Insight into naphthoquinone metabolism: β-glucosidase-catalysed hydrolysis of hydrojuglone β-D-glucopyranoside

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In plants, the naphthoquinone juglone is known to be involved in pathogenic defence mechanisms, but it may also take part in plant developmental processes. This naphthoquinone can accumulate in a glycosylated form, namely hydrojuglone β-D-glucopyranoside. The structural configuration of this compound was shown to be 1,5-dihydroxy-4-naphthalenyl-β-D-glucopyranoside by means of MS, NMR and nuclear Overhauser effect spectroscopy analyses. A hydrojuglone β-D-glucopyranoside β-glucosidase (EC 3.2.1.21) was purified to homogeneity from *Juglans regia* L. The enzyme catalysed the release of juglone from hydrojuglone β-D-glucopyranoside with high specificity and showed Michaelis–Menten kinetics with K_m = 0.62 mM and V_{max} = 14.5 μkat/mg of protein. This enzyme also showed a higher activity towards β-D-fucosyl than β-D-glucosyl bonds. The purified enzyme had an apparent M_r of 64000 by SDS/PAGE and a pI 8.9 by isoelectrofocusing PAGE. The purified enzyme was inhibited by several bivalent cations, such as Cu^{2+}, Fe^{2+}, Hg^{2+}, and by D-glucono-1,5-lactone, showing non-competitive inhibition of the mixed type.

INTRODUCTION

In plants, phenolic compounds are widely encountered as β-D-glucosyl conjugates. These glucosides belong to various chemical families, such as benzoic or hydroxycinnamic acids, coumarins, flavonoids (for review see [1]), and can generally be found in large amounts stored in vacuoles [2,3]. Both heteroglucosides and their corresponding aglycones are found in plant tissues, with the heteroglucosides considered not to be physiologically active, whereas the corresponding aglycones are. This was proved with salicylic acid, a compound responsible for systemic acquired resistance in many plants [4], which is able to bind a target protein, whereas its glucoside is not [5]. Two other lines of evidence are given by: (1) the ability of the monolignol coniferyl alcohol, and not its glucoside precursor coniferin, to be oxidized before its integration in lignins [6,7], and (2) the ability of the flavonol quercetin, and not its glycoside rutin, to inhibit several enzymic activities [8,9]. In addition, other non-phenolic heteroglucosides were also reported to be physiologically inactive, such as glucosyl-*O*-zeatin [10,11] or glucosyl-saponins [12,13]. Hence, we can argue that the enzymes responsible for the conversion from one form to another may play an important regulatory role in biological processes [14], although the cellular mechanisms involved in this conversion remain unknown.

The hydrolysis of glucosides, leading to physiologically active compounds, is catalysed by β-glucosidases (EC 3.2.1.21). In addition, plant β-glucosidases are generally specific for their natural glucosides [15,16], and recent work carried out on these enzymes reports the isolation of β-glucosidases with high activity towards natural substrates isolated from the same tissue [13, 17,18]. This is likely to be the case with naphthoquinones. These phenolics occur in about 20 plant families [19] and are derived from the shikimic acid and *o*-succinoylbenzoic acid biosynthetic pathway [20]. Amongst the naphthoquinones, juglone (5-hydroxy-1,4-naphthoquinone) is of great interest due to its chemical reactivity. Juglone is known to undergo reversible oxido-reduction reactions with the concomitant formation of free radicals [21] and shows electrophilic reactivity towards cellular nucleophiles such as thiol groups [22]. Recent studies have shown that juglone could penetrate the plasma membrane and induce depolarization by blocking K^+ channels [23]. Hence, it has been proposed that naphthoquinones could act as plant growth regulators [24] or as protective agents against microorganisms [25], although the underlying control mechanisms are somewhat obscure. Furthermore, juglone has been shown to promote cell division, cell elongation and adventitious root formation in blackberry [26], and 1,4-naphthoquinone, a compound related to juglone, has also been shown to promote callus growth in the tomato and to induce root formation in mung bean cuttings [24].

Although juglone was first believed to occur as an artefact of extraction procedures, its presence as a genuine, endogenous compound has now been revealed [20,27]. Metabolic studies have shown that juglone formation is the result of 1,4,5-trihydroxynaphthalene oxidation and that it could also occur as a glucoside, namely hydrojuglone β-D-glucopyranoside (HJG) [28]. In early reports, the structural configuration of the 1,4,5-trihydroxynaphthalene glucosyl conjugate was given with the β-D-glucosyl moiety bonded in position 5 on the naphthalene ring [29,30]. Later it was given with the β-D-glucosyl moiety bonded in position 4 [25,31], but with no data to support the structural configuration. The possibility of an enzymic hydrolysis of the 1,4,5-trihydroxynaphthalene glucosyl conjugate has never been reported. We decided to address this question in walnut, where HJG accumulates in roots, shoots and young leaves of walnut trees [32–34] and amounts to as much as 6–8 % of the total dry mass in young tissues and organs such as buds of male catkin and fruits [35].

The aims of the experiments carried out in the present study are (1) to resolve the structural configuration of 1,4,5-tri-

Abbreviations used: HJG, hydrojuglone-β-D-glucopyranoside or 1,5-dihydroxy-4-naphthalenyl-β-D-glucopyranoside; IEF, isoelectrofocusing; 4-MU, 4-methylumbellyferyl; *p*-NP, *p*-nitrophenyl; WBG8.9, walnut β-glucosidase pI 8.9 or hydrojuglone-β-D-glucopyranoside-β-glucosidase.

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hydroxynaphthalene glucosyl conjugate by means of MS and NMR (the conjugate is also used as a natural substrate for the purified enzyme reported here), and (2) to purify and characterize biochemically a hydrojuglone β-D-glucopyranoside β-glucosidase from *Juglans regia* L.

MATERIALS AND METHODS

Reagents and materials

Reagents were purchased from Sigma (L'Isle d'Abeau Chesnes, France) unless otherwise stated. Walnut cotyledon fragments (*Juglans regia* L.) were cultured *in vitro* as previously described [36] and harvested after 96 h of culture. Only the cotyledon petioles [37] were used for the protein-extraction experiments. After dissection, cotyledon petioles were immediately dipped in liquid nitrogen, then freeze-dried (Lyovac GT2, Leybold-Heraeus, Les Ulis, France) and stored *in vacuo* until extraction with Silicagel (Sigma) as a water trap.

