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ORIGINAL PAPER

Extracellular monoenzyme deglycosylation system of 7-O-linked flavonoid β-rutinosides and its disaccharide transglycosylation activity from *Stilbella fimetaria*

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Abstract We screened for microorganisms able to use flavonoids as a carbon source; and one isolate, nominated *Stilbella fimetaria* SES201, was found to possess a disaccharide-specific hydrolase. It was a cell-bound ectoenzyme that was released to the medium during conidiogenesis. The enzyme was shown to cleave the flavonoid hesperidin (hesperetin 7-O-α-rhamnopyranosyl-β-glucopyranoside) into rutinose (α-rhamnopyranosyl-β-glucopyranose) and hesperetin. Since only intracellular traces of monoglycosidase activities (β-glucosidase, α-rhamnosidase) were produced, the disaccharidase α-rhamnosyl-β-glucosidase was the main system utilized by the microorganism for hesperidin hydrolysis. The enzyme was a glycoprotein with a molecular weight of 42224 Da and isoelectric point of 5.7. Even when maximum activity was found at 70°C, it was active at temperatures as low as 5°C, consistent with the psychrotolerant character of *S. fimetaria*. Substrate preference studies indicated that the enzyme exhibits high specificity toward 7-O-linked flavonoid β-rutinosides. It did not act on flavonoid 3-O-β-rutinoside and 7-O-β-neohesperidosides, neither monoglycosylated substrates. In an aqueous medium, the α-rhamnosyl-β-glucosidase was also able to transfer rutinose to other acceptors besides water, indicating its potential as biocatalyst for organic synthesis. The monoenzyme strategy of *S. fimetaria* SES201, as well as the enzyme substrate preference for 7-O-β-flavonoid rutinosides, is unique characteristics among the microbial flavonoid deglycosylation systems reported.

Keywords Glycoside hydrolase · Diglycosidase · Hesperidin · α-Rhamnosyl-β-glucosidase

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Introduction

Glycosyl hydrolases (EC 3.2.1.-) are a widespread group of enzymes that hydrolyze the glycosidic bond; and from a biotechnological point of view, they find extended applications for biotransformations of plant-based foods. An important fraction of aroma-precursors of tea, wine and other foods is constituted by glycosylated molecules that become volatile after removing the sugar moieties (Ibarz et al. 2006; Ma et al. 2001; Mizutani et al. 2002; Spagna et al. 2002; Yamamoto et al. 2002). On the other hand, the flavonoids in citrus are a major class of secondary metabolites that have significant impact on nearly every aspect of citrus fruit production and processing, contributing to the bitter taste and also to juice clouding (Manthey and Grohmann 1996; Polaina and MacCabe 2007). An enzymatic deglycosylation can be carried out by adding commercially available preparations to hydrolyze aroma-precursors as well as flavonoids, for releasing volatile compounds and debittering and clarifying fruit juices, respectively, (Genovés et al. 2005; Hemingway et al. 1999; Wang et al. 2001).

The major sugar moieties of the plant-based compounds mentioned above are disaccharides such as α-L-arabinofuranosyl-, α-L-rhamnopyranosyl-, β-D-xylopyranosyl-, and

β-D-apiofuranosyl-β-D-glucopyranose (Wang et al. 2001; Williams et al. 1982). A sequential enzymatic mechanism involving two mono-glycoside hydrolases is reported as the most common deglycosylation system among microorganisms (Orrillo et al. 2007; Spagna et al. 2002). For oenological purposes, various enzymatic cocktails are obtained from *Aspergillus* spp., which contain several monoglycosidases. β-Glucosidase and α-arabinofuranosidase are usually the most abundant while α-rhamnosidase and particularly β-apiosidase activities are either very low or absent (Barbagallo et al. 2004; Sarry and Gunata 2004; Spagna et al. 2002). Nowadays, such glycosidases are finding applications in fine chemistry for modification of biologically active compounds owing to their stereoselectivity and regioselectivity. As an example, Monti et al. (2005) generated an α-L-rhamnosidases library by screening 16 fungal strains, which was then used for selective modification of the saponin asiaticoside (triterpene carrying a trisaccharide unit).

Recently, quite a few disaccharide-specific hydrolases (diglycosidases) from microorganisms belonging to the genera *Arthrobacter*, *Aspergillus* and *Penicillium*, acting on p-nitrophenyl β-primeveroside (6-O-β-D-xylopyranosyl-β-D-glucopyranoside) and quercetin 3-O-β-rutinosides (6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside) were described in detail (Ahn et al. 2004; Mizutani et al. 2002; Nakanishi et al. 2005; Yamamoto et al. 2002). This work deals with the monoenzyme deglycosylation strategy of 7-O-linked β-rutinosides of the fungus *Stilbella fimetaria* SES201, isolated from South Patagonia, Argentina. The production and characterization of the diglycosidase responsible for the activity is described, addressing the potential of the enzyme as an industrial biocatalyst.

Materials and methods

Chemicals

p-Nitrophenyl β-D-glucopyranoside (pNGP), p-nitrophenyl α-L-rhamnopyranoside (pNRP), hesperidin, hesperetin, rutin, naringin, hesperidin methylchalcone and diosmin were purchased from Sigma Chemical (St. Louis). Eriocitrin, neohesperidin and narirutin were a generous gift from Dr. María Rita Martearena (Universidad Nacional de Salta, Argentina). Molecular biology chemicals were acquired from Invitrogen. All other chemicals were from standard sources.

