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Transglycosylation specificity of *Acremonium* sp. α-rhamnosyl-β-glucosidase and its application to the synthesis of the new fluorogenic substrate 4-methylumbelliferyl-rutinoside

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ABSTRACT

Transglycosylation potential of the fungal diglycosidase α-rhamnosyl-β-glucosidase was explored. The biocatalyst was shown to have broad acceptor specificity toward aliphatic and aromatic alcohols. This feature allowed the synthesis of the diglycoconjugated fluorogenic substrate 4-methylumbelliferyl-rutinoside. The synthesis was performed in one step from the corresponding aglycone, 4-methylumbelliferone, and hesperidin as rutinose donor. 4-Methylumbelliferyl-rutinoside was produced in an agitated reactor using the immobilized biocatalyst with a 16% yield regarding the sugar acceptor. The compound was purified by solvent extraction and silica gel chromatography. MALDI-TOF/TOF data recorded for the $[M+Na]^+$ ions correlated with the theoretical monoisotopic mass (calcd $[M+Na]^+$: 507.44 *m*/*z*; obs. $[M+Na]^+$: 507.465 *m*/*z*). 4-Methylumbelliferyl-rutinoside differs from 4-methylumbelliferyl-glucoside in the rhamnosyl substitution at the C-6 of glucose, and this property brings about the possibility to explore in nature the occurrence of endo-β-glucosidases by zymographic analysis.

1. Introduction

The study of the degradation of natural polysaccharides or glycoconjugates by an enzyme consortium—and even the purified proteins—is a complex task. Endo-glycosidases usually present an extended binding site cleft composed of several subsites for monomer units. As a consequence, substrate specificity depends on the recognition of more than one glycosidic moiety, sometimes an aglycone moiety, and the linkage(s) between them.[1] Both polymers and commercial substrates are utilized to study substrate specificity of these enzymes.[2] The techniques that measure release of a chromophoric aglycone upon enzymatic hydrolysis of a glycoconjugate allow the easy monitoring of the reaction course by continuous UV–vis or fluorescence spectroscopy.[3] However, commercial substrates are not always available and it is particularly true in the case of the screening of enzymatic hydrolysis of some sugar moieties found in secondary metabolites of plants. These compounds are nowadays gaining attention because of their biological activities; and their absorption and metabolism are different depending on the sugar moieties due to differences in structural features which determine solubility, stability in the body, and also transport pathways.[4,5]

The disaccharidic moiety rutinose (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) is an important constituent of flavonoids, terpenoids, anthocyanins, and other secondary metabolites of plants. The most commonly reported deglycosylation mechanism involves an α-rhamnosidase (EC 3.2.1.40) and a β-glucosidase (EC 3.2.1.21) that act in a sequential mode[9] (Table 1). We recently reported the diglycosidase activity α-rhamnosyl-β-glucosidase (EC 3.2.1.168) that acts on hesperidin and other bioactive flavonoids, splitting off a rutinose residue (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) in one step (Table 1).[10] It is not clear, however, which of the mechanisms is the dominant one in nature because of the lack of tools for the screening of α-rhamnosyl-β-glucosidase activity. Comparable problems are faced when searching for other diglycosidases (for a review, see Ref. 11). Yamamoto et al.[12] designed an ingenious plate screening system for β-primeverosidases consisting of the use of the chromogenic substrates 4-nitrophenyl-β-primeveroside (6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) (medium P) and 4-nitrophenyl-β-xylopyranoside (medium X). The microorganisms that were more deeply colored in medium P than medium X were selected as promising candidates. In this work we studied the *Acremonium* sp. α-rhamnosyl-β-glucosidase transglycosylation ability using different acceptors bearing OH

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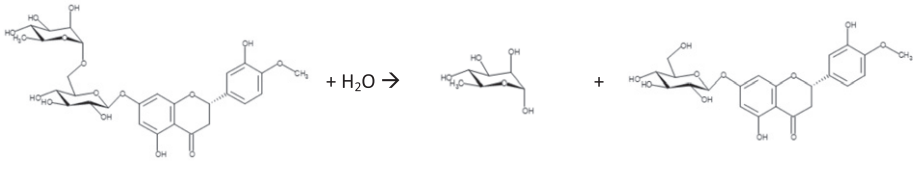
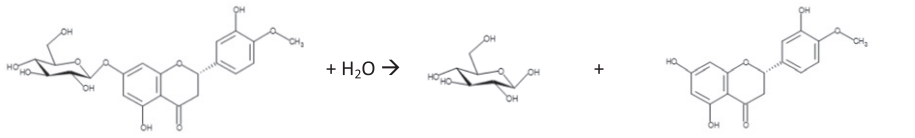
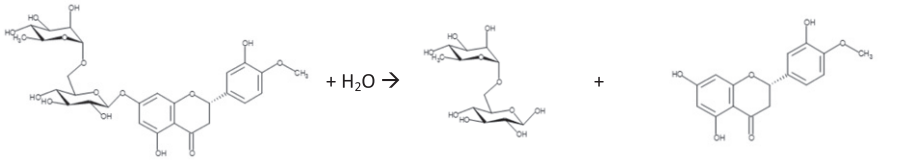
Table 1
Enzymatic activities acting on hesperidin, and substrates used for their detection

