyranoside	β(1,)Glc	100	100
p-NP-β-D-cellobioside	β(1,4)Glc	16.8	22.3
o-NP-β-D-xylopyranoside	β(1,4)Xyl	13.1	13.6
p-NP-α-D-glucopyranoside	α(1,4)Glc	4.7	6.1
Saccharides			
Gentiobiose	β(1,6)Glc	100	100
Amygdalin	β(1,6)Glc	80.2	67.8
Laminarin	β(1,3)Glc	38.7	53.6
Salicin	β(1,4)Glc	36.0	81.2
Cellobiose	β(1,4)Glc	27.6	74.4
Lichenan	β(1,4)Glc and β(1,3)Glc at approximate ratio of 2:1	22.5	46.8
Arbutin	β(1,4)Glc	7.6	9.9

[a] No activity was detected against *p*-NP-α-D-galactopyranoside, *o*-NP-β-D-galactopyranoside, *p*-NP-α-D-arabinofuranoside, trehalose, methyl-β-D-glucopyranoside, CM-cellulose and Avicel.

[b] Depending on the type of substrate, activity was detrmined by measuring the release of glucose (glucose oxidase/peroxidase assay kit) or of *o*-NP and *p*-NP (410 nm). The relative initial rates of hydrolysis of aryl-glycosides and saccharides are expressed as percentanges of the initial rates of hydrolysis obtained with *p*-NP-β-D-glucopyranoside and gentiobiose. Both enzymes were used at 13 mU $ml^{-1}_{reaction\ volume}$. All substrate concentrations used were 1 mM except CM-cellulose, Avicel and lichenan which were 1% (w/v).

3.4. Kinetic and specificity studies against aryl-glycosides and saccharides

The relative initial rates of hydrolysis of several aryl-glycosides and saccharides by purified G_I and G_{II} are presented in Table 2. Both G_I and G_{II} were optimally active against the *p*-NP-β-D-glucopyranoside with only 5% of the corresponding activity appearing against *p*-NP-α-D-glucopyranoside. Significant activity was also recorded against *p*-NP-β-D-cellobioside and *p*-NP-β-D-xylopyranoside (about 20 and 13%, respectively, of the activity against *p*-NP-β-D-glucopyranoside). Both enzymes were inactive against *p*-NP-α-D-galactopyranoside, *o*NP-β-D-galactopyranoside and *p*-NP-α-D-arabinofuranoside.

G_I could efficiently hydrolyze natural oligosaccharides having α(1,4)-glucosidic linkages such as laminarin, salicin, cellobiose, lichenan (glucose polysaccharide—mixture of α(1,4)- and α(1,3)-glucosidic linkages at approximate ratio of 2:1), but the enzyme exhibited higher activity against oligosaccharides having α(1,6)-glucosidic linkages such as gentiobiose and amygdalin. Gentiobiose proved to be the best substrate for G_{II} which additionally showed 50 to 150% higher initial rates of hydrolysis than that of G_I against glucosides with α(1,3) and α(1,4) bonds such as laminarin, cellobiose and lichenan. Arbutin was poorly hydrolyzed by both β-glucosidases which were also inactive on trehalose, methyl-β-D-glucopyranoside, CM-cellulose and Avicel.

The affinity of G_I and G_{II} was determined for *p*-NP-β-D-glucopyranoside and various saccharides and the results are summarized in Table 3. The K_m values of G_I and G_{II} against *p*-NP-β-D-glucopyranoside (0.013 and 0.07 mM, respectively) were the lower ones reported in literature for fungal β-glucosidases [29–34,36–39]. The comparison of k_{cat}/K_m ratio revealed that G_{II} hydrolyzed *p*-NP-β-D-glucopyranoside and other tested saccharides 3–10 times more efficiently than G_I.

The purified G_I and G_{II} from *P. decumbens* exhibited broad substrate specificity, since they can hydrolyze a range of (1,3)-, (1,4)- and (1,6)-β-glucosides as well as aryl β-glucosides. β-Glucosidases with very broad specificity have been isolated from many fungi such as *A. oryzae* [29], *Aspergillus tubingensis* [30], *A. persicinum* [31], *Fusarium oxysporum* F3 [32], *Aspergillus niger* [36]and *B. cinerea* [37].

3.5. Kinetic studies against flavonoid glycosides

The ability of G_I and G_{II} to hydrolyze a variety of glucosides was assesed using a variety of aglycone structures that were linked to sugars through various position on the aglycone (Fig. 1 and Table 3). The flavanols tested namely, quercetin-3-glucoside, kaempferol-3-glucoside and quercetin-3-rhamnoside, were glycosylated at 3 position, although flavanols glycosylated at 7 and 4′ positions may also be found in some plants, e.g. onions [10]. All other flavonoids examined such as the flavones (apigetrin), flavanones (naringenin-7-glucoside) and isoflavones (daidzin) were glycosylated at 7 position.