Microbial sources

Sediment and soil samples were collected from Antarctica and South Patagonia (Argentina). One gram was suspended in 5 ml of sterile 0.16 M NaCl and used for microorganism isolation. Selection medium (g l^{-1}): 5.0 hesperidin, 1.0 milk peptone, 2.0 yeast extract, 15 agar and 100 mM Na_2CO_3 (pH 10). Serial dilutions were streaked out on selection medium and the plates were incubated at 5°C during 6 weeks. The strains were cultured 7 days in an orbital shaker (250 rev/min, 25°C) in 20 ml fermentation medium and supernatants were collected after culture centrifugation (10 min–12000*g*) for enzyme activity determination. The isolates were stored in 12% v/v glycerol (prokaryotes) and agar slants (eukaryotes) at −18 and 5°C, respectively.

Characterization and identification of strain SES201

DNA extraction was performed by chemo-mechanical disruption of the fungal cells according to Cassago et al. (2002). For amplification of 18S rDNA, a combination of the specific eukaryotic primers was used: 18S–42F (CTCA ARGAYTAAGCCATGCA), 18S–1498R (CACCTACGG AAACCTTGTTA) (Slapeta et al. 2005). The conserved fungal primer pairs were used for 5.8S, 28S rDNA and for the internal transcribed spacer regions (ITS) amplification: ITS1 (TCCGTAGGTGAACCTGCGG) and NL4 (GGTCC GTGTTTCAAGACGG), and ITS1 (TCCGTAGGTGAAC CTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). PCR amplifications were carried out according to Martínez et al. (2002). Amplicons were sequenced at MACROGEN (Korea) and analyzed using the programs ChromasPro and DNAman.

Culture conditions

Stilbella fimetaria SES201 was cultured in media containing (g l^{-1}): 5.0 carbon source (hesperidin, naringin or rutin) or 2.5 hesperetin, 1.0 milk peptone, 2.0 yeast extract and 15 agar. The pH of the media was adjusted by adding 100 mM solution containing sodium citrate (pH 4–5), sodium phosphate (pH 6–8) and Tris–HCl (pH 9) buffers, and Na_2CO_3 (pH 10). Effects of temperature, initial pH, NaCl concentration and carbon source were determined from the radial rates of colony growth (K_r) during 12 days. Mycelial growth was measured using the software ImageJ (National Institutes of Health, USA). Submerged cultures were performed at 28°C in a medium containing (g l^{-1}): 5.0 carbon source (hesperidin, naringin and rutin), 1.0 milk peptone, 2.0 yeast extract and 100 mM citrate buffer (pH 5), in an orbital shaker (250 rev/min).

Fermentations

A 1.0 l working volume fermentor (Braun, Stuggart, Germany) was employed for culturing at different pH values (manipulated between 5.0 and 9.0), using fermentation

medium (g l^{-1}): 5.0 hesperidin; 1.0 milk peptone, 2.0 yeast extract; controlled pH at 5.0 with 0.5 M H_2SO_4 and 1 M NaOH at an agitation speed of 250 rev/min, aeration of 0.4 vvm at 25°C. Aliquots were taken periodically and centrifuged (10 min–12000*g*). The pellets (approx 70 mg dry weight) were washed twice with 0.16 M NaCl and suspended in 1 ml of 0.1 M Tris–HCl pH 7.5 buffer. Then, they were disrupted by ultrasound (5 cycles of 10 s) and centrifuged (10 min–12000*g*) to eliminate cell debris, and the supernatants were used to measure intracellular enzymatic activities.

Purification of α-rhamnosyl-β-glucosidase

The culture broth was filtered with Whatman filter paper No 1. Ammonium sulfate was added to 1 l; of supernatant at 5°C until a 1.5 M final concentration was reached. The solution was incubated for 1 h and filtered again with Whatman filter paper No 1. The filtrate was loaded on a column packed with butyl-agarose previously equilibrated with 10 mM sodium citrate buffer pH 5.0 containing 1.5 M ammonium sulfate. After enzyme loading, the column was washed with 2 volumes of equilibration solution, and elution was carried out by a gradient of 1.5–0 M ammonium sulfate containing 10 mM sodium citrate buffer pH 5.0. The active fractions were pooled and diafiltrated in a 10 kDa cutoff membrane against 5 mM sodium citrate buffer pH 5.0 containing 1 mM 2-mercaptoethanol until the conductivity reached 2.5 mS/cm. Then, the solution was further loaded on a QAE column and eluted with a gradient of 0–1.0 M NaCl in 5 mM sodium citrate buffer pH 5.0 containing 1 mM 2-mercaptoethanol. The active fractions were freeze-dried and stored at −18°C.