Enzymatic activity	Commercial substrates for activity detection
α-Rhamnosidase (3.2.1.40)	4-nitrophenyl-α-L-rhamnopyranoside 4-methylumbelliferyl-α-L-rhamnopyranoside
 Hesperidin + H_2O → α-L-Rhamnose + Hesperetin 7-*O*-(β-D-glucopyranoside) Hesperetin 7-*O*-(6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside)	
β-Glucosidase (3.2.1.21)	4-nitrophenyl-β-D-glucopyranoside 4-methylumbelliferyl-β-D-glucopyranoside
 Hesperetin 7-*O*-(β-D-glucopyranoside) + H_2O → β-D-Glucose + Hesperetin	
α-Rhamnosyl-β-glucosidase (3.2.1.168)	None
 Hesperidin + H_2O → Rutinose + Hesperetin	

groups. Such ability was exploited for the one step synthesis of 4-methylumbelliferyl-rutinoside using kinetic control of the reaction. This compound can be used as a fluorogenic substrate for screening of α-rhamnosyl-β-glucosidase activity by zymographic staining.

2. Experimental

2.1. Chemicals and stock solutions

4-Nitrophenol, 4-methylumbelliferone (4-MU), 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-*Glc*), hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) and hesperidin (hesperetin 7-*O*-(6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) were purchased from Sigma Chemical (St. Louis). HPLC grade methanol LiChrosolv® was obtained from Merck (Darmstadt). The other chemicals were from standard sources.

To prepare stock solutions for enzymatic reactions, hesperidin and 4-methylumbelliferone were solubilized in dimethylsulfoxide (180 mM) and suspended in water. To adjust pH 100 mM sodium citrate (pH 5) and sodium phosphate (pH 8) buffers were used. Standards for HPLC were prepared in dimethylsulfoxide (180 mM) and suitably diluted in the mobile phase before injection.

2.2. Enzyme source

Acremonium sp. DSM 24697 was cultured using hesperidin as carbon source for induction of α-rhamnosyl-β-glucosidase and the enzyme was purified as described previously.[10]

2.3. Enzyme reactions

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 30 min at 60 °C and stopped by adding 500 μL of 3,5-dinitrosalicylic acid (DNS).[13] 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 rutinose per min. For transglycosylation activity, 10%$_{v/v}$ alcohols or 1.8 mM 4-methylumbelliferone was added to the reaction mixture and incubated at 30 °C with 0.0023 U/mL of α-rhamnosyl-β-glucosidase activity for 1 h (otherwise indicated in text). Log*P* of the alcohols where obtained from PhysProp (http://www.syrres.com/what-we-do/databaseforms.aspx?id=386).

The immobilized enzyme on chitosan composites was performed as previously described.[14] The products of enzymatic reaction were analyzed by thin layer chromatography (Silica Gel 60 W) using ethyl-acetate/2-propanol/water (3:2:2) as mobile phase and stained with anthrone reagent. Total activity (hydrolysis + transglycosylation) was quantified by measuring hesperetin released at 320 nm.[15] Spectra of analytes were obtained using a USB4000 UV–vis spectrophotometer (Ocean Optics).

2.4. Analytical assays

Reaction substrates and products were analyzed by high performance liquid chromatography (HPLC) using a KONIK-500-A series

HPLC system attached to a KONIK Uvis 200 detector. The column was a reversed-phase LiChroCART® 125-4 (12.5 cm length, 4 mm internal diameter) LiChrospher® 5 μm, RP 18 (pore size 100 Å). The elution consisted of an isocratic flow of 40%$_{v/v}$ methanol and 60%$_{v/v}$ water at a flow rate of 1.0 mL/min at 40 °C. Peak areas and extinction coefficient were calculated from chromatograms of authentic standards detected at 285 nm. For 4-methylumbelliferyl-β-D-rutinoside quantification, 4-methylumbelliferyl-β-D-glucopyranoside was used as standard. Triplicate samples were used. Samples were deproteinized according to Contin et al.[16]

2.5. Production and purification of 4-methylumbelliferyl-β-D-rutinoside

For production of 4-methylumbelliferyl-β-D-rutinoside, a 60 mL-working volume reactor was used under low stirring at 30 °C. The reaction mixture contained 1.8 mM hesperidin and 1.8 mM 4-methylumbelliferone as sugar donor and acceptor, respectively, and 2%$_{v/v}$ DMSO in 50 mM sodium citrate buffer pH 5.0. The immobilized catalyst was added at a final concentration of 0.0023 U/mL (2 mL chitosan composites beads). The reaction mixture (60 mL) was filtered with Double Rings filter paper No. 103. A solvent extraction was performed twice by adding 0.5 volume of ethyl-acetate. Aqueous phase was freeze-dried and suspended in ethyl-acetate/2-propanol/water (3:2:2). Separation of the products was performed by column chromatography using Silica Gel 60 W (10 cm length × 1 cm internal diameter) as the stationary phase and ethyl-acetate/2-propanol/water (3:2:2) as the mobile phase.