Extraction and purification of HJG

HJG was extracted from fresh walnut husks in 80 % acetone at 4 °C. Acetone was evaporated under reduced pressure to produce an aqueous extract. In order to remove the photosynthetic pigments, the aqueous extract was subjected to phase-partitioning three times against the same volume of petroleum spirit and three times against the same volume of ethyl acetate. De-pigmented aqueous phase was then concentrated to dryness *in vacuo* and the solid residue was partly solubilized in absolute methanol. The remaining insoluble material was pelleted by centrifugation at 10000 ***g*** for 2 min. HJG was purified twice from the resulting methanolic supernatant by means of preparative reverse-phase HPLC (System Gold, Beckman, Gagny, France) using a C_{18} matrix (LiChrospher 7 μm, 1.0 cm × 25.0 cm; Merck, Nogent sur Marne, France). The mobile phase consisted of a linear gradient of A [acetic acid/H_2O (1:99)] and B [methanol/acetonitrile (1:1)] starting from 30 % (v/v) B to 100 % (v/v) B in 24 min at a flow rate of 2 ml/min. The purity of HJG was verified by analytical reverse-phase HPLC (LiChrospher 5 μm, 0.4 cm × 24.4 cm; Merck) coupled with a diode-array scan monitor (168 module, Beckman), using a previously described chromatography gradient [38].

Identification of HJG

The identification of the purified HJG was confirmed by MS and NMR methods. MS experiments were conducted on an R10-10C apparatus (Nermag, Paris, France). Spectra were obtained by electron-impact MS and chemical-impact MS in ammonia or methane. NMR experiments were conducted on an AM series spectrometer (Brucker, Wissembourg, France) operating at a frequency of 300.13 MHz for the proton channel. Data were all processed using Brucker DISNMR software package version 87, working on an Aspect 3000 computer fitted with an array Fourier transform coprocessor. Identification of the spin–spin coupling network and measurements of the *J* coupling magnitude were achieved on a 0.5 mM sample of HJG in DMSO-d6 at 25 °C by two-dimensional (4096 × 256 data point matrix), proton double-quantum filtered correlation spectroscopy (DQF-COSY) in the phase-sensitive mode [39] according to the procedure described by Marion and Wüthrich [40]. Steady-state nuclear Overhauser effects [41] were evaluated on a de-gassed 1 mM sample in the same solvent at 30 °C with a 2 s nuclear Overhauser effects generation period and 5 s of relaxation for each irradiation. Free induction decay values (960 scans each) were then subtracted to a reference before Fourier transformation. The structure of HJG was constructed using the structure-generation facilities of Chem-x software version 86 on an Evans and Sutherland PS 390 three-dimensional graphical workstation hosted by a Digital Equipment micro VAX-II computer operating under the VAX-VMS system version (Molecular Design Ltd., Oxford, U.K.). [*f*,*y*] energetic space was evaluated over the whole angular scale by 7.5 ° (48 points) increments using Van der Waals potential function excluding tortionals, exo-anomeric effect and intramolecular H-bonding. Iso-energy contours were mapped with 1 kcal/mol interpolation relative to the global minimum; 10 kcal/mol was selected as the outer limit. *f* and *y* tortional angles were defined according to IUPAC–IUBMB conventions.

Extraction and purification of HJG β-glucosidase

All steps of purification were performed at 4 °C in a cold room. Dry material (500 mg) was reduced to a fine powder using a mortar and pestle in the presence of white sand. Dry powder was then soaked in 50 ml of 0.1 M sodium phosphate buffer (buffer A) at pH 7.0 and supplemented with 20 mM $Na_2S_2O_5$, 20 mM cysteine, 5 mM PMSF and 4 % (w/v) polyvinylpolypyrrolidone for 1 h. The homogenate was centrifugated for 10 min at 15000 ***g*** and the supernatant was saved. The pellet was re-extracted under the same conditions. Crude extract consisted of the pooled supernatants resulting from both extractions. Crude extract was then soaked for 1 h with 2 g of polyvinylpolypyrrolidone to remove the remaining phenolic compounds. After centrifugation, the supernatant was subjected to overnight dialysis (cellulose membrane, Sigma) against 5 litres of buffer A, and the volume was reduced to 5 ml by ultrafiltration (Ultrafree 15, 5 kDa molecular mass cut-off; Millipore, Saint Quentin Yvelines, France). Purification of the β-glucosidase was performed according to a three-step chromatography procedure. (1) Dialysed extract was loaded on to a S-200-Sephacryl gel-filtration matrix (1.5 cm × 70 cm; Sigma) equilibrated with buffer A. Fractions (55–65 ml) containing β-glucosidase activity were pooled, concentrated by ultrafiltration and dialysed against 10 mM diethanolamine buffered with HCl at pH 8.8 (buffer B). (2) This protein solution was further applied to a DEAE-trisacryl anion-exchange matrix (1.0 cm × 3.0 cm; BioSepra, Villeneuve La Garenne, France) equilibrated with buffer B. The matrix was washed with 20 ml of buffer B and the non-retained fractions containing the β-glucosidase activity were pooled. The protein solution was dialysed against 10 mM glycine buffered with NaOH at pH 8.8 (buffer C) and concentrated to 1 ml by ultrafiltration. (3) The protein fraction was loaded on to a CM-Fractogel cation-exchange matrix (1.0 cm × 3.0 cm; Merck, Nogent sur Marne, France) equilibrated with buffer C. The β-glucosidase activity was eluted with 20 ml of buffer C supplemented with 80 mM NaCl. The purified β-glucosidase was stored at 4 °C.

Analytical gel-filtration

Gel-filtration chromatography was performed using an S-100-Sephacryl matrix (1.5 cm × 70 cm; Sigma) equilibrated with buffer A. The matrix was calibrated with the following proteins (Sigma): fructose-6-phosphate kinase (M_r 84000), BSA (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000) and cytochrome *c* (M_r 12400).