Enzyme assays

For monoglycosidase activities (α-rhamnosidase and β-glucosidase), 5 µl of the corresponding substrate (70 mM pNRP or pNGP in dimethylformamide) and 895 µl of 50 mM sodium citrate buffer pH 5.0 (pH 6.0 for intracellular samples) were incubated with 100 µl of enzyme solution. The reaction was performed for 1 h at 40°C and stopped by adding 100 µl of 0.1 M NaOH. Absorbance was measured at 420 nm, and the amount of p-nitrophenol released was calculated using its extinction coefficient ($\varepsilon_{420nm} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$) (Orrillo et al. 2007). One enzyme unit was defined as the amount of enzyme that released 1 µmol of p-nitrophenol in 1 min at the indicated temperature. For quantification of α-rhamnosyl-β-glucosidase activity, each reaction (1 ml) contained 450 µl of substrate (0.11% w/v hesperidin in 50 mM sodium citrate buffer pH 5.0) and 50 µl of suitably diluted enzyme solution. The reaction was performed for 1 h at 60°C (40°C for screening) and stopped by adding 500 µl of 3,5-dinitrosalicylic acid (DNS) (Miller 1959). The tubes were placed in a boiling water bath for 10 min and cooled before measuring the absorbance at 540 nm. One unit of α-rhamnosyl-β-glucosidase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars (as maltose) per min. For activity determination at low temperatures, the reaction time was extended until detectable amounts of products were released. Kinetic parameters were calculated from plots of enzymatic activity against substrate concentration using the Michaelis–Menten equation and the least squares method. For transglycosylation activity, 5% v/v ethanol was added to the reaction mixture. The products of enzymatic reaction were analyzed by thin layer chromatography (Silicagel 60 W) using ethyl-acetate/2-propanol/water (3:2:2) as mobile phase and stained with anthrone reagent. The 32-bit colour images were split into red, green and blue (RGB) components using the software ImageJ 1.38× (National Institutes of Health, USA). Images corresponding to the red component were chosen, due to the highest signal to noise ratio. Then, integrated optical density units were used for semiquantification of sugar and sugar-derivatives. Total activity (hydrolysis + transglycosylation) was quantified by measuring hesperetin released at 320 nm (Lai et al. 1992).

Analytical assays

Protein concentration was determined by the method of Lowry, using hen egg white lysozyme as standard. SDS–PAGE (10% w/v bis/acrylamide) was performed according to Laemmli (1970) using prestained broad range molecular markers and developed by silver staining (Oakley et al. 1980). Periodic acid-Schiff staining for glycoproteins detections was done according to Møller and Poulsen (1996). For reverse phase liquid chromatography-tandem mass spectrometry (LC–MS/MS), 46 kDa-band was excised from the SDS–PAGE gel and the in-gel digestion performed with proteomics sequencing grade trypsin gold (Promega), according to the supplier protocol (Promega 2008). Mass and tandem mass spectrometry for molecular mass estimation of the entire protein and the tryptic peptides, respectively, were performed by means of a Qstar spectrometer (Applied Biosystems). The whole pattern of peptides was analyzed with Mascot search engine (http://www.matrixscience.com). The peptides signals were *de novo* sequenced using the program Peaks (Bioinformatics solutions Inc.) and N-terminus underwent Edman degradation. Both assays were performed at Interdisciplinary Center for Biotechnology Research (University of Florida). Multiple alignment analysis against β-glycosidases was performed using CLUSTALW neighbor joining method (http://www.uniprot.org/align). Isoelectric focusing was

done with an IPGphor system (Pharmacia Biotech) using pH 3–10 IPG strips and Byolite ampholytes (Biorad). Melting temperature of the enzyme (90 μg ml^{-1}) was calculated by measuring the intrinsic fluorescence in a gradient of temperatures from 25 to 95°C at a scan rate of 60°C h^{-1}. The assay was carried out in a spectrofluorometer outfitted with a peltier cell for temperature control (Ocean Optics USB4000) using an excitation wavelength of 285 nm and emission wavelength of 340 nm. Activation energy for thermal unfolding of the enzyme was calculated from the slope of the Arrhenius plot of ln k (inactivation rate constant) versus T^{-1}.

Maldi-TOF/TOF and ESI MS analysis

Samples of substrates and products of the enzymatic reaction were analyzed by ultraviolet (UV)–Maldi–TOF/TOF MS analyses performed on the Bruker Ultraflex Daltonics mass spectrometer in positive and negative ion modes. For matrix preparation, *nor*-harmane, 2,5-dihydrobenzoic acid, 2′,4′,6′-Trihydroxyacetophenone and α-cyanohydroxycinnamic acid triethylamine solutions (2 mg/ml) were prepared by dissolving selected compound in acetonitrile/water (1:1 v/v) solution. Carbon nanotubes prepared as described by Nonami et al. (1997) were also utilized as matrix. Dry droplet sample preparation method was used according to Gholipour et al. (2008). Desorption/Ionization was obtained by using a 337-nm nitrogen laser. The accelerating potential was 20 kV. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively. The sugar products of the hesperidin hydrolysis were separated by TLC, eluted with milli-Q water and analyzed by Electrospray-MS at Universidad de Buenos Aires.

Nucleotide sequences accession numbers

The nucleotide sequences of 18S rDNA and adjacent ITS regions have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) under the accession numbers FJ939395 and FJ939394, respectively.