2.6. MALDI-TOF/TOF 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-cyano-4-hydroxycinnamic 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.[17] were also utilized as matrix. Dry droplet sample preparation method was used according to Gholipour et al.[18] Desorption/ionization was obtained by using a 355-nm solid laser. The accelerating potential was 20 kV. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively.

3. Results and discussion

3.1. Acceptor specificity of α-rhamnosyl-β-glucosidase

The acceptor specificity of the fungal diglycosidase α-rhamnosyl-β-glucosidase was investigated using different acceptors bearing hydroxyl groups, and hesperidin as rutinose donor. Primary and secondary alcohols were shown to be glycosylated by the enzyme, and hydrolytic activity was suppressed with the exception of amyl- and benzyl-alcohols. In the last case, the reaction mixture formed a two phase-system because of the low water solubility of the alcohol. For the reason that hydrolytic activity was suppressed, released hesperetin was stoichiometrically equivalent to the amount of synthesized alkyl-rutinosides and it was used for estimation of the transglycosylation yield (Table 2). The short-chain alcohols (C_1–C_3) were found to give the highest yields of alkyl-rutinosides. On the other hand, *tert*-butanol was not glycosylated. This result is in agreement with the behavior of most retaining glycoside hydrolases, which are reported to be inert to sterically hindered alcohols.[19] Recently, Zhow et al.[20] described the syntheses of *R*-β-rutinosides by means of a crude rutin-degrading solution prepared from the seeds of *Fagopyrum tataricum*. α-Rhamnosyl-β-glucosidase is able to use hesperidin instead of rutin as a rutinose donor due to its specificity for 7-*O*-flavonoid-rutinosides. Advantageously, 7-*O*-flavonoid-rutinosides are the major flavonoids in some citrus industry by-products, constituting an inexpensive raw material.[21]

Table 2
Transglycosylation activity of α-rhamnosyl-β-glucosidase using different alcohols as acceptors

Acceptor	Transglycosylation activity ($\times 10^{-3}$ U/mL)
Methanol	4.6 ± 1.7
Ethanol	5.6 ± 0.6
Propanol	4.0 ± 1.3
Isopropanol	4.1 ± 0.4
Butanol	2.9 ± 2.0
Isobutanol	2.7 ± 0.4
tert-Butanol	ND
Amyl-alcohol	<0.5
Benzyl-alcohol	<0.5

ND. Not detected.

An inverse relationship was found between the transglycosylation activity for primary and secondary aliphatic alcohols and the alcohols log*P* (Fig. 1). In all cases, activity was enhanced (2–4-fold) in comparison with the hydrolysis, achieving the highest yields with methanol and ethanol as acceptors. Since hesperidin solubility is low in water, and increases in presence of water-soluble solvents, addition of short-chain alcohols is expected to enhance the whole activity (transglycosylation + hydrolysis) of the enzyme.

Retaining glycoside hydrolases usually catalyze the glycosylation of short alkyl alcohols while examples of aromatic alcohols being used as acceptors are less common. Since the alcohols act as substrates, the affinity interaction with the surrounding environment of the active site is likely to be different depending on the alcohol. Studies performed with crystallized glycoside hydrolases have demonstrated the importance of hydrophobic residues for the aglycone binding pocket.[22,23] This could explain the fact that the enzyme preferably transfers the sugar moiety to –OH acceptors other than water, even when the water concentration is up to 50 times higher. Another factor to be considered is the protein stability in aqueous-alcoholic systems. Because alcohols destabilize the protein by lowering the dielectric constant of the medium, the product yield will depend on the equilibrium of the variables mentioned above.

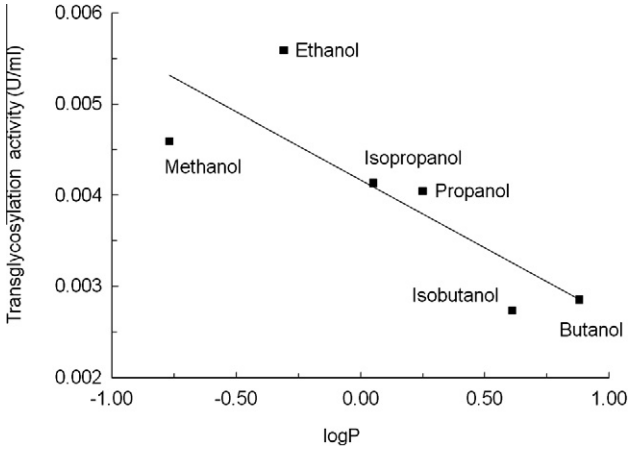


Figure 1. Transglycosylation activity of α-rhamnosyl-β-glucosidase as a function of acceptor log*P*.

