JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

α-Rhamnosyl-β-glucosidase-Catalyzed Reactions for Analysis and Biotransformations of Plant-Based Foods

Marisol Minig,[†] Laura S. Mazzaferro,[†] Rosa Erra-Balsells,[‡] Gabriela Petroselli,[‡] and Javier D. Breccia*[,†]

[†]INCITAP-CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa (UNLPam), Av. Uruguay 151, (6300) Santa Rosa, La Pampa, Argentina

[‡]CIHIDECAR-CONICET, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, 3er P., Ciudad Universitaria, (1428) Buenos Aires, Argentina

ABSTRACT: Most aroma compounds exist in vegetal tissues as disaccharide conjugates, rutinose being an abundant sugar moiety in grapes. The availability of aroma precursors would facilitate analytical analysis of plant-based foods. The diglycosidase α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM 24697 efficiently transglycosylated the rutinose moiety from hesperidin to 2-phenylethanol, geraniol, and nerol in an aqueous−organic biphasic system. 2-Phenethyl rutinoside was synthesized up to millimolar level with an 80% conversion regarding the donor hesperidin. The hydrolysis of the synthesized aroma precursors was not detected in an aqueous medium. However, in the presence of ethanol as a sugar acceptor, the enzyme was able to transfer the disaccharide residue forming the alkyl-rutinoside. The aroma precursors were significantly hydrolyzed (up to 3−4% in 2 h at 30 °C), which indicated the potential use of the enzyme for biotechnological applications, for example, in aroma modulation of fermented foods.

KEYWORDS: α-Rhamnosyl-β-glucosidase, rutinosylated terpenoids, hesperidin, transglycosylation

INTRODUCTION

The aroma profile in wine mainly consists of monoterpenes, alcohol-esters, and benzene derivatives. A large proportion of these compounds is glycosidically conjugated; therefore, it does not contribute to the flavor of wine. The diglycosides present in grape are mainly acuminosides (6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides), vicianosides (6-*O*-α-L-arabinopyranosyl-β-D-glucopyranosides), primeverosides (6-*O*-β-D-xylopyranosyl- β-D-glucopyranosides), and rutinosides (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides), which represent over 50% of total glycoconjugates (according to grape variety) (Figure 1).[1] Particularly, rutinosides were also found in several fruits other than grape, roots, seeds, and leaves.[2−4]

The glycosidically bound aroma fraction mentioned above, upon hydrolysis, can give rise to odorous volatiles able to generate odor-active compounds during wine storage through glycosidase-catalyzed reactions. Considering that it is not desired to release all of the bound flavors into the volatile form, an enzymatic preparation can be added as part of a final blend for aroma modulation. Nevertheless, the enzyme action must be carefully controlled and stopped after 1−4 months, depending on the desired effect.

Most commercially available aroma compounds are obtained by extraction or chemical synthesis using the Koenigs−Knorr modified method.[5] Compounds required for synthesis are not always available or easily prepared, and several steps of protection−deprotection are needed. On the other hand, stereo- and regiospecificity can be easily achieved by the use of enzymes, with a minimum environmental injure.[6] Some glycosidases have long been known to catalyze not only the hydrolysis of glycosidic bonds but also their formation. They catalyze the synthesis of glycosides or oligosaccharides by a kinetically controlled reaction between a glycosylated donor and an alcoholic acceptor.[7] A few enzymatic syntheses by transglycosylation of aroma precursors possessing di- or trisaccharides have been recently reported.[8−10] Tsuruhami et al.[11] described the synthesis of a series of β-primeverosides as aroma precursors by a β-primeverosidase from *Penicillium multicolor* IAM7153. Tramice et al.[10] performed a series of enzymatic transglycosylation reactions using the xylosidase/xylanase activity from *Thermotoga neapolitana*. They obtained β-1,4-xylooligosaccharides of different aglycones such as 1-hexanol, 9-fluorene methanol, 1,4-butanediol, and geraniol.

Previously, we described an α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM 24697, which is able to transfer the rutinose moiety (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) to OH acceptors in an aqueous medium.[12] This work deals with the enzymatic synthesis and identification of rutinosylated aroma precursors, to be used as standards for food analysis. The potential use of the enzyme for aroma modulation of plant-based foods (enzymatic alcoholysis of the precursors) is also demonstrated.