Results and discussion

Isolation of flavonoid-degrading microorganisms from Antarctica and South Patagonia

In environments with cold topographic conditions, carbon accumulates in soils because of the low temperatures and water logging keep decomposition rates low relative to the rate of input of organic material from surface vegetation (Schimel et al. 2006). Flavonoids, which are constituents of higher plant's debris, together with lignocellulosic material, represent to those microorganisms in possession of appropriate catabolic enzymes, a carbon-rich source (Shaw et al. 2006). Hence, these conditions constitute an adequate enrichment environment for cold-adapted microorganisms involved in flavonoid degradation (Orrillo et al. 2007). Soil and sediment collected from Fire Land, El Calafate National Park and Antarctica (Argentina) were used for microbial isolation at 10°C. From approximately 250 isolates, sixteen were selected according to their prominent flavonoid degradation ability. Since glycosidases play a major role in the initial phases of the decomposition of organic compounds by acting on glycosidic bonds of glycosides, polysaccharides and complex carbohydrates (Klose and Tabatabai 2002), the isolates were grown on hesperidin (hesperetin 7-O-rhamnoglucoside) and evaluated for extracellular α-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21) activities. The fungal isolate SES201 was the only one capable of deglycosylating hesperidin while α-rhamnosidase and β-glucosidase activities were not detected in the supernatant. When it was grown in a 1.0 l fermentor with hesperidin as carbon source, the substrate deglycosylating activity was cell-bound up to the stationary phase and was released in a soluble form to the extracellular medium during conidiogenesis reaching a specific activity of 0.14 U mg^{-1}. Additionally, intracellular traces of monoglycosidase activities (β-glucosidase 35×10^{-5} U mg^{-1}; α-rhamnosidase 6×10^{-5} U mg^{-1}) were found.

Yamamoto et al. (2002) reported the screening of β-primeverosidase-producing strains by combining the artificial substrates p-nitrophenyl-β-xyloside and home synthesized p-nitrophenyl-β-primeveroside. In our case, the clear degradation of hesperidin in the absence of extracellular monoglycosidases suggests a way of deglycosylation different from the sequential mechanism.

Identification and cultural features of strain SES201

The colonies of the isolated fungus SES201 grown on potato dextrose agar were initially white, becoming light-orange at maturity. They were powdery to granular, lacked dark pigments and did not change color in presence of neither KOH nor lactic acid. The hyphae were septate and hyaline, and the conidia were aseptate, hyaline and ellipsoidal. When hesperidin was used as the sole carbon source, the strain SES201 showed optimum growth temperature at 25°C ($K_r = 0.98 \pm 0.05$ mm day^{-1}). It had a psychrotolerant character since it was able to grow at lower temperatures ($K_r = 0.064 \pm 0.003$ mm day^{-1} at 10°C) and stressed cells were observed above 30°C. It was able to grow at initial pH values between 4.5 and 10, the lag period was extended at lower pH values and it showed a plateau of maximum growth rate within the pH

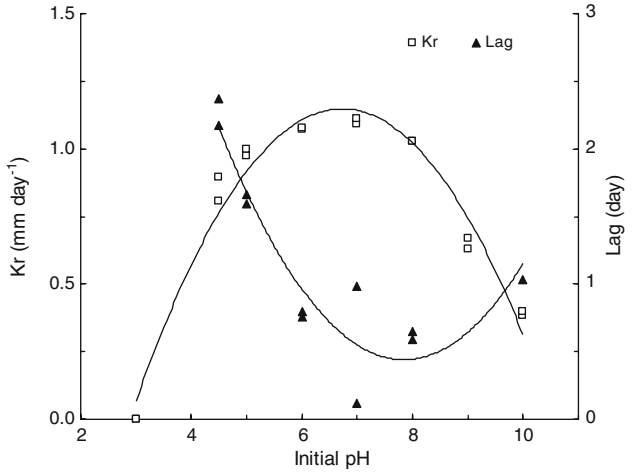


Fig. 1 Effect of the initial pH values on the radial growth rate (K_r) and lag phase extension of *Stilbella fimetaria* SES201 cultured with hesperidin as carbon source

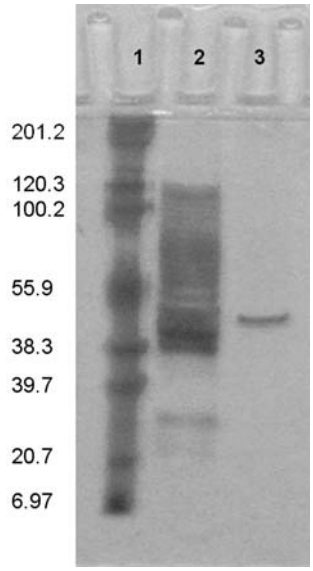


Fig. 2 SDS–PAGE of the purified α-rhamnosyl-β-glucosidase developed by silver staining. *Lane 1* prestained molecular mass markers. *Lane 2* Extracellular proteins of *Stilbella fimetaria* SES201. *Lane 3* α-rhamnosyl-β-glucosidase