Electrophoretic methods

SDS/PAGE was performed as described by Laemmli [42] using a 4.5 % (w/v) acrylamide stacking gel and an 8 % (w/v) acrylamide running gel (7.5 cm × 5.0 cm). M_r standards (Sigma)

were: myosin (205000), β-galactosidase (116000), phosphorylase *b* (97000), fructose-6-phosphate kinase (84000), BSA (66000), glutamate dehydrogenase (55000), ovalbumin (45000) and glyceraldehyde-3-phosphate dehydrogenase (36000). Gels were stained for protein with silver nitrate [43]. Isoelectrofocusing (IEF) PAGE was performed as described by Wrigley [44] in a Multiphor II unit (Pharmacia, Saclay, France), using a 5% (w/v) acrylamide slab gel (24.5 cm × 11 cm) with 2.5% (v/v) ampholytes (Ampholine, Pharmacia), pH range 3–10. The IEF protein standards (Sigma) were: trypsinogen (pI 9.3); basic lentil lectins (pI 8.8), lentil lectin (pI 8.6), acidic lentil lectin (pI 8.2), basic myoglobin (pI 7.2), acidic myoglobin (pI 6.8), carbonic anhydrase I (pI 6.6), basic carbonic anhydrase II (pI 5.9), β-lactoglobulin (pI 5.1). Before silver staining for protein [43], gels were soaked in a solution containing 20% (w/v) trichloroacetic acid and 5% (w/v) sulphosalicylic acid for 30 min to remove the ampholytes. Glycosyl hydrolase activities were revealed with 4-MU-β-D-Glc*p* or 4-MU-β-D-Fuc*p* in 0.1 M phosphate buffer at pH 6.0 [45], where 4-MU is 4-methylumbellyferyl.

Enzyme assays and protein content

Under standard conditions, enzyme activity was measured using 1 mM substrate in 0.1 M phosphate buffer, pH 6.0, at 25 °C. Chromatography effluents were measured for β-glucosidase activity with *p*-NP-β-D-Glc*p* as substrate, where *p*-NP is *p*-nitrophenyl, and for protein content by Bradford's method [46]. Hydrolysis of *p*-NP glycosides was performed by measuring the release of *p*-nitrophenolate ion at 405 nm (MR 5000 microplate reader; Dynatech, Guyancourt, France). Upon 4-MU-glycoside hydrolysis, the released free 4-MU was monitored by spectrofluorimetry at an excitation wavelength of 360 nm and an emission wavelength of 460 nm (Fluoscan II microplate reader; Biosearch, Millipore). The hydrolysis of natural substrates was assessed by HPLC (System GOLD, Beckman) using a C_{18} matrix (LiChrospher 5 μM, 0.4 cm × 24.4 cm; Merck) and monitored by absorbance measurement at 280 nm. For each substrate, a calibration curve was determined by plotting peak area versus quantity of the injected molecule. Calibration curves were then used to calculate the quantity of substrate consumed during the course of reaction. Hydrolysis of HJG was assessed by measuring the decrease in absorbance at 306 nm, whereas juglone formation was monitored at 423 nm using a spectrophotometer (Uvikon 930, Kontron, Montigny le Bretonneux, France). For determination of the kinetic parameters, the quantity of enzyme was adjusted to keep the initial velocities linear during the time of reaction. K_m and V_{max} were calculated from linear regression of Hanes–Woolf plots. The inhibitory effects of selected compounds on enzyme activity were tested with 4-MU-β-D-Glc*p* and 4-MU-β-D-Fuc*p* used as substrates, and owing to the poor solubility of some metallic ions in 0.1 M phosphate buffer at pH 6.0, enzymic reactions were performed in 0.2 M acetate buffer, pH 5.5, at 25 °C instead. Kinetic studies for D-glucono-1,5-lactone inhibition were performed with HJG as substrate in 0.1 M phosphate buffer pH 6.0. The kinetic parameters for inhibition, K_{IE} and K_{IES}, were calculated from Lineweaver–Burk plots. All assays were carried out in triplicate and values shown represent the means of three trials.

RESULTS

Chemical structure of HJG

Before MS and NMR analyses, HJG was purified by means of reverse-phase preparative HPLC. In order to avoid degradation by oxidation, analyses were done rapidly after elution. A soft chemical-impact MS (ammonia, probe) determined the mass of this compound, *m/z* (relative intensity): 339 $[M+H]^+$ (56), 356 $[M+NH_4]^+$ (40), 180 $[(163-H)+NH_4]^+$ (100), 177 $[M-(163+H)+H]^+$ (68). The ion *m/z* 180 shows the presence of glucose. Another investigation by chemical-impact MS (methane, probe), 70 eV gives *m/z* (relative intensity): 339 $[M+H]^+$ (12), 177 $[M-(163+H)+H]^+$ (30). An electron-impact MS (probe), 70 eV gives *m/z* (relative intensity); 338 $[M]^+$ (1), 176 $[M(-163+H)]^+$ (100). All these data suggest that $M = 338$. The one-dimensional proton NMR spectrum of HJG in DMSO-d6 exhibits six broad and easily chemically exchangeable resonances which can be attributed to hydroxyl signals. They show similar half-height width, except for the one at $\delta = 9.37$ p.p.m., which is much narrower than the others. This is related to the slower exchange rate of this hydroxyl position. This high chemical-shift value and the one at $\delta = 9.78$ p.p.m. indicate two free phenolic hydroxyls in the molecule with different chemical environments. One-dimensional analysis showed two coupled aromatic spin systems: AM ($\delta = 7.20$ and 6.71 p.p.m.) and AMX ($\delta = 7.57$, 7.29 and 6.88 p.p.m.), characteristic of a mono-O-substituted 1,4,5-trihydroxynaphthalene. The other resonances are mainly crowded under the residual water signal at $\delta = 3.32$ p.p.m. or ^{2}HOH ($\delta =$ 3.49 p.p.m.), when introducing deuterium oxide. The doublet resonance at $\delta = 4.50$ p.p.m. is characteristic of an anomeric proton of a sugar residue. Full identification is nevertheless achieved by analysis of the two-dimensional DQF-COSY spectrum of HJG. Starting from the AB spin system at $\delta = 3.78$ and 3.50 p.p.m. with a large ^2J value of 11.9 Hz, attributed to the terminal methylene group of a hexose, it is possible to identify vicinal proton (H5′, where the prime indicates a sugar residue) at $\delta = 3.36$ p.p.m. with ^3JH5′, H4′ of 9.5 Hz, similar to ^3JH4′, H3′. Finally, the value of ^3JH1′, H2′ (8 Hz) proves the full trans-diaxial protonic disposition of a β-glucopyranosyl-type structure. The degeneracy of H2′–H3′ at $\delta = 3.30$ ($\Delta\delta \approx 0$), inducing virtual coupling with associated spin systems H1′ and H4′, should be noted. Full assignments are listed in Table 1.