MATERIALS AND METHODS

Materials. Aroma compounds (purity 97−100%) (linalool, geraniol, nerol, and 2-phenylethanol), rutinose, hesperidin, anthrone reagent, *nor*-harmane, β-cyclodextrin (cyclomaltoheptaose), and 2,5-dihydrobenzoic acid were obtained from Sigma-Aldrich Chemical (St. Louis). All other chemicals were from standard sources.

Enzyme Source. *Acremonium* sp. DSM 24697 was cultured using hesperidin as the carbon source for induction of α-rhamnosyl-β-glucosidase, and the enzyme was purified as described before.[12] The strain *Acremonium* sp. DSM24697 was deposited in the public German microbial culture collection

Received: April 14, 2011
Accepted: August 11, 2011
Revised: August 11, 2011
Published: August 12, 2011

ACS Publications © 2011 American Chemical Society

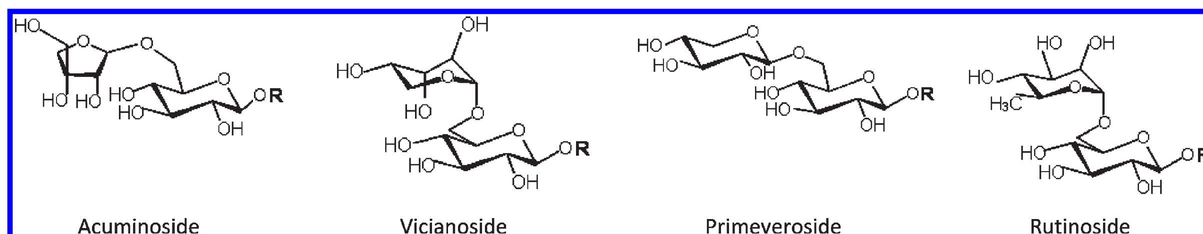

Figure 1. Structure of diglycoconjugated aroma precursors found in *Vitis vinifera* (common grape wine). R: The aglycon part is formed by terpenols, terpenic polyols, norisoprenoids, volatile phenols, or phenolic acids.

DSMZ under the Budapest treaty (see http://www.dsmz.de/microorganisms/main.php?menu_id=2).

α-Rhamnosyl-β-glucosidase Activity. For quantification of α-rhamnosyl-β-glucosidase activity, each reaction (1 mL) contained 495 μL of substrate (0.11% w/v hesperidin in 50 mM sodium citrate buffer, pH 5.0) and 5 μL of suitably diluted enzyme solution. The reaction was performed for 60 min at 30 °C and stopped by adding 500 μL of 3,5-dinitrosalicylic acid.[13] The samples were incubated at 100 °C for 10 min, and the concentration of reducing sugars was measured at 540 nm using rutinose as standard. One enzyme unit was defined as the amount of enzyme that released 1 μmol of rutinoside per minute.

Transglycosylation Reactions. The transglycosylation reactions (500 μL) were performed using 1.8 mM hesperidin as the disaccharide donor and 2% (v/v) aroma compounds as acceptors (linalool, geraniol, nerol, and 2-phenylethanol). For the optimization of the acceptor concentration, 0−60% (v/v) of 2-phenylethanol was used.

Hydrolysis of the Rutinoside Compounds. It was carried out in presence of 12% (v/v) ethanol as the OH acceptor. Each reaction (100 μL) contained 0.23 mM substrate products: neryl-, geranyl-, or 2-phenylethyl-rutinoside and 0.4 U/mL α-rhamnosyl-β-glucosidase in 50 mM sodium citrate buffer (pH 5.0) for 2 h (otherwise indicated in text) in an orbital shaker (250 rev/min) at 30 °C.