range close to neutrality (pH 6–8) (Fig. 1). The growth was not affected by sodium chloride at concentrations below 0.22 M, nevertheless higher concentrations reduced growth rates and it was completely inhibited above 1.3 M (Data not shown). The fungus was not able to grow on the aglycone hesperetin as sole carbon source. Other flavonoids, rutin ($K_r = 1.08 \pm 0.11$ mm day^{-1}) and naringin ($K_r = 0.90 \pm 0.03$ mm day^{-1}), were used as carbon sources without any significant differences in the growth rates in comparison with hesperidin, indicating the importance of the glycosidic moiety for flavonoid degradation by the isolate SES201. The nucleotide sequences alignment (18S, 28S, 5.8S, ITS1 and ITS2 rDNA) combined with cultural and morphological features allowed the identification of strain SES201 as *Stilbella fimetaria* (Seifert 1985). *Stilbella,* a genus of the coprophilous fungal community, was reported as a producer of several bioactive compounds such as tyrosine kinases inhibitors, antibiotics polypeptides and prostaglandins (Jaworski and Brückner 2001; Kafanova et al. 2008; Lehr et al. 2006; Puder et al. 2005). Although *Stilbella* spp. are widespread soil fungi, knowledge of their enzymatic systems for degradation of natural or xenobiotic compounds is very limited.

α-Rhamnosyl-β-glucosidase characterization

The enzyme responsible for hesperidin deglycosylation was purified to homogeneity by fractionation with ammonium sulfate followed by two chromatographic steps: butyl-agarose and QAE-Sephadex (Table 1). The enzyme was identified as a glycoprotein with an isoelectric point at pH 5.7, and the molecular mass (M_r) was estimated to 46 kDa by SDS–PAGE (Fig. 2) and 42224 Da by mass spectrometry.

The enzyme exhibited high activity in the pH range 4.0–8.0 and its optimum was found at pH 5.0 (Fig. 3). The apparent optimum temperature was 70°C when the reaction was extended for 30 min, but it was active at temperatures as low as 5°C (Fig. 4). Kinetics data of thermal inactivation at pH 5.0 were used to calculate activation energy (E_a) for α-rhamnosyl-β-glucosidase denaturation, and it was found to be 183.3 ± 5 kJ mol^{-1} (Fig. 5). Thermodynamic denaturation revealed a melting temperature (T_m) of 74.6°C. Even when the α-rhamnosyl-β-glucosidase activity was significant at reduced temperatures (5–10°C), it was low in comparison with other reported cold-active enzymes; and

Table 1 Purification of α-rhamnosyl-β-glucosidase from *Stilbella fimetaria* SES201

Purification step	Volume (ml)	Activity (U ml^{-1})	Sp act (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	900	1.00	0.14	100	1
$(NH_4)_2SO_4$ precipitation	900	0.86	0.17	86.7	1.2
HIC (Butyl-agarose)	150	1.82	6.58	30.4	45.7
Anion exchange (QAE-Sephadex)	28	1.86	27.50	5.8	190.9

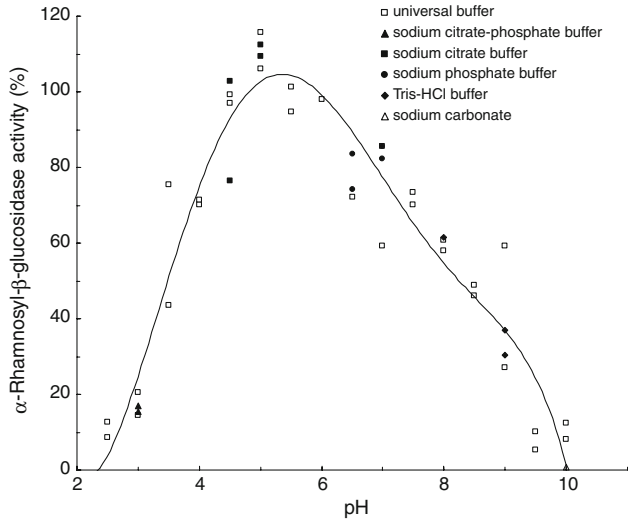


Fig. 3 α-Rhamnosyl-*β*-glucosidase activity displayed at different pH values. 100% Activity corresponded to 0.24 U ml^{-1}

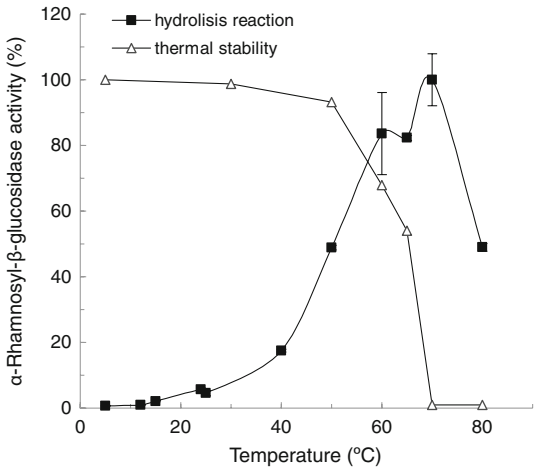


Fig. 4 Effect of temperature on hydrolysis reaction and stability of α-rhamnosyl-*β*-glucosidase using hesperidin as substrate. Stability was measured by the residual activity exhibited at 60°C after 30 min incubation at different temperatures. 100% Activity corresponded to 1.02 U ml^{-1}

the E_a and T_m showed a rather high thermal stability, comparable to the enzymes of mesophilic microorganisms (Russell 2000). Regarding the influence of divalent metal ions, manganese was the only which did not affect α-rhamnosyl-*β*-glucosidase activity, other metal ions assayed as well as the chelating agent EDTA reduced the activity in the range of 10–50% (Ba, Ca, Zn, Mg, Ni, Fe) while Cu and Hg were deleterious for the enzyme.