Table 1 Chemical shifts and *J* coupling assignment of proton resonances of HJG

Analysis was conducted at 25 °C with 0.5 mM HJG in DMSO-d6. *Primes refer to the sugar moiety; †undeuterated residue of DMSO as internal reference = 2.49; ‡values given ± 0.25 Hz.

Position*	Multiplicity	Shift (p.p.m.)†	Coupling (Hz)‡
OH (1)	s	9.78	–
OH (2)	s	9.37	–
H 6	dd	7.57	^3J 6, 7 = 6, 8; ^4J 6, 8 = 1, 5
H 7	dd	7.29	^3J 6, 7 = 6, 8; ^3J 7, 8 = 7, 5
H 3	d	7.20	^3J 3, 2 = 8, 1
H 5	dd	6.88	^3J 8, 7 = 6, 8; ^4J 8, 6 = 1, 5
H 2	d	6.71	^3J 2, 3 = 8, 1
OH	s	5.73	-
OH	s	5.18	-
OH	s	5.10	-
H 1′	d	4.50	^3J 1′, 2′ = 8, 0
OH	s	4.69	-
H 6′	dd	3.78	^2J 6′, 6″ = 11, 9; ^3J 6′, 5 = 2, 0
H 6″	dd	3.50	^2J 6″, 6′ = 11, 9; ^3J 6″, 5 = 6, 2
HOD	s	3.49	-
H 5′	ddd	3.36	^3J 5′, 4′ = 9, 5
H 20	s	3.32	-
H 3′/2′	m	3.30	-
H 4′	t	3.17	^3J 4′, 3′ = ^3J 4′, 5′ = 9, 5

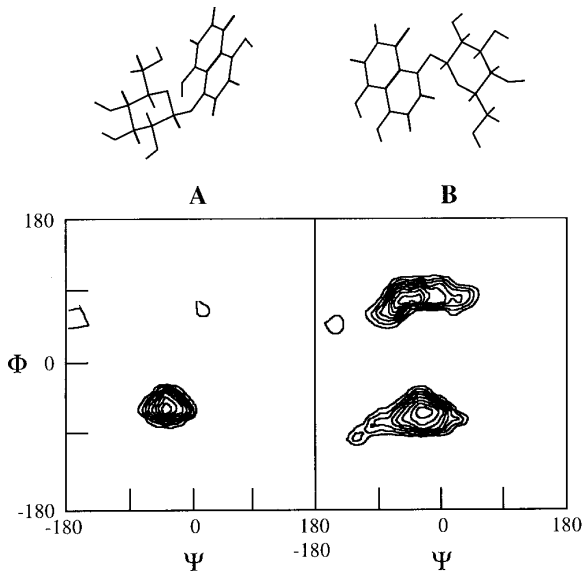


Figure 1 Isoenergy map (ϕ, ψ) of HJG

(**A**) 1,5-dihydroxy-4-naphthalenyl-β-D-glucopyranoside and (**B**) its isomer. Relative iso-energy contours are drawn at intervals of 1 kcal/mol. ϕ refers to the O15–C11–O41–C3 torsion and ψ as the C11–O41–C3–C4 torsion.

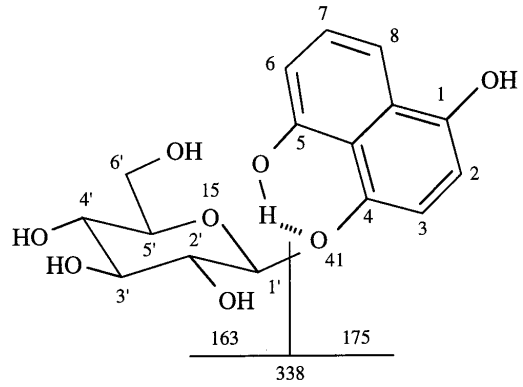


Figure 2 Molecular structure of HJG

The major fragmentation and the relative molecular mass of each part are indicated.

Determination of the glucosyl substitution position on naphthol can now be achieved by evaluation of the H1′-space proximity of any proton in the range 200–400 p.p.m. through steady-state NOESY [41]. When irradiating H1′, the difference spectrum shows a strong positive effect (enhancement) on the aromatic resonance at $\delta = 7.20$ p.p.m. The reciprocal experiment gives similar results on H1′ and on the coupled resonance at $\delta =$ 7.20 p.p.m. which is in the range 200–250 p.p.m. from H1′. If we attribute this resonance to H3, the glucosyl bond is then located at position 4 of naphthol. Confirmation of this postulate is given by evaluation of the [ϕ, ψ] conformation matrix of the supposed structure. Figure 1 (A) shows isoenergy contour maps obtained from the [ϕ, ψ] conformation matrix of the supposed structure. A local minimum occurs at $\phi = -45°$ and $\psi = -68°$. In this conformer the H1′–H3 distance is 224 p.p.m., in full agreement with the observed nuclear Overhauser effect. As an ultimate verification, the same computation was performed on the 1-isomer of HJG, performed in case of misassignment of H3. The iso-energy map (Figure 1B) shows several large minima around $\phi = 90°$, $\psi = -50°$ and $\phi = -30°$, $\psi = -80°$ with uncorrelatable H1′–H3 distances in the range 250–400 p.p.m. Therefore, we can confidently conclude the structural assignment of HJG as 1,5-dihydroxy-4-naphthalenyl-β-glucopyranoside (Figure 2).