3.2. Synthesis of 4-methylumbelliferyl-rutinoside

α-Rhamnosyl-β-glucosidase was incubated with the aromatic alcohol 1.8 mM 4-methylumbelliferone (4-MU) as acceptor and 1.8 mM hesperidin as rutinose donor. Thin layer chromatography (TLC) of the enzymatic reaction mixture gave a weak fluorescent spot (R_f = 0.72), similar to that of 4-MU-glucoside (4-MU-*Glc*, R_f = 0.80). The spot was found to be a glycoconjugate that, taking into account the enzyme mechanism,[10] strongly suggested the synthesis of 4-MU-rutinoside (Fig. 2). The yield of transglycosylation was 28% after the first hour of reaction at 30 °C (Fig. 3). Subsequently, the amount of transglycosylation product remained constant for at least 3 h, while the hydrolysis was shown to proceed up to completion. This behavior is in agreement with that observed for other glycosidase-catalyzed synthesis. Transglycosylation rate is usually higher than free sugar formation rate during the first stage of the reaction and, later on, transglycosylation product concentration reaches a plateau or diminishes because it acts as a substrate of the

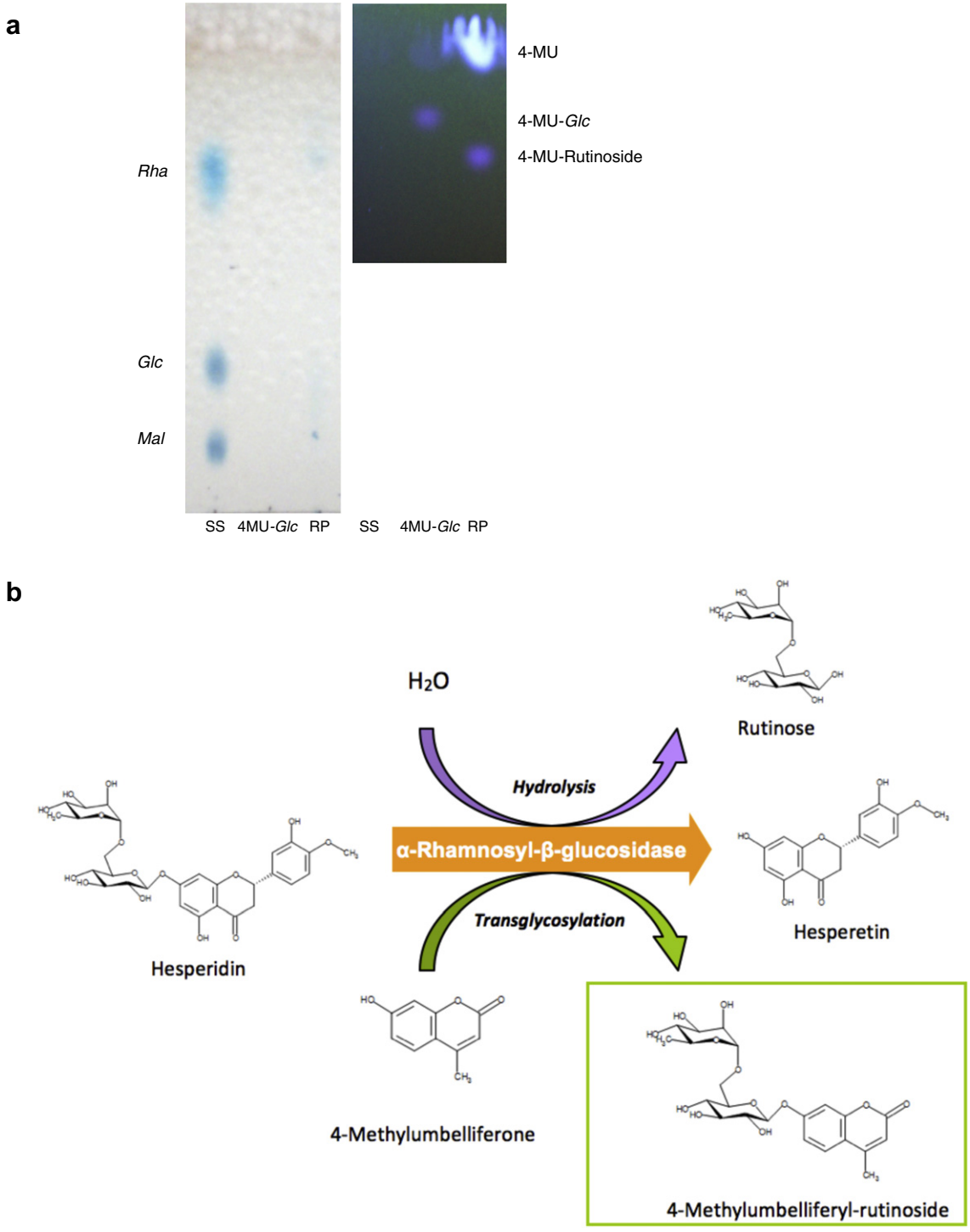


Figure 2. (a) Rutinose transglycosylation from hesperidin to 4-methylumbelliferone performed by α-rhamnosyl-β-glucosidase. SS sugar standards (*Glc* glucose, *Rha* rhamnose, *Mal* maltose), 4-MU-*Glc* 4-methylumbelliferyl-β-D-glucopyranoside, RP reaction products. (b) Hydrolysis and transglycosylation reactions catalyzed by α-rhamnosyl-β-glucosidase.

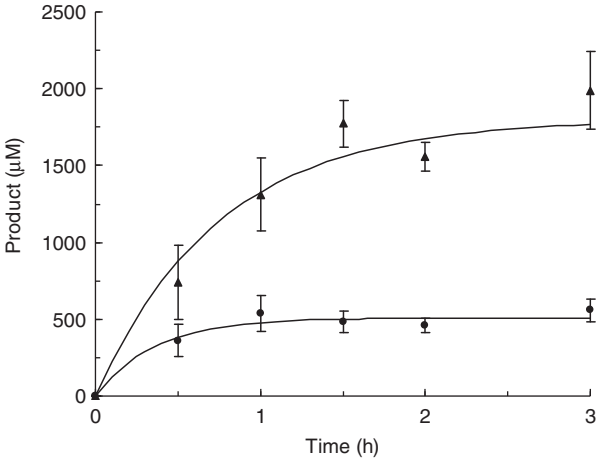


Figure 3. Time course of enzymatic reaction measured by production of (▲) hesperetin and (●) 4-MU-rutinoside.

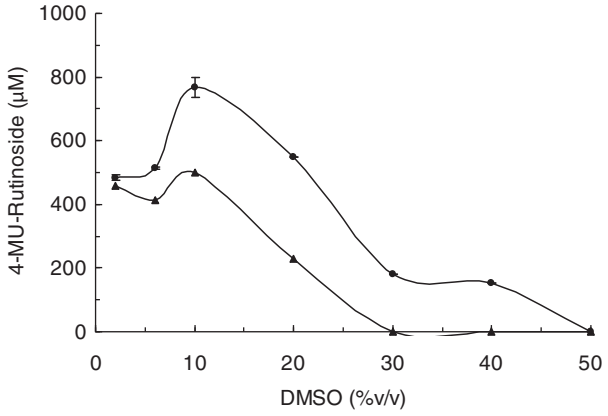


Figure 4. Effect of DMSO addition on 4-MU-rutinoside yield after (▲) 1 h and (●) 3 h.