Mode of reaction and substrate preferences

The reaction products obtained from enzymatic hydrolysis of hesperidin with purified enzyme consisted of two sugar spots, which R_f differed from those corresponding to the

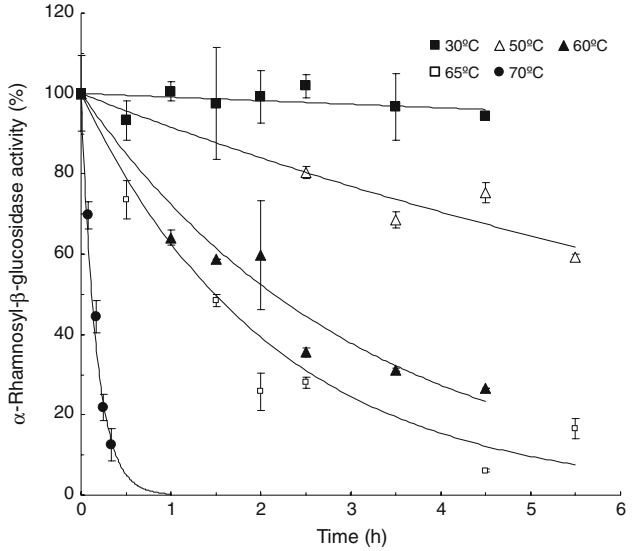


Fig. 5 Kinetics of thermal inactivation of α-rhamnosyl-*β*-glucosidase. The 100% activity corresponded to 0.71 U ml^{-1}

monosaccharides glucose and rhamnose (Fig. 6). The main spot was identified as rutinose by direct acid hydrolysis into both constituent monosaccharides and by MALDI–TOF/TOF (calc. [Rutinose + Na]$^+$: 349.29 m/z; obs. [Rutinose + Na]$^+$: 349.26 m/z) and ESI MS analysis. The MALDI–TOF/TOF spectra are shown in support material (Supplemental material I). Hence, α-rhamnosyl-*β*-glucosidase cleaved the flavonoid in an endo-manner into rutinose

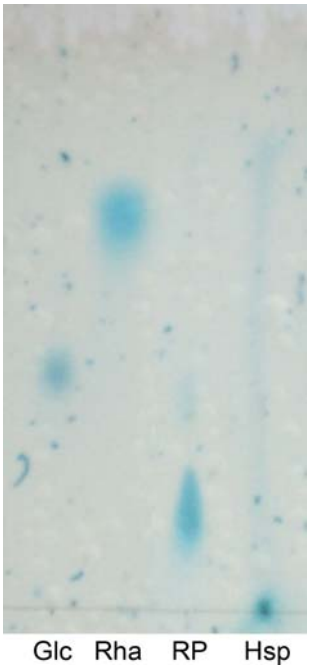


Fig. 6 Evaluation of hesperidin hydrolysis by α-rhamnosyl-*β*-glucosidase from *Stilbella fimetaria* SES201. *Glc* glucose, *Rha* rhamnose, *RP* reaction products, *Hsp* hesperidin (substrate control)

Table 2 Plant and microbial diglycosidases

Enzyme	Main substrate	Organism	Molecular mass (kDa)	References
Rutinosidase	Quercetin 3-O-β-rutinoside (6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside)	*Arthrobacter sp.*	42	(Sang-Joon et al. 1990)
Rutinosidase	Quercetin 3-O-β-rutinoside	*Penicillium rugulosum*	65	(Narikawa et al. 2000)
Primeverosidase	p-Nitrophenyl β-primeveroside (6-O-β-D-xylopyranosyl-β-D-glucopyranoside)	*Aspergillus fumigatus*	47	(Yamamoto et al. 2002)
Primeverosidase	p-Nitrophenyl β-primeveroside	*Penicillium multicolor*	50	(Ma et al. 2001; Tsuruhami et al. 2006)
Primeverosidase	2-Phenyl ethyl β-primeveroside	*Camelia sinensis*	61	(Mizutani et al. 2002)
Rutinosidase	Quercetin 3-O-β-rutinoside	*Fagopyrum esculentum*	74.5	(Baumgertel et al. 2003)
Furcatin hydrolase	Furcatin (p-allylphenyl 6-O-β-D-apiofuranosyl-β-D-glucopyranoside)	*Viburnum furcatum*	56	(Ahn et al. 2004; Chuankhayan et al. 2005)
Primeverosidase	Lucidin 3-O-β-primeveroside	*Rubia tinctorum L.*	68	(Nakanishi et al. 2005)
β-apiosyl-β-glucosidase	Isoflavonoid 7-O-β-glucosides	*Dalbergia nigrescens*	62	(Chuankhayan et al. 2005)

and hesperetin. Quite a few enzyme-releasing disaccharide units have been identified from plants and from microorganisms belonging to the genera *Arthrobacter*, *Aspergillus* and *Penicillium* (Table 2). The last ones are widespread flavonoid-degrading genera at low pH values, but they usually produce extracellular monoglycosidases (β-glucosidase and α-rhamnosidase) as main systems for flavonoid deglycosylation (Manzanares et al. 2001; Monti et al. 2004; Orrillo et al. 2007; Polaina and MacCabe 2007; Spagna et al. 2002). Until now, the search strategy for diglycosidases was based on the use of p-nitrophenyl derivatives (Yamamoto et al. 2006). Some microbial genera were shown to produce such enzymes but, in many cases, the screening resulted in enzymes with unknown natural substrates or which cleaved both mono and diglycosides (Yamamoto et al. 2006; Chuankhayan et al. 2005; Ma et al. 2001; Sarry and Gunata 2004).