Protein purification

Crude extract obtained from the petioles of walnut cotyledon fragments cultivated *in vitro* was shown to possess hydrolytic activity towards the natural endogenous substrate HJG (Table 2). In the crude extract, six polypeptide bands which hydrolysed 4-MU-β-D-Glc*p* were detected in native IEF-PAGE analysis, with pI values ranging from 8.9 to 4.1 (results not shown). A three-step chromatography procedure was developed to separate these enzymes and to purify the putative HJG β-glucosidase. For this purpose, β-glucosidase activity from chromatography effluents was first measured with *p*-NP-β-D-Glc*p* as substrate (Figure 3). Then each of the pooled β-glucosidase fractions was measured to determine the fraction showing the highest HJG-β-glucosidase specific activity (Table 2). In a first chromatography step, crude extract was fractionated on a S-200-Sephacryl matrix and β-glucosidases activities were recovered in two peaks (Figure 3A): peak 1.1 close to the matrix void volume contains the majority of the proteins, and peak 1.2 contained 80% of the initial HJG-β-glucosidase activity. Fractions from peak 1.2 were pooled and further loaded on to a DEAE-trisacryl anion-exchange matrix. β-Glucosidase activities were recovered in four peaks after selective elution (Figure 3B). The majority of HJG β-glucosidase activity was found in peak 2.1, with the highest specific activity. Protein IEF-PAGE analysis of peak 2.1 showed a major band at pI 8.9 and two other bands at pI 6.8 and pI 6.2 (results not shown). Fractions from peak 2.1 were pooled and further fractionated on a CM-Fractogel cation-exchange matrix. β-Glucosidase activities were then recovered in two peaks (Figure 3C). Pooled fractions from peak 3.2 were shown to have the highest HJG β-glucosidase specific activity and to be the protein with pI 8.9; this protein was named walnut glucosidase pI 8.9 (WBG8.9). This three-step purification procedure allowed a 27-fold purification of WBG8.9, with 23.2% of activity recovery assessed with HJG used as substrate (Table 2). The purified enzyme stored at 4 °C did not show any significant loss of activity for at least 1 month.

Electrophoretic analysis and gel filtration of WBG8.9

The pooled fractions from peak 3.2 containing WBG8.9 gave a single polypeptide band upon SDS/PAGE with an M_r of 64000 (Figure 4A), as well as upon IEF-PAGE with pI 8.9 (Figure 4B). Furthermore, this same polypeptide band was evidenced in IEF-PAGE by the fluorescence of 4-MU, which was released by its enzymic activity from 4-MU-β-D-Glc*p* or 4-MU-β-D-Fuc*p* used as substrate (Figure 4B). Taken together, these results confirmed the homogeneity of the purified enzyme and showed its activity towards the β-D-fucosyl bond. Upon S-100-Sephacryl gel filtration, purified WBG8.9 gave a single symmetrical peak corresponding to an M_r of 68000 after calibration with standard proteins. As this protein gave a single polypeptide band with an M_r of 64000 on SDS/PAGE (Figure 4A), we conclude that this enzyme has a monomeric structure.

Enzymic hydrolysis of HJG

Upon hydrolysis by the purified WBG8.9, 1,5-dihydroxy-4-naphthalenyl-β-D-glucopyranoside yielded a compound pre-

Table 2 Purification of WBG8.9 from *J. regia* L. cotyledon petioles

Freeze-dried petioles (500 mg) were used for purification. β-Glucosidase was assayed with 1 mM HJG at pH 6.0 and 25 °C. Peak numbers are shown in Figure 3.

Fraction	Peak number	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Purification factor	Yield (%)
Crude extract		21.60	3686	171	1.0	100.0
S-200-Sephacryl	1.1	9.10	510	56	0.3	13.8
	1.2	6.40	2947	461	2.7	79.9
DEAE-Trisacryl	2.1	0.88	1223	1398	8.2	33.2
	2.2	0.67	293	437	2.6	7.9
	2.3	1.52	418	275	1.6	11.3
	2.4	1.53	103	67	0.4	2.8
CM-Fractogel	3.1	0.37	283	767	4.5	7.6
	3.2	0.19	856	4531	26.6	23.2

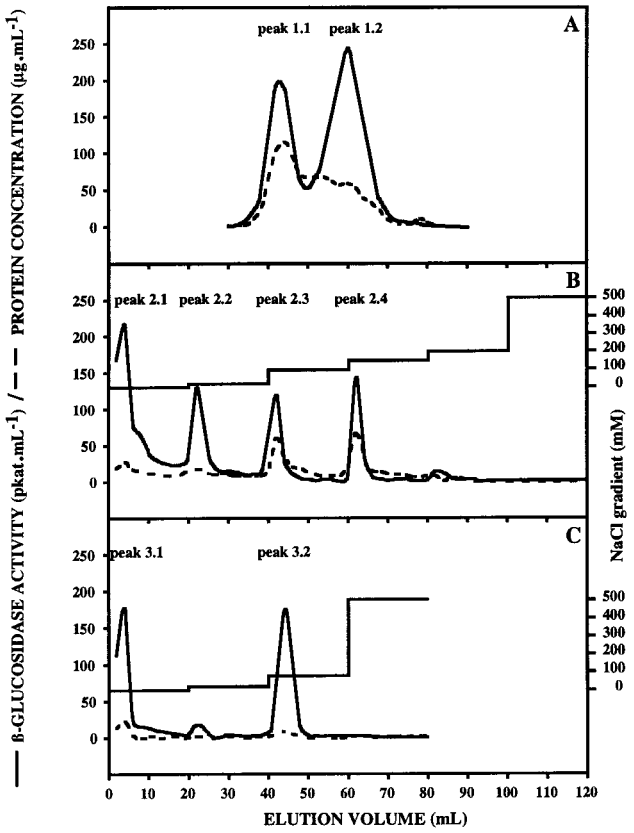


Figure 3 Chromatography procedure for purification of WBG8.9 from *J. regia* L. cotyledon petioles

Elution profiles of β-glucosidase activity from: (**A**) S-200–Sephacryl gel filtration of crude protein extract; (**B**) DEAE-trisacryl anion-exchange chromatography of peak 1.2; (**C**) CM-Fractogel cation-exchange chromatography of peak 2.1. WBG8.9 corresponds to the pooled fractions from peak 3.2. β-Glucosidase activity was measured with *p*-NP-β-Glc*p* as the substrate under standard conditions. See the Materials and methods section for details.