Analytical Assays. The products of enzymatic reaction were analyzed by thin-layer chromatography (TLC). The reaction products (5 μL) were loaded onto silica gel TLC 0.2 mm layer thickness with medium pore diameter (60 Å) (Fluka Chemika GmbH, Switzerland). The mobile phase was ethyl-acetate/2-propanol/water (3:2:2, [v/v]). The chromatograms were stained using anthrone reagent according to the procedure described for Witham et al.[14] The blue complex produced for each glycosidic compound was quantified as integrated optical density units using rutinose as the standard. The TLC images were analyzed using the software ImageJ 1.38x (National Institutes of Health, United States; http://rsb.info.nih.gov/ij/). The 32-bit color images were split into red, green, and blue (RGB) components. Images corresponding to the red component were chosen, due to the highest signal-to-noise ratio, and integrated optical density units were used for quantification of rutinose and rutinosylated compounds.

Synthesis of Aroma Precursors (Scale-up). Bench-scale syntheses of rutinosides were performed in an agitated reactor (10 mL) containing 0.4 U/mL α-rhamnosyl-β-glucosidase, 1.8 mM hesperidin, and 10% (v/v) aromatic compound (geraniol, nerol, or 2-phenylethanol) as the OH acceptor in 50 mM sodium citrate buffer (pH 5.0) for 16 h at 30 °C. The reaction was stopped by placing the samples in a water bath for 10 min at 100 °C.

Purification of Rutinosylated Compounds and UV-MALDI-TOF/TOF MS and UV-LDI-TOF/TOF MS Analysis. The rutinosides were purified by solid-phase extraction using a C18 Reversed-Phase cartridge (Varian Bond Elut LAC C18). Reaction mixtures were loaded on the cartridge and washed with water (3 volumes). Elution was performed with 60% (v/v) ethanol (3 volumes). The alcohol content in samples containing the purified rutinosides was reduced by evaporation in vacuum. After that, the samples were freeze-dried and stored at 4 °C.

The purified products were analyzed by ultraviolet matrix-assisted laser desorption−ionization mass spectrometry (UV-MALDI-MS) and by ultraviolet laser desorption−ionization mass spectrometry (UV-LDI-MS) performed on the Bruker Ultraflex Daltonics TOF/TOF mass spectrometer in positive and negative ion modes. For UV-MALDI-MS, matrix solutions were prepared by dissolving *nor*-harmane and 2,5-dihydrobenzoic acid (DHB) (2 mg/mL) in acetonitrile/water (1:1 [v/v]) solution. Analyte solutions were prepared with methanol (approximately 0.7 mg/mL). For UV-MALDI-MS experiments, the dry droplet sample preparation or sandwich method was used according to Nonami et al.,[15] loading successively 0.5 μL of matrix solution, analyte solution, and matrix solution after drying each layer at normal atmosphere and room temperature. For UV-LDI-MS experiments, two portions of analyte solution (0.5 μL × 2) were loaded on the probe, as two dry layers, desorption/ionization was obtained by using a 355 nm solid laser. The accelerating potential was 20 kV. External mass calibration was made using β-cyclodextrin (MW 1134) with *nor*-harmane as the matrix in positive and negative ion mode. The matrix mass was used as an additional standard for calibration. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively.

RESULTS AND DISCUSSION

Rutinosides Syntheses. The enzyme α-rhamnosyl-β-glucosidase was used to transfer the rutinose moiety from hesperidin (sugar donor) to various alcoholic aroma acceptors in aqueous medium (30 °C, 2−22 h). Linalool, nerol, geraniol, and 2-phenylethanol were chosen on the basis of their abundance in grape. The reaction products were analyzed by TLC and a new sugar spot (R_f values ∼ 0.82−0.84) corresponding to the transglycosylation product was detected for each acceptor except for the tertiary alcohol, linalool (Figure 2a). These results are in agreement with those reviewed by van Rantwijk et al.,[6] who describe absolute selectivity of glycosidase with regard to the stereochemistry at the anomeric center and show a high degree of chemoselectivity for different hydroxyl groups in the order of reactivity: primary > secondary alcohols > phenols, with tertiary alcohols being unreactive. The transglycosylation reaction was higher than the hydrolysis when 2-phenylethanol was utilized as the sugar acceptor, with the transglycosylation/hydrolysis ratio being 6/1, respectively. Figure 2B shows an scheme of the transglycosylation reaction using 2-phenylethanol as the acceptor and the hydrolysis of hesperidin by *Acremonium* sp. α-rhamnosyl-β-glucosidase. This acceptor was selected to establish the reaction conditions to acheive transglycosylation products up to a millimolar scale.