enzyme.[24,25] The increment of the reaction temperature up to the near-optimal for hydrolysis (60 °C) was shown to diminish the yield of 4-MU-rutinoside, probably by favoring the hydrolysis of the transglycosylation product. The highest yield was obtained for the acceptor donor ratio in the range 0.8–1:1 (data not shown).

Several authors reported the synthesis of 4-nitrophenyl- and 4-methylumbelliferyl-dissacharides and oligosaccharides[26–28] using glycosidases that catalyze the transfer of one sugar unit from a polysaccharide to the corresponding monoglycoside. In that way, Kadi and Crouzet[28] synthesized phenyl-β-primeveroside) by means of *Trichoderma longibrachiatum* xylanase, which was capable to transfer a xylose moiety from xylan to phenyl-glucoside. However, synthesis of rutinosides starting from 4-MU-*Glc* would only be possible by reverse hydrolysis since α-L-rhamnosidases (3.2.1.40) reported up to now are inverting enzymes, and so cannot catalyze transglycosylation.[29] Remarkably, use of α-rhamnosyl-β-glucosidase represents a shortcut for the one step synthesis of the fluorogenic substrate 4-methylumbelliferyl-rutinoside.

3.3. Water-soluble organic solvents

The inclusion of an organic co-solvent in the reaction medium has frequently been used to increase the synthetic efficiency of glycosidase-catalyzed processes; the two most commonly employed organic co-solvents in this respect being dimethylsulfoxide (DMSO) and acetone.[30] Acetone addition (50%$_{v/v}$) to α-rhamnosyl-β-glucosidase was shown to promote the protein aggregation, with a concomitant lost in the enzyme activity. When DMSO and dimethylformamide (10%$_{v/v}$) were included in the transglycosylation reaction mixture (0.5 h-reaction) the first did not increase the product yield (Fig. 4), while the latter was deleterious for the enzyme.