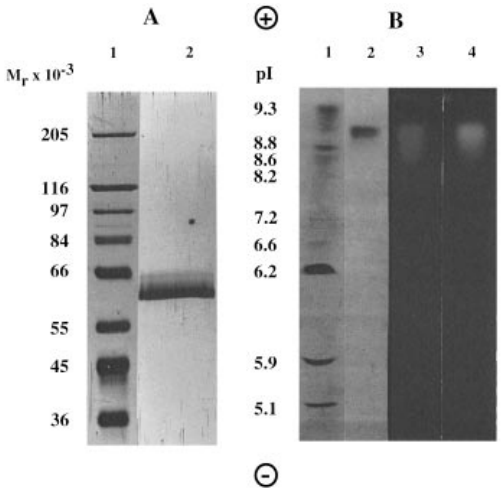


Figure 4 Analytical electrophoresis of WBG8.9

The fraction eluted from CM-trisacryl corresponding to peak 3.2 was analysed. (**A**) SDS/PAGE (8% acrylamide) after silver-staining. Lane 1, protein markers (see the Materials and methods section for details); lane 2, WBG8.9 (0.5 μg). (**B**) IEF-PAGE was performed with carrier ampholytes in the pH range 3–10. Lane 1, protein markers stained with silver nitrate: trypsinogen (pI 9.3), basic lentil lectins (pI 8.8), lentil lectin (pI 8.6), acidic lentil lectin (pI 8.2), basic myoglobin (pI 7.2), acidic myoglobin (pI 6.8), carbonic anhydrase I (pI 6.6), basic carbonic anhydrase II (pI 5.9), β-lactoglobulin (pI 5.1); lane 2, WBG8.9 (0.5 μg) stained with silver nitrate; lane 3, WBG8.9 (3 μg) screened for activity with 4-MU-β-Glc*p*; lane 4, WBG8.9 (3 μg) screened for activity with 4-MU-β-D-Fuc*p*.

senting the typical juglone UV/visible spectrum with absorbance maxima at 249 nm and 423 nm (Figure 5A) [47]. In addition, the product of the reaction showed the same retention time as a genuine sample of juglone in our HPLC procedure. Both these results indicate that juglone is formed upon HJG hydrolysis, rather than the expected 1,4,5-trihydroxynaphthalene. To confirm this conclusion, the hydrolysis of HJG (measured at 306 nm) and the formation of juglone (measured at 423 nm) were monitored throughout the enzyme reaction. The results show a tight correlation between HJG disappearence and juglone formation (Figure 5B).

Specificity and kinetic properties of WBG8.9

Optimal activity of WBG8.9 towards *p*-NP-β-D-Glc*p* and HJG was observed at pH 6.0. Thus, all enzymic activity studies were performed at pH 6.0. Hydrolysis of various *p*-NP-glycosides was performed in order to test the specificity of WBG8.9 for the glycone moiety. Amongst the synthetic substrates tested, *p*-NP-α-glycosides were poorly hydrolysed, showing a specificity of WBG8.9 for the β-anomeric position of the hydroxyl group on C-1 (Table 3). From the β-glycosides tested, *p*-NP-β-D-Fuc*p* was the most readily hydrolysed (Table 3). Michaelis–Menten kinetic

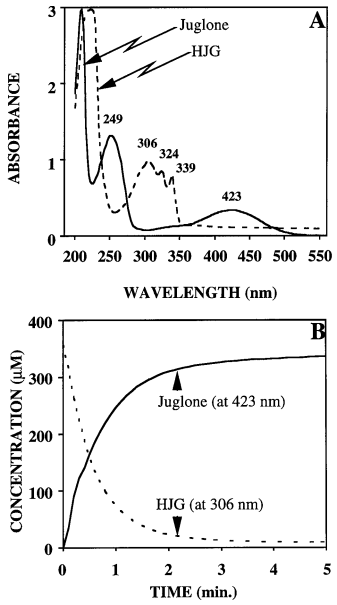


Figure 5 Enzymic release of juglone from HJG

(**A**) UV/visible spectra of juglone and HJG. Spectra were determined in 0.1 M phosphate buffer, pH 6.0, at 25 °C. Numbers indicate the characteristic absorption maxima of both compounds. (**B**) WBG8.9-catalysed hydrolysis of HJG. HJG disappearance and juglone appearance were monitored at 306 nm and 423 nm respectively. Enzymic reaction was performed under standard conditions with purified WBG8.9.

Table 3 Hydrolysis of synthetic substrates by purified WBG8.9

Enzymic assays were performed with 1 mM glycosides and approx. 0.2 μg of purified enzyme in 0.1 M phosphate buffer at pH 6.0 and 25 °C. *p*-NP or 4-MU formation was quantified to measure the rates of hydrolysis. *Calculated from absorbance measurements at 405 nm; †calculated from fluorescence measurements; ‡amount of *p*-NP produced was below the limit of detection.

Substrate	*p*-NP* or 4-MU† released (pkat)
p-NP-β-D-fucopyranoside	58.90
4-MU-β-D-fucopyranoside	27.10
p-NP-β-D-glucopyranoside	1.44
4-MU-β-D-glucopyranoside	13.10
p-NP-β-D-galactopyranoside	0.41
p-NP-β-D-xylopyranoside	0.23
p-NP-β-D-mannopyranoside	0.02
p-NP-β-L-arabinopyranoside	0.01
p-NP-β-D-glucopyranoside-uronic acid	0.01
p-NP-β-D-cellobioside	0.01
p-NP-α-L-arabinopyranoside	0.06
p-NP-α-L-fucopyranoside	0.02
p-NP-α-D-glucopyranoside	0.02
p-NP-α-D-galactopyranoside	0.01
p-NP-α-D-mannopyranoside	0.01
p-NP-α-D-xylopyranoside	< 0.01‡
p-NP-α-L-rhamnopyranoside	< 0.01‡

parameters were determined for selected synthetic heterosides (Table 4) and showed lower K_m values for the β-D-fucosides than for the corresponding β-glucosides, indicating a better specificity of WBG8.9 for β-D-fucosyl rather than β-D-glucosyl bonds. Furthermore, WBG8.9 showed lower K_m values for the 4-MU-glycosides than for the corresponding *p*-NP-glycosides, indicating

Table 4 Kinetic properties of purified WBG8.9

Enzymic assays were performed in 0.1 M phosphate buffer at pH 6.0 and 25 °C. *p*-NP or 4-MU release or HJG disappearence was measured to quantify initial velocities of hydrolysis. Michaelis–Menten kinetic parameters were calculated on the basis of Hanes–Woolf plots.