Acceptor Concentration and Time Course. The effect of 2-phenylethanol concentration (0−60% [v/v]) on transglycosylation yield was studied using a fixed hesperidin concentration (1.8 mM) (2 h, 30 °C). 2-Phenylethanol solubility in water is around 2% (v/v), and as a consequence, the reaction mixture was a monophasic system in the range of 0−1% (v/v) acceptor and a

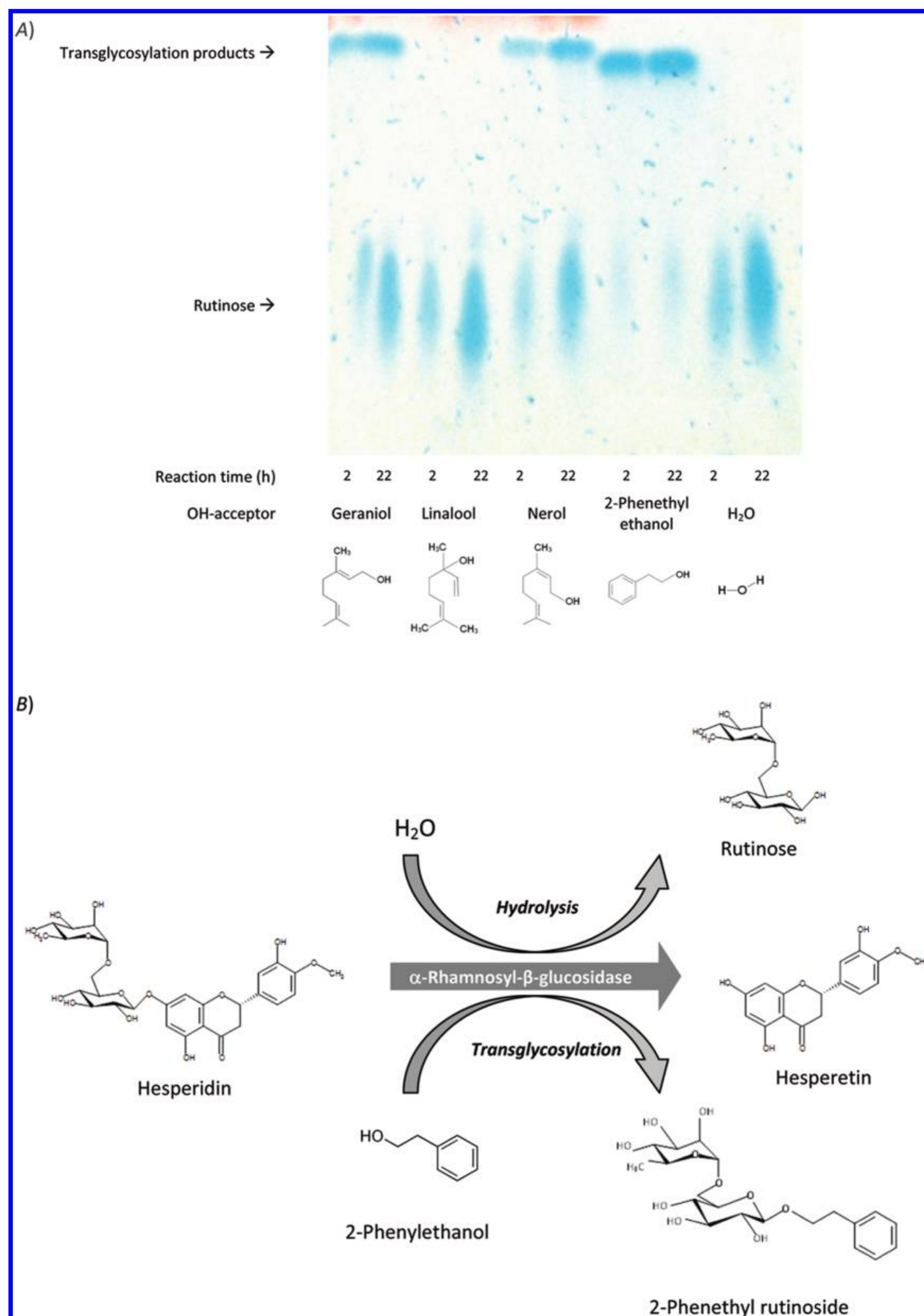

Figure 2. (A) Rutinose transglycosylation from hesperidin to various OH acceptors performed with α-rhamnosyl-β-glucosidase (2 or 22 h of reaction at 30 °C). Lanes 1 and 2, geraniol; lanes 3 and 4, linalool; lanes 5 and 6, nerol; lanes 7 and 8, 2-phenylethanol; and lanes 9 and 10, hydrolysis. (B) Scheme of hesperidin hydrolysis and transglycosylation reaction using 2-phenylethanol as an acceptor by α-rhamnosyl-β-glucosidase.