The solvent DMSO was added to the reaction mixture in concentrations in the range of 2%$_{v/v}$ (the minimum required for substrates stock preparation) to 50%$_{v/v}$. The addition of 6–10%$_{v/v}$ DMSO did not change the 4-MU-rutinoside yield in comparison with 2%$_{v/v}$ DMSO, for 1 h-reaction (Fig. 4). However, it effectively increased the yield after 3-h reaction. The conversion percentages diminished at higher DMSO concentrations, and no product was formed above 30%$_{v/v}$ likely due to the destabilization of the biocatalyst. The increasing concentration of organic co-solvent usually promotes protein aggregation decreasing the enzymatic activity. Thus, the achievement of high activity caused by higher substrate solubility is counterbalanced by the instability of the protein structure.[31]

The enhancement of transglycosylation activity by employment of organic solvents (2–20%$_{v/v}$) might be caused by suppression of hydrolytic activity, increase of substrates solubility, or a combination of both factors.[32] During MUR synthesis, α-rhamnosyl-β-glucosidase was shown to produce both transglycosylation product and free sugar even when high DMSO concentration (30–40%$_{v/v}$) was used. Even though, there was a trend to increase the ratio between the transglycosylation product and free sugar with increasing DMSO concentration in the range 2–20%$_{v/v}$ (data not shown). To probe that the major effect of DMSO was due to the solubilization of the substrates with a consequent increment in the overall activity of the enzyme, hydrolytic activity was tested with 1.8 mM hesperidin as a substrate in presence of DMSO at different concentrations (Table 3). Hydrolytic activity was not modified by addition of 2%$_{v/v}$ DMSO and was significantly diminished when DMSO concentration was increased up to 10%$_{v/v}$. The fact that an increment in the solubility of hesperidin by DMSO addition did not raise the reaction rate is explained by its water solubility

Table 3
Effect of DMSO addition on hesperidin hydrolysis

	Enzyme activity (30 °C, U/mL)	Yield (18 h-incubation, %)
Control	0.401 ± 0.005	26 ± 4
2%$_{v/v}$ DMSO	0.399 ± 0.005	43 ± 22
10%$_{v/v}$ DMSO	0.318 ± 0.003	52 ± 15

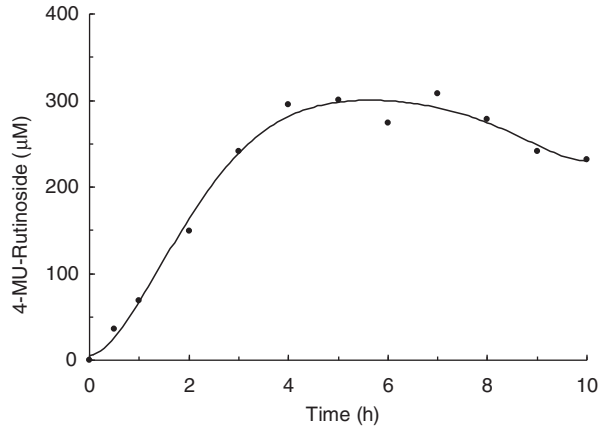


Figure 5. Time course of transglycosylation reaction in a bench-scale reactor. One-hundred percent activity corresponded to 0.01 U/mL.

(0.324 mM),[33] which is high enough in comparison with hesperetin concentration reached in the reaction mixture (0.12 mM after 30 min reaction). Consistently, hydrolysis yield was increased upon addition of DMSO in the range studied, with hesperetin final concentrations in the range 0.47–0.95 mM after 18 h-reaction. This result suggested that when the reaction proceeds, hesperidin solubilization rate becomes limiting for the enzymatic reaction.

3.4. Enzymatic reactor for 4-methylumbelliferyl-rutinoside production

The production of 4-methylumbelliferyl-rutinoside was performed in a bench-scale agitated reactor (60 mL) making use of the performed optimization: temperature was set at 30 °C and acceptor:donor ratio was 1:1. The enzyme α-rhamnosyl-β-glucosidase was immobilized in chitosan composites beads in order to separate and re-use the biocatalyst. Since agitation increases the solubilization rates of the substrates, DMSO concentration was set at suboptimal concentration (2%$_{v/v}$). The kinetics of the transglycosylation reaction was monitored during 10 h (Fig. 5). The 4-methylumbelliferyl-rutinoside concentration was shown to reach a maximum after 4 h. It remained near a constant value of ca. 300 μM up to 7 h-reaction-representing 16% conversion of the acceptor-, and later on it slightly decreased to ca. 250 μM after 10 h. Reaction was kinetically controlled by separation of the catalyst, and the reaction mixture was filtered to remove suspended solids.