Substrate	K_m (mM)	V_{max} (μkat/mg)	V_{max}/K_m
p-NP-β-D-fucopyranoside	5.77	2.16	374.0
p-NP-β-D-glucopyranoside	19.90	0.14	7.1
4-MU-β-D-fucopyranoside	0.81	0.23	295.0
4-MU-β-D-glucopyranoside	0.80	0.11	141.0
HJG	0.62	14.50	23387.0

Table 5 Hydrolysis of natural substrates by purified WBG8.9

Enzymic assays were performed with approx. 0.2 μg of purified enzyme and 1 mM glycosides in 0.1 M phosphate buffer at pH 6.0 and 25 °C. Substrate disappearance was measured by means of HPLC, except for HJG, whose disappearance was monitored directly at 306 nm. *Substrate hydrolysis was not detected.

Substrate	Activity (pkat)
HJG	965.0
Indican	58.1
Phloridzin	56.4
Esculin	24.1
Fraxin	17.3
Arbutin	< 0.3*
Salicin	< 0.3*
Isoquercitrin	< 0.3*
Glucosyl-*O*-zeatin	< 0.3*
p-NP-β-D-glucopyranoside	1.44

a better specificity of WBG8.9 for the dicyclic aromatic structure of 4-MU than for the monocyclic one of *p*-NP. In order to test this hypothesis, hydrolysis of different natural heterosides, presenting either monocyclic or dicyclic aromatic aglycone moieties, was performed. WBG8.9 showed the highest activity towards HJG with a K_m of 0.62 mM (Tables 4 and 5). Indican, which had the second highest rate of hydrolysis, showed a 17-fold lower rate of hydrolysis than HJG (Table 5). Hydrolysis of heterosides with a monocyclic aromatic aglycone moiety, such as arbutin or salicin, was not detected under the experimental conditions used (Table 5). No hydrolysis of a walnut glucosyl-flavonol, such as isoquercitrin, was observed.

Effect of inhibitors on WBG8.9 activity

The effect of putative inhibitors, such as bivalent cations, *p*-hydroxymercuribenzoate, D-glucono-1,5-lactone and EDTA, on the hydrolysis of 4-MU-β-D-Glc*p* and 4-MU-β-D-Fuc*p* was investigated (Table 6). An inhibition of 90% and 70% was found respectively for Hg^{2+} and Fe^{2+} at 1 mM. When tested at 10 mM, Cu^{2+}, Mn^{2+}, Pb^{2+}, *p*-hydroxymercuribenzoate and D-glucono-1,5-lactone also exhibited a strong inhibition of both β-glucosidase and β-D-fucosidase activities to approximately the same extent. The chelating agent EDTA showed no significant inhibitory effects. In order to investigate further the inhibition mechanism of the sugar analogue D-glucono-1,5-lactone, kinetic studies were undertaken with HJG as substrate. Total inhibition could not be achieved even at 100 mM D-glucono-1,5-lactone with 1 mM substrate. Kinetics revealed non-competitive in-

Table 6 Effect of various putative inhibitors on WBG8.9 activity

Enzyme was preincubated with each compound for 30 min before being assayed with either 1 mM 4-MU-β-Glc*p* or 1 mM 4-MU-β-D-Fuc*p* in 0.2 M acetate buffer, pH 5.5, at 25 °C. Relative activity is expressed as a percentage of control activity in 0.2 M acetate buffer alone. Control activity corresponds to a hydrolysis rate of 0.7 pkat for 4-MU-β-Glc*p* and of 1.4 pkat for 4-MU-β-D-Fuc*p*.

		Residual activity (%)	
Inhibitors	Final concentration (mM)	β-Glucosidase	β-D-Fucosidase
Control		100	100
$CaCl_2$	1	92	84
	10	98	93
$CoCl_2$	1	90	99
	10	71	81
$CuSO_4$	1	53	68
	10	10	16
$FeSO_4$	1	27	34
	10	2	2
$HgCl_2$	1	7	13
	10	1	1
$MgCl_2$	1	112	87
	10	84	93
$MnCl_2$	1	66	63
	10	35	44
$NiCl_2$	1	99	90
	10	75	80
$Pb(NO_3)_2$	1	53	72
	10	12	19
EDTA	1	106	90
	10	90	95
D-Glucono-1,5-lactone	1	98	81
	10	27	42
p-Hydroxymercuribenzoate	1	99	102
	10	43	54

hibition of the mixed type, with $K_{IE} = 8.3$ mM and $K_{IES} = 2.2$ mM.

DISCUSSION

In this study, we have verified the structural configuration of HJG used as the natural substrate for WBG8.9 and provide clear evidence that the glucosyl moiety is bonded in position 4 on the naphthalene skeleton, corresponding to 1,5-dihydroxy-4-naphthalenyl-β-D-glucopyranoside (Figure 1). This finding is in support of that reported by Müller and Leistner [31] but differed from that first reported by Daglish [29] and Ruelius and Gauhe [30], with the glucosyl moiety bonded in position 5 (see Figure 1).

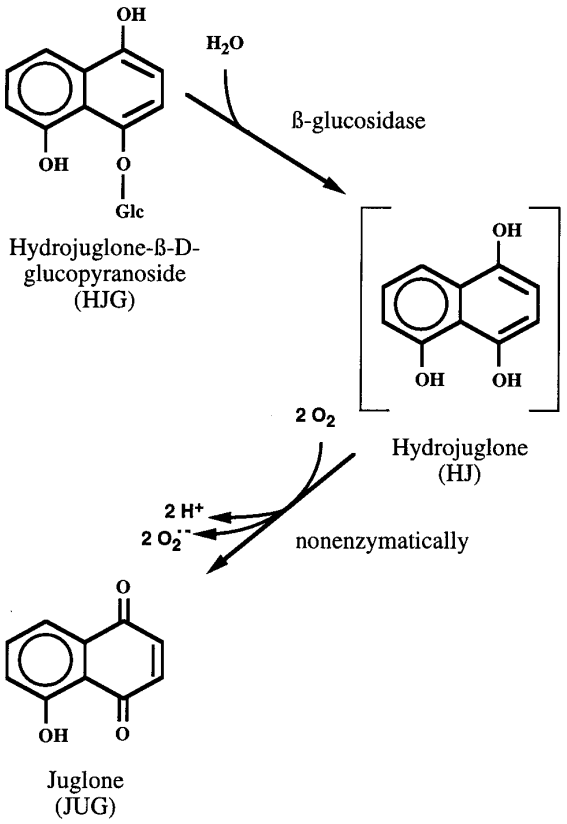
A crude protein extract from walnut cotyledon petioles contained several β-glucosidase activities with distinct pI values. This was supported by the separation of four fractions showing β-glucosidase activity on DEAE-trisacryl anion-exchange chromatography (Figure 4B). Among these enzymes, we purified WBG8.9, which showed a high activity towards HJG. We have also obtained another chromatography fraction enriched with β-glucosidase activity (see Table 2, peak 3.1), which mainly contains two polypeptides of pI 6.8 and pI 6.2. The further purification and biochemical characterization of these two polypeptides is now in progress. This would allow us to state whether they are isoforms of WBG8.9 or distinct isoenzymes. The presence of several β-glucosidases in the same plant can result from multi-allelism [48,49] or post-translational modification of a single enzyme, leading to several isoforms [50]. Furthermore, it is possible that several specific β-glucosidases involved in different metabolic pathways, encoded by a distinct set of genes, can exist in the same organ [16].