biphasic system between 5 and 60% (v/v) acceptor. The 2-phenylethyl rutinoside production profile resembled a rectangular hyperbola (Figure 3). For the reaction in aqueous medium, the yield was increased with increasing acceptor concentration. The yield was maximum and constant (89% based on the amount of donor) for the biphasic system. Previously, Tsuruhami et al.[11]

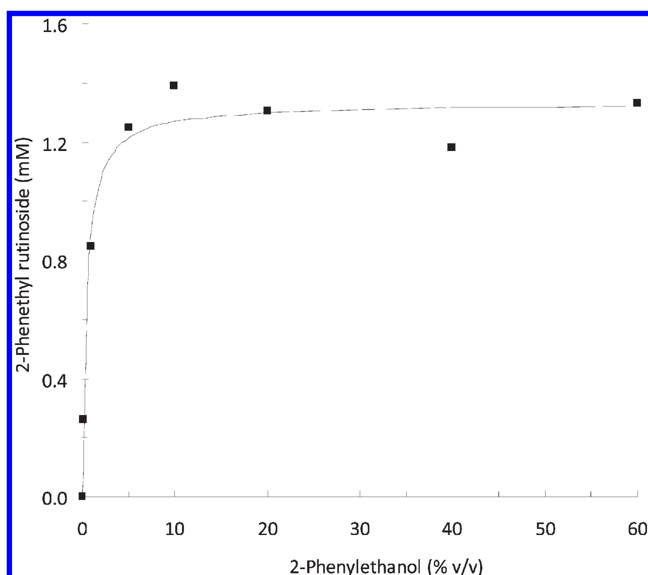

Figure 3. Transglycosylation reaction yield using an increasing concentration of 2-phenylethanol (0−60%, v/v). The reaction was performed by *Acremonium* sp. α-rhamnosyl-β-glucosidase with hesperidin as the rutinose donor (2 h, 30 °C).

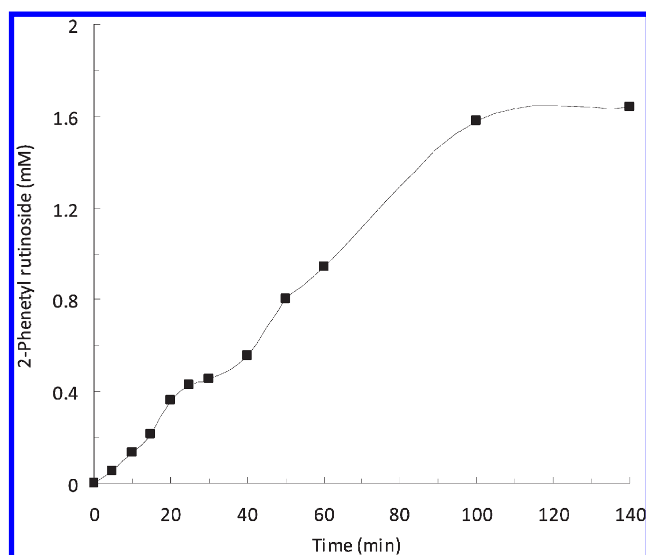

Figure 4. Time course of the synthesis of 2-phenethyl rutinoside catalyzed by α-rhamnosyl-β-glucosidase (pH 5.0, 30 °C).

used a fungal β-primeverosidase to synthesize 2-phenylethyl primeveroside with similar yield (70%).