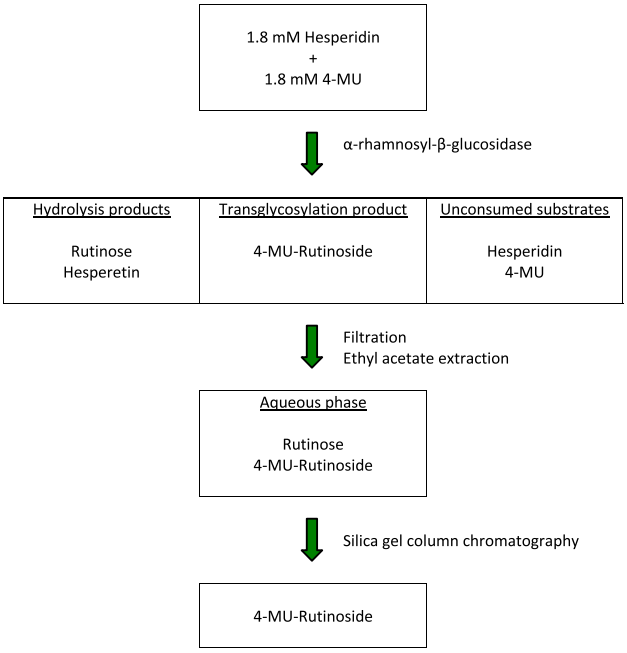


Figure 6. Schematic diagram of the enzymatic synthesis and purification of 4-MU-rutinoside.

3.5. Isolation and identification of 4-methylumbelliferyl-rutinoside

Liquid–liquid extraction was applied for isolation of the synthesized compound: the filtrate was extracted twice with ethyl-acetate to separate the aglycones. The freeze-dried aqueous phase underwent preparative chromatography for separation of the sugar products (Fig. 6). The transglycosylation product was subjected to MALDI-TOF/TOF. Data recorded for the $(M+Na)^+$ ions indicate that the observed mass correlates with the calculated monoisotopic mass of the expected product, 4-MU rutinoside (Fig. 7, calcd $[M+Na]^+$: 507.44 *m*/*z*; obs. $[M+Na]^+$: 507.465 *m*/*z*). UV–vis spectrum (pH 8) was recorded for the obtained compound, which showed a peak at 318 nm, being similar to the maximum wavelength for the commercial substrate 4-MU-*Glc* (320 nm). When the product was incubated with the enzyme the typical peak of 4-MU was observed (365 nm) due to the hydrolysis of the newly synthesized artificial substrate.

Yamamoto et al.[12] reported the screening of β-primeverosidase-producing microbial strains by combining the artificial substrates 4-nitrophenyl-β-xylopyranoside and their own synthesized 4-nitrophenyl-β-primeveroside. Among commercial substrates, 4-nitrophenol is a good leaving group for glycosidases and it is mostly employed for activity detection in liquid mixtures of enzymes. On the contrary, 4-methylumbelliferone is the aglycone of choice for zymographic analysis. While the last gives focused UV-fluorescent bands on analytical polyacrylamide gel electrophoresis or electrofocusing, the first diffuses through out the gel and activity

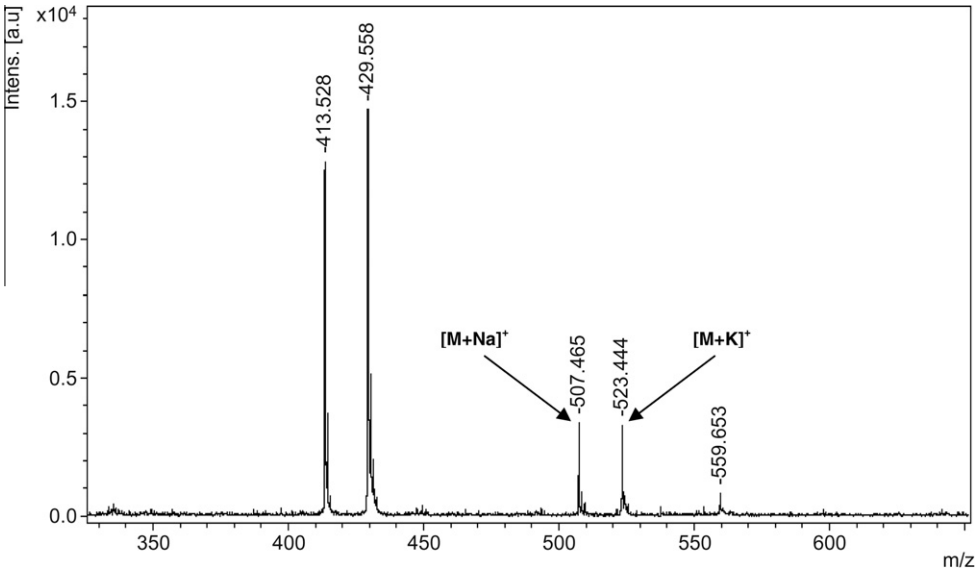


Figure 7. MALDI-TOF/TOF spectrum of reaction product obtained using *nor*-harmane matrix, positive ion mode, 44% laser.

bands cannot be clearly recognized. The fluorogenic substrate 4-methylumbelliferyl-rutinoside is a new contribution for the study of diglycosidases since it allows the detection of enzymes specific for rutinose in one step by zymographic analysis.