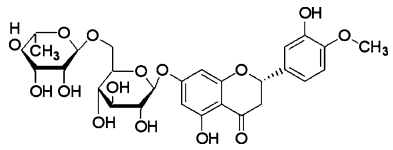
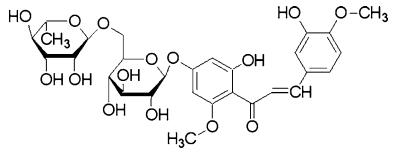
Several diglycoconjugated flavonoids were tested as substrates of the purified α-rhamnosyl-β-glucosidase based on structural similarities. The substrates yielding the highest activities were the β-rutinosides hesperidin and eriocitrin (Table 3). Unfortunately, both flavonoids are co-purified and they are usually contaminated with each other in commercial preparations, causing difficulties to reach a straightforward conclusion. Narirutin, hesperidin methylchalcone and diosmin were also hydrolysed by α-rhamnosyl-β-glucosidase but in a lesser extent, while it did not display activity against the 3-O-linked β-rutinoside, rutin. This behavior could be due to aglycone recognition and/or steric hindrance. Relating the sugar moiety, the enzyme was not able to hydrolyze naringin, neohesperidin (both 7-O-linked β-hesperidosides), and the artificial substrates pN-β-glucoside and pN-α-rhamnoside. Glycosidases are well reported to have stringent substrate specificities concerning both the sugar and the aglycone moieties (Henrissat et al. 2008). The ability of *S. fimetaria* SES201 to grow with rutin and naringin as carbon sources together with the α-rhamnosyl-β-glucosidase specificity for 7-O-linked flavonoid rutinosides implies the presence of different enzymatic systems for degradation of related flavonoids. Relating to the sugar moiety, reported diglycosidases are similar in sequence and

Table 3 Substrate specificity of α-rhamnosyl-β-glucosidase from *Stilbella fimetaria* SES201

Substrate	Aglycone	Relative activity (%)
7-O-β-Rutinosides		
Hesperidin	Hesperetin	100
Eriocitrin	Eriodictyol	89.7
Narirutin	Naringenin	61.0
Hesperidin methylchalcone	Hesperetin methylchalcone	58.5
Diosmin	Didymin	3.3
7-O-β-Neohesperidosides		
Naringin	Naringenin	ND
Neohesperidin	Hesperetin	ND
3-O-β-Rutinoside		
Rutin	Quercetin	ND
Artificial substrates		
p-NRP	p-Nitrophenol	ND
p-NGP	p-Nitrophenol	ND

ND No detectable activity

Table 4 Kinetic parameters of α-rhamnosyl-β-glucosidase on two substrates

Name	Molecular structure	Solubility (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
Hesperidin		0.324[a]	1.77 (±0.41)	32.4 (±2.9)	18.3
Hesperidin methylchalcone		>300	8.73 (±1.95)	68.2 (±8.8)	7.8

[a] Water solubility of hesperidin (Mauludin and Müller 2008)

tertiary structure to β-glucosidases, and they cleave the same sugar bond, but substrate specificity is greatly different between them (Daiyasu et al. 2008). *S. fimetaria* SES201 α-rhamnosyl-β-glucosidase also cleaves β-glucosidic bonds, but it is noteworthy that it is the only 7-O-linked flavonoid β-rutinosidase described up till now, since the other microbial diglycosidases are specific for 3-O-linked flavonoid β-diglycosides (Table 2).

Kinetic studies

α-Rhamnosyl-β-glucosidase followed Michaelis–Menten kinetics for hesperidin despite its low water solubility, with a K_M value of 1.77 mM (Table 4). This indicated that the solubilization rate of the substrate was either high enough not to reduce the catalytic rate or that the enzyme was able to hydrolyze the solid substrate. On the other hand, hesperidin methylchalcone, a water soluble substrate, presented a higher K_M value. Moreover, the catalytic efficiency of hydrolysis was one fold lower for hesperidin methylchalcone than for hesperidin, indicating the importance of the aglycone, and consequent spatial conformation of the substrate in the biocatalysis (Table 4). The saturation kinetics is shown in support material (Supplemental material II).