Transglycosylation and hydrolysis courses were studied using 10% (v/v) 2-phenylethanol as acceptor (140 min, 30 °C, pH 5.0) (Figure 4). The 2-phenylethyl rutinoside concentration increased linearly during the first 100 min of incubation, after which the concentration was maintained constant. Usually, glycosidase-catalyzed syntheses have low yields due to the fact that the product of transglycosylation is also a substrate of the enzyme.[16] Both the high yield and the continuous increment of product concentration obtained in this process indicated that 2-phenylethyl rutinoside would not be a good substrate for the enzyme.

Isolation and Characterization of Transglycosylation Products. Rutinosylated compounds were synthesized in an agitated bench-scale reactor (10 mL) from hesperidin and alcoholic aroma acceptors by transglycosylation using the *Acremonium* sp. enzyme α-rhamnosyl-β-glucosidase. The transformed products neryl-, geranyl-, and 2-phenylethyl-rutinoside were obtained in high yields (58, 76, and 89%, respectively, based on the amount of donor added). The rutinosylated products were purified from the reaction mixture by adsorption on reverse phase (C18) cartridges and eluted with 60% (v/v) aqueous ethanol. The molecular weight of each β-rutinoside was measured by UV-MALDI-TOF/TOF MS and by UV-LDI-TOF/TOF MS, and their structures were characterized by MS/MS experiments. From all of the conditions tested, the clearest signals were obtained in UV-MALDI-MS experiments by using DHB in positive ion mode and in the same ion mode in UV-LDI-MS measurements.

By UV-MALDI-TOF/TOF MS analysis of 2-phenetyl rutinoside, geranyl rutinoside, and neryl rutinoside in positive ion mode by using DHB as matrix, the molecular ions were detected with m/z values that supported the structure assigned to each product. Furthermore, as is shown in Figure 5A by UV-LDI-MS, the 2-phenethyl rutinoside intact molecular ion was detected as monopotasiated $[M + K]^+$ and monosodiated $[M + Na]^+$ species at m/z 469 and 453, respectively. From the former, the typical fragmentations due to the presence in the molecular structure of an aromatic functional group, the phenethyl moiety, were observed yielding the daughter ions $[(M + Na) - C_6H_5]^+$ and $[(M + Na) - CH_2C_6H_5]^+$ at m/z 377 and 362. The alkenic character of geranyl rutinoside and neryl rutinoside explains the absence of a clear fragmentation pattern although with no doubt both UV-LDI mass spectra are different (Figure 5B,C). In both spectra, the intact molecular ions as $[M + K]^+$ and $[M + Na]^+$ species at m/z 501 and 485 were observed. Finally, it is necessary to point out that the signals at m/z 430 and 414 are contaminations included in the samples during the workup followed for the products isolation. Those peaks were also found in control samples developed with same purification procedure performed without the synthesized compounds.

Hydrolysis of Aroma Precursors. α-Rhamnosyl-β-glucosidase was incubated with the purified aromatic and terpenyl rutinosides as substrates (Table 1). In an aqueous medium, the hydrolysis was not detected. Similar results were found for other fungal diglycosidases, which present poor hydrolysis of aroma precursors. *P. multicolor* IAM7153 β-primeverosidase was shown to hydrolyze 2-phenylethyl primeveroside only slightly (0.1% respective to the artificial substrate *p*-nitrophenyl β-primeveroside).[11] *Aspergillus fumigatus* AP-20 was able to split off eugenol from the corresponding primeveroside, although efficiency was also low (1.8% with respect to the artificial substrate *p*-nitrophenyl β-primeveroside).[17] On the contrary, plant diglycosidases usually hydrolyze their own secondary metabolites, which are located in different cellular compartments. In that way, *Camellia sinensis* β-primeverosidase catalyzed the deglycosylation of 2-phenyletlhyl primeveroside with a hydrolysis rate of 185% relative to the *p*-nitrophenyl derivative.[18]