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References

1. Malet, C.; Planas, A. *Biochemistry* **1997**, *36*, 13838–13848.
2. Fukamizo, T.; Hayashi, K.; Tamoi, M.; Fujimura, Y.; Kurotaki, H.; Kulminskaya, A.; Kitaoka, M. *Arch. Biochem. Biophys.* **2008**, *478*, 187–194.
3. Van Tilbeurgh, H.; Claeyssens, M.; de Bruyne, C. K. *FEBS Lett.* **1982**, *149*, 152–156.
4. Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. *J. Agric. Food Chem.* **2001**, *49*, 1546–1551.
5. Niesen, I. L. F.; Dragsted, L. O.; Ravn-Haren, G.; Freese, R.; Rasmussen, S. E. *J. Agric. Food Chem.* **2003**, *51*, 2813–2820.
9. Sarry, J. E.; Gunata, Z. *Food Chem.* **2004**, *87*, 509–521.
10. Mazzaferro, L.; Piñuel, L.; Minig, M.; Breccia, J. D. *Arch. Microbiol.* **2010**, *192*, 383–393.
11. Mazzaferro, L. S.; Breccia, J. D. *Biocatal. Biotransform.* **2011**, *29*, 103–112.
12. Yamamoto, S.; Okada, M.; Usui, T.; Sakata, K. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 801–807.
13. Miller, G. L. *Anal. Chem.* **1959**, *31*, 426–428.
14. Piñuel, L.; Mazzaferro, L. S.; Breccia, J. D. *Process Biochem.* **2011**, *46*, 2330–2335.
15. Lai, L. B.; Gopalan, V.; Glew, R. H. *Anal. Biochem.* **1992**, *2*, 365–369.
16. Contin, M.; Mohamed, S.; Albani, F.; Riva, R.; Baruzzi, A. *J. Chromatogr., B* **2008**, *873*, 129–132.
17. Nonami, H.; Fukui, S.; Erra-Balsells, R. *J. Mass Spectrom.* **1997**, *32*, 287–296.
18. Gholipour, Y.; Nonami, H.; Erra-Balsells, R. *Anal. Biochem.* **2008**, *383*, 159–167.
19. Simerska, P.; Kuzma, M.; Monti, D.; Riva, S.; Mackova, M.; Kren, V. *J. Mol. Catal. B: Enzym.* **2006**, *39*, 128–134.
20. Zhow, L.; Lu, C.; Wang, G.-L.; Geng, H.-L.; Yang, J.-W.; Chen, P. *J. Asian Nat. Prod. Res.* **2009**, *11*, 18–23.
21. Peterson, J. J.; Dwyer, J. T.; Beecher, G. R.; Bhagwat, S. A.; Gebhardt, S. E.; Haytowitz, D. B.; Holden, J. M. *J. Food Compos. Anal.* **2006**, *19*, S66–S73.
22. Tang, S.-Y.; Yang, S.-J.; Cha, H.; Woo, E.-J.; Park, C.; Park, K.-H. *Biochim. Biophys. Acta* **2006**, *1764*, 1633–1638.
23. Tribolo, S.; Berrin, J.-G.; Kroon, P. A.; Czjzek, M.; Juge, N. *J. Mol. Biol.* **2006**, *370*, 964–975.
24. Spangenberg, P.; André, C.; Dion, M.; Rabiller, C.; Mattes, R. *Carbohydr. Res.* **2000**, *329*, 65–73.
25. Maugeri, F.; Hernalsteens, S. *J. Mol. Catal. B: Enzym.* **2007**, *49*, 43–49.
26. Zeng, X.; Yoshino, R.; Murata, T.; Ajisaka, K.; Usui, T. *Carbohydr. Res.* **2000**, *325*, 120–131.
27. Borris, R.; Krah, M.; Brumer, H.; Kerzhner, M. A.; Ivanen, D. R.; Eneyskaya, E. V.; Elyakova, L. A.; Shishlyannikov, S. M.; Shabalin, K. A.; Neustroev, K. N. *Carbohydr. Res.* **2003**, *338*, 1455–1467.
28. Kadi, N.; Crouzet, J. *Food Chem.* **2006**, *98*, 260–268.
29. Martearena, M.; Daz, M.; Ellenrieder, G. *Biocatal. Biotransform.* **2007**, *26*, 177–185.
30. Heidecke, C. D.; Parsons, T. B.; Fairbanks, A. J. *Carbohydr. Res.* **2009**, *344*, 2433–2438.
31. Doukyu, N.; Ogino, H. *Biochem. Eng. J.* **2010**, *48*, 270–282.
32. Fan, J.-Q.; Takegawa, K.; Iwahara, S.; Kondo, A.; Kato, I.; Abeygunawardana, C.; Lee, Y. C. *J. Biol. Chem.* **1995**, *270*, 17723–17729.
33. Mauludin, R.; Müller, R. H. *Pharmacogenet./Pharmacogenomics Virtual J.* **2008**, *10S2*. http://www.aapsj.org.