Partial sequence analysis

Tandem mass spectrometry (LC–MS/MS) of the tryptic peptides was performed, and highly specific stringent search was applied as a first layer screen to identify either known (i.e. present in a database) proteins or unknown proteins sharing identical peptides with related database sequences (MASCOT). Any peptide matches were assigned to protein hits. Eight peptides were de novo sequenced and aligned together with representative members of glycoside hydrolases. Considering both the substrate specificity and the cleaved glycosidic bond of *S. fimetaria* SES201 α-rhamnosyl-β-glucosidase, plant and fungal β-glucosidases belonging to families 1 (GH1) and 3 (GH3) were selected. In spite of the lack of sequenced fungal diglycosidases, those of plant origin acting on β-glucosidic bonds were included: *Camellia sinensis* β-primeverosidase (EC 3.2.1.149) and *Dalbergia nigrescens* β-apiosyl-β-glucosidase, (EC 3.2.1.161). N-terminal (APQAAYLDFK) and short peptides (less than 10 amino acids) were not significantly aligned. One peptide tag (ASGGGAHGNSK) showed homology with both families, while three peptide tags (YASYLTTQDLNQFAQAGLNVLR, NSTAATTPNVLR, LPVMLQGSFK) were aligned to conserved regions of glycosyl hydrolase family 1 suggesting that *S. fimetaria* SES201 α-rhamnosyl-β-glucosidase also belongs to this family. In the three cases, the peptides showed higher homology to GH1 fungal β-glucosidases than to plant diglycosidases indicating that the enzyme may have evolved from a common monosaccharide β-glucosidase.

Transglycosylation reaction

Family 1 glycoside hydrolases present a retaining hydrolysis mechanism and are therefore potential transglycosylating enzymes (Cantarel et al. 2009). The enzyme α-rhamnosyl-β-glucosidase was incubated with ethanol (5% v/v) as acceptor and hesperidin and hesperidin methylchalcone as sugar donors, and the reaction was followed by TLC. In addition to the hydrolytic product (rutinose), another glycosylated product was found, which was identified with MALDI-TOF/TOF analysis as ethyl rutinoside (calc. $[M + Na]^+$: 377.34 m/z; obs. $[M + Na]^+$: 377.07 m/z), indicating that α-rhamnosyl-β-glucosidase is able to transfer disaccharide units to OH-acceptors in an aqueous medium. The spectra are shown in support material (Supplemental material III). In the initial stages of the reaction, the enzyme preferentially catalyzed the glycosylation of

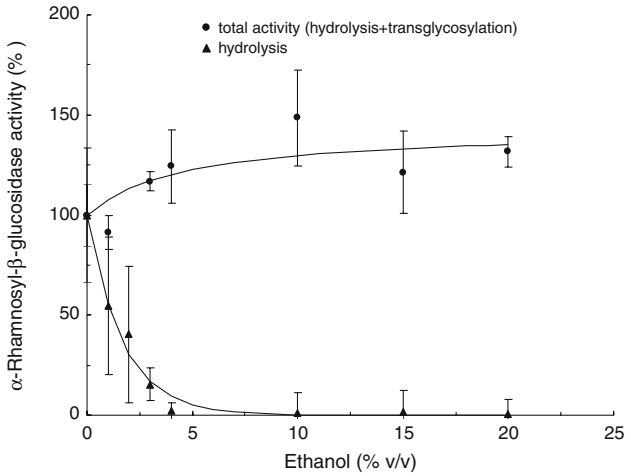


Fig. 7 Hydrolysis and transglycosylation reactions of α-rhamnosyl-β-glucosidase using ethanol as sugar acceptor. The 100% activity corresponded to 0.14 U ml^{-1} (based on the hydrolysis reaction)

ethanol for hesperidin and the hydrolysis for hesperidin methylchalcone (data not shown).

Increasing ethanol concentration (0–20% v/v) was shown to diminish hesperidin hydrolysis, but total activity was increased (Fig. 7). β-Glucosidases from *Pichia anomala, Saccharomyces cerevisiae* and *Oenococcus oeni* were also reported to be activated by ethanol although transglycosylation activity was not demonstrated (Barbagallo et al. 2004; Grimaldi et al. 2000), since the standard assay for β-glucosidase activity based in the quantification of released p-nitrophenol—hide the fate of the glycone moiety. The main physiological function of glycosidases is to produce saccharides that are utilized as carbon and energy sources. However, transglycosylation activities also play physiologically important roles in carbohydrate metabolism, as an example sophorose (2-O-glucopyranosyl glucose), the strongest cellulase inducer of *Trichoderma reesei*, can be formed from cello-oligosaccharides by transglycosylation (Kato et al. 2002). Although there is no evidence that transglycosylation activity plays any physiological role in vivo for *S. fimetaria*, it might be possible that this ability confers on the microorganism the advantage of modifying harmful chemicals in the surrounding environment.

Regarding the biotechnological applications, the increased activity of α-rhamnosyl-β-glucosidase in the presence of ethanol suggests its potential to act in fermented food products. On the other hand, hesperidin is the flavonoid at highest concentration in many citrus varieties and is usually precipitated during peel processing, constituting an inexpensive by-product of the citrus industry (Manthey and Grohmann 1996). Combined use of monoglycosidases can be a tool for modification of complex glycoconjugates (Monti et al. 2005). Recently, Martearena et al. (2007) reported the synthesis of rutinose and rutinosides by α-L-rhamnosidase reverse hydrolysis of the corresponding glucosides. On the contrary, α-rhamnosyl-β-glucosidase could be used for one-step synthesis of rutinosides starting from every source of hesperidin and non-glycosylated OH-acceptors.

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