Regarding the technological applications, plant enzymes are not usually the first option. Above, we suggested that the reason of the high yield for the synthesis was that rutinosides are not good substrates for the enzyme. To find a system where hydrolysis would be possible, we searched for an acceptor better than water. In a previous study, a series of alkyl-alcohols (C_1-C_5) were used to investigate the acceptor specificity of the enzyme, and short chain alcohols were shown to be preferably used as

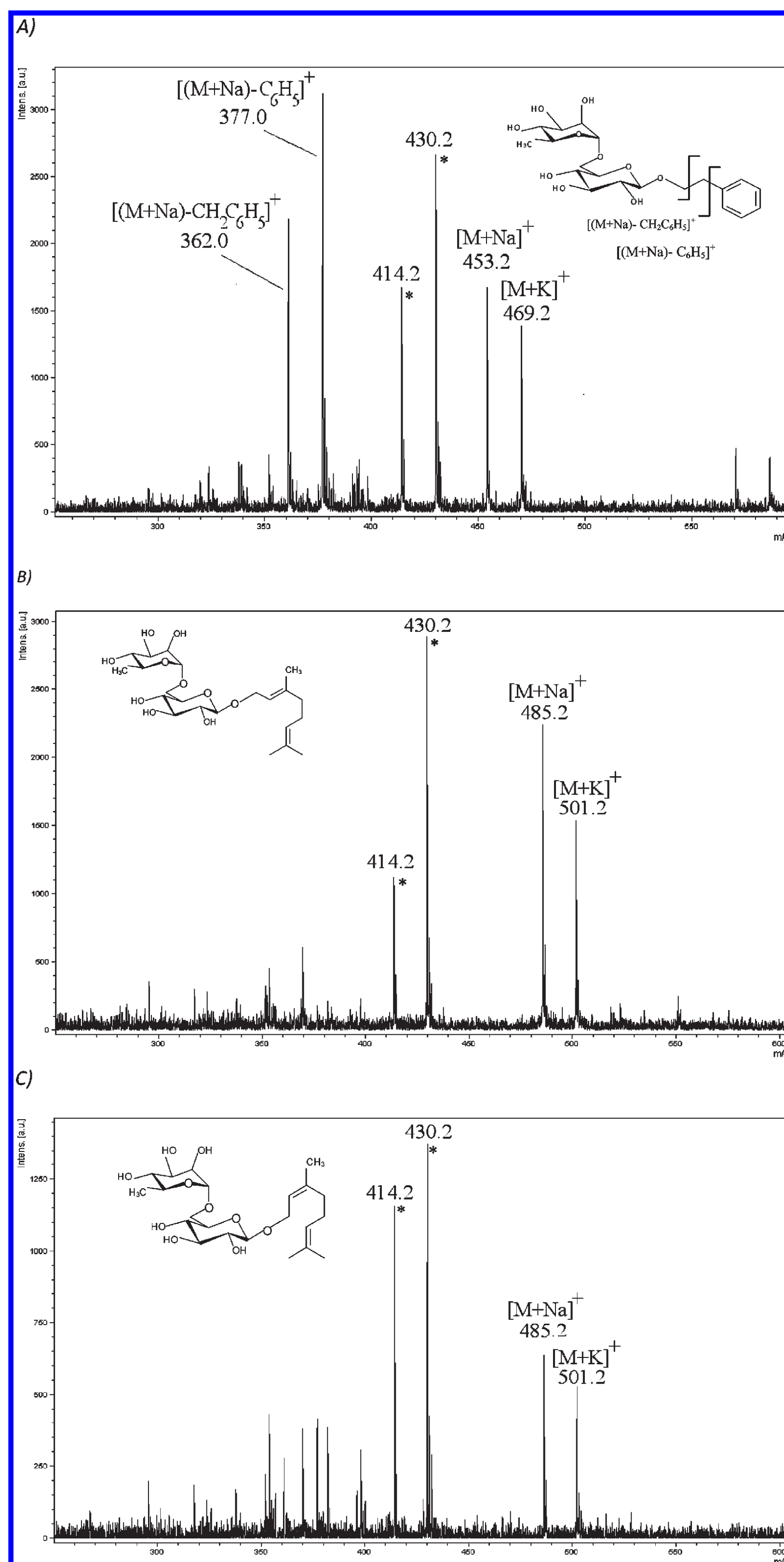

Figure 5. UV-LDI-TOF/TOF spectrum of reaction product obtained: (A) 2-phenethyl rutinoside (calculated molecular mass $[+Na]^+$: 453.28 m/z), (B) geranyl rutinoside (calculated molecular mass $[+Na]^+$: 485.37 m/z), and (C) neryl rutinoside (calculated molecular mass $[+Na]^+$: 485.37 m/z). Positive ion mode; * denotes workup contamination.