Both enzymes were capable of hydrolyze the flavonoids glycosylated at the 7 position (Table 3). G_I exhibited higher apparent affinity (K_m) against daidzin compared to apigetrin and naringenin-7-glucoside. On the other hand, the apparent affinities for G_{II} against the above-mentioned flavonoids were much lower. G_{II} exhibited approximately seven times

Table 3
Summary of the kinetic constants of the two purified β-glucosidases of *P. decumbens* against various substrates

Substrate	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	
	G_I	G_{II}	G_I	G_{II}	G_I	G_{II}
Aryl-glycosides						
p-NP-β-D-glucopyranoside	0.013	0.007	0.357	1.527	27.46	218.14
Oligosaccharides						
Cellobiose	0.475	0.400	0.100	0.507	0.210	1.267
Gentiobiose	0.834	0.355	0.425	0.598	0.509	1.685
Simple phenolics						
Salicyl alcohol-Glc (salicin)	0.455	0.487	0.100	0.663	0.219	1.361
Hydroquinone-Glc (arbutin)	0.228	0.109	0.030	0.110	0.132	1.009
Cyanogenic glycosides						
Mandelonitrile-general (amygdalin)	0.466	0.126	0.237	0.413	0.508	3.278
Isoflavones (phytoestrogens)						
Daidzein-7-Glc (daidzin)	0.970	0.651	0.325	1.072	0.335	1.647
Flavonols						
Quercetin-3-Rha (quercitrin)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Kaempferol-3-Glc	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Quercetin-3-Glc (isoquercitrin)	N.D.	0.066	N.D.	6.7×10^{-4}	N.D.	0.010
Flavones						
Apigenin-7-Glc (apigetrin)	0.541	0.402	0.165	0.605	0.305	1.505
Flavanones						
Naringenin-7-Glc	0.669	0.452	0.190	0.632	0.284	1.398

N.D.: not detected.

higher specificity against flavonoids glycosylated at 7 position compared to G_I.

Similar behavior against flavonoids glycosylated at 7 position was also reported for cell-free extracts of human small intestine and liver with β-glucosidase activity [21], a cytosolic β-glucosidase from pig liver [42], a cytosolic β-glucosidase from human liver (hCBG) [22,23], an *A. niger* β-glucosidase [25]and lactase-phlorizin hydrolase (LPH) isolated from sheep small intestine [43].

Of the flavanols tested, both enzymes were incapable of hydrolyze quercetrin indicating that they do not have any rhamnosidase activity. Furthermore, no activity was detected against kaempferol-3-glucoside by G_I and G_{II}. These results are in accordance with those reported for an *A. niger* β-glucosidase [25].

The main difference between G_I and G_{II} was the ability of G_{II} to hydrolyze the quercetin-3-glucoside. The K_m and k_{cat}/K_m values of G_{II} for quercetin-3-glucoside were 0.066 mM and 0.01 $mM^{-1}\,s^{-1}$, respectively. Dietary flavonoids are more frequently found with a sugar attached in the 3 position, compared to the 7 and 4′ positions. Human cytolosic β-glucosidase appears incapable of hydrolyze 3-linked glucosides (e.g. quercetin-3-glucoside), hence necessitating the existence of another β-glucosidase with the required specificity such as LPH which is specifically located to the intestinal epithelial cells [24,43]. To our knowledge, the only fungal β-glucosidase capable of hydrolyze quercetin-3-glucoside was an *A. niger* β-glucosidase purified from a commercial enzyme preparation [25].

4. Conclusions

Two intracellular β-glucosidases (G_I and G_{II}) from *P. decumbens* were purified to homogeneity by ammonium sulfate fractionation, ion-exchange and hydrophobic interaction chromatographies. The two enzymes seem to differ very much in their structure, since, significant differences both in the molecular weight as well as in the tertiary structure have been identified. The purified G_I and G_{II} exhibited broad substrate specificity, since they hydrolyzed a range of (1,3)-, (1,4)- and (1,6)-β-glucosides as well as aryl β-glucosides. Furthermore, G_I and G_{II} were capable of hydrolyzing flavones (apigetrin), flavanones (naringenin-7-glucoside) and isoflavones (daidzin), e.g. flavonoids glycosylated at the 7 position, but G_{II} hydrolyzed them five times more efficiently than G_I. Both enzymes were incapable of hydrolyzing flavanols like quercetrin or kaempferol-3-glucoside. The main difference between G_I and G_{II}, was the ability of G_{II} to hydrolyze the quercetin-3-glucoside. This data indicate that β-glucosidases could be useful tools for altering the form and properties of naturally occurring plant polyphenols in order to positively affect their bioavailability and/or their biological activities in humans.

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