The purified WBG8.9 was shown to be homogenous, as judged with SDS/PAGE and IEF-PAGE after silver-staining. In addition, several peptide sequences obtained after tryptic digestion of the purified protein showed strong similarities to β-glucosidase sequences belonging to family 1 of the glycosyl hydrolases, providing further evidence for protein homogeneity (results not shown). WBG8.9 was purified 27-fold when measured with HJG as the substrate, and this enzyme may represent 4 % of the total soluble protein content of walnut cotyledon petioles. This is in keeping with other reports where abundant β-glucosidases (representing 4 %–8 % of the total protein in the extract) were purified to homogeneity by a factor of 10–40-fold [13,18,51,52]. The purified WBG8.9 appeared to consist of a single subunit of 64000 in SDS/PAGE, similar to many other plant β-glucosidases, which generally exhibit M_r values in the range 50000–65000 [12,18,51–53]. However, we cannot reject the possibility that WBG8.9 could exist in an aggregated form [12,52,54], since a β-glucosidase fraction hydrolysing HJG passed through the S-200-Sephacryl matrix (Table 2, peak 1.1). The purified WBG8.9 showed a higher activity towards β-D-fucosyl rather than β-D-glucosyl bonds. This property is shared by many other β-glucosidases purified from plant tissues [12,50,52,55]. Moreover, it seems likely that the same catalytic site is responsible for both β-D-glucosidase and β-D-fucosidase activities, as has been shown for an enzyme purified from Thai rosewood [56]. The occurrence of β-D-fucosidase activity in plants is unexpected, since to our knowledge no β-D-fucosides have been identified in plant tissues. Therefore, it is difficult to assess a physiological role for β-D-fucosidase activity, although it has been claimed that a strict β-D-fucosidase from lettuce could release fucose from cell-wall extracts [57]. This result requires further investigation, as the natural chirality of fucose in plant xyloglucans is not β-D-fucose but α-L-fucose [58,59]. However, its seems unlikely that WBG8.9 is involved in cell-wall polysaccharide hydrolysis, as this enzyme exhibited a strong activity towards aryl-β-glucosides.

We have shown that WBG8.9 had a lower K_m for 4-MU-glycosides than for the corresponding *p*-NP-glycosides. Thus, it appears that a dicyclic aromatic ring could fit the aglycone catalytic subsite better than a monocyclic aromatic ring. This finding was confirmed with the use of natural heteroglucosides, where arbutin and salicin, both presenting a monocyclic aromatic ring, were not hydrolysed. In accordance with our hypothesis, WBG8.9 exhibited a high activity towards the walnut naphthoquinone HJG.

Daglish [29] showed that upon acidic hydrolysis, HJG yielded 1,4,5-trihydroxynaphthalene, which could be further chemically oxidized to form juglone. It is likely that a similar mechanism, involving a reaction intermediate, occurs during the enzymic hydrolysis of HJG by WBG8.9, since we have shown that the product of the enzymic reaction is juglone rather than the expected 1,4,5-trihydroxynaphthalene. Thus, we propose that the formation of juglone from HJG, catalysed by WBG8.9, is a two-step mechanism (Scheme 1), with the first enzymic step leading to the formation of 1,4,5-trihydroxynaphthalene and the second, non-enzymic, step leading to the formation of juglone. Identical two-step mechanisms have been shown in the formation of saponins [13] and coumarins [16] from their respective glucosides.

A K_m of 0.62 mM, obtained for HJG, was 5–10-fold higher than values generally found for other plant β-glucosidases for their natural substrates [13,60,61]. Although this K_m value appears rather high, it remains in accordance with the estimated concentration of 15–30 mM HJG in the cotyledon petiole tissues. However, further purification and characterization of the other



Scheme 1 Juglone synthesis from HJG

Juglone synthesis from HJG is a two-step process: (1) a β-glucosidase catalyses the hydrolysis of HJG and (2) fast chemical oxidation of hypothetic hydrojuglone by oxygen leads to the formation of juglone, a more stable quinone.

β-glucosidase fractions extracted from walnut tissues would provide us with a greater insight into the role of these enzymes with respect to HJG and juglone metabolism. The sensitivity of WBG8.9 to inhibition by Hg^{2+}, Fe^{2+} and *p*-hydroxymercuribenzoate may indicate that these compounds react with a cysteine required for activity, as found in other glycosidases [18,52,62]. The WBG8.9 was weakly inhibited by D-glucono-1,5-lactone and the inhibition was of the mixed type. Although D-glucono-1,5-lactone is known to be a specific and potent competitive β-glucosidase inhibitor [63–65], several plant β-glucosidases were only weakly inhibited [61,66,67] and some non-competitively [17,68]. However, further kinetic investigations should be undertaken in order to explain this atypical property of WBG8.9.

Finally, we have achieved the purification of a walnut β-glucosidase, which hydrolyses the hydrojuglone-β-glucopyranoside with high specificity. We are now focusing on the molecular characterization of WBG8.9 and the cloning of its corresponding gene. The study of WBG8.9 gene expression in walnut tissues should provide us with information about the role of WBG8.9 with respect to juglone metabolism *in vivo*.

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