acceptors.[16] For the reason that ethanol is a common constituent of several foods and beverages, it was chosen as an additive for the reaction mixture (Table 1). The aroma precursors were significantly hydrolyzed (up to 3−4% within 2 h at 30 °C), which

Table 1. Hydrolysis of Rutinosyl Derivatives by α-Rhamnosyl-β-glucosidase[a]

substrate	substrate conversion (%)	
	aqueous medium	12% (v/v) ethanol
hesperidin	14.3	21.7
2-phenethyl rutinoside	ND	3.8
geranyl rutinoside	ND	2.9
neryl rutinoside	ND	2.9

[a] ND, not detected.

indicated the potential use of the enzyme for biotechnological applications, for example, in aroma modulation of wines. α-Rhamnosyl-β-glucosidase differs from β-glucosidases in that the lasts on their own are ineffective for the release of the aroma components from the diglycosylated aroma precursors. Sugar breakdown of rutinosides is usually sequential, and the rhamnose moiety must be removed first by an α-rhamnosidase. The ability of α-rhamnosyl-β-glucosidase to release the disaccharide moieties from aroma precursors in one single step makes it a promising candidate for winemaking processes.

AUTHOR INFORMATION

Corresponding Author

*Tel: +54 2954 436787 ext. 26. Fax: +54 2954 432535. E-mail: javierbreccia@exactas.unlpam.edu.ar.

Funding Sources

This work was funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Universidad de Buenos Aires, and Universidad Nacional de La Pampa (UNLPam).

REFERENCES

(1) Maicas, S.; Mateo, J. J. Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: A review. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 322–335.

(2) Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochemistry* **1982**, *21*, 2013–2020.

(3) Ueda, J.; Tezuka, Y.; Banskota, A. H.; Tran, Q. L.; Tran, Q. K.; Saiki, I.; Kadota, S. Constituents of the vietnamese medicinal plant *Streptocaulon juventas* and their antiproliferative activity against the human HT-1080 fibrosarcoma cell line. *J. Nat. Prod.* **2003**, *66*, 1427–1433.

(4) Zarzuelo, A.; Gámez, J. M.; Utrilla, P.; Jiménez, J.; Jiménez, I. Luteolin 5-rutinoside from *Salvia lavandulifolia* ssp. oxyodon. *Phytochemistry* **1995**, *40*, 1321–1322.

(5) Kanaoka, D.; Miyakoshi, T. Synthesis of terpene-glycosides by modified Koenigs-Knorr method. *Nippon Kagakkai Koen Yokoshu* **2002**, *82*, 186.

(6) van Rantwijk, F.; Woudenberg-van Oosterom, M.; Sheldon, R. A. Glycosidase-catalysed synthesis of alkyl glycosides. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 511–532.

(7) Blanchard, J. E.; Withers, S. G. Rapid screening of the aglycone specificity of glycosidases: Applications to enzymatic synthesis of oligosaccharides. *Chem. Biol.* **2001**, *8*, 627–633.

(8) Kadi, N.; Belloy, L.; Chalier, P.; Crouzet, J. Enzymatic synthesis of aroma compound xylosides by transfer reaction of *Trichoderma longibrachiatum* xylanase. *J. Agric. Food Chem.* **2002**, *50*, 5552–5557.

(9) Kadi, N.; Crouzet, J. Enzymatic synthesis of primeverosides using transfer reaction by *Trichoderma longibrachiatum* xylanase. *Food Chem.* **2006**, *98*, 260–268.

(10) Tramice, A.; Pagnotta, E.; Romano, I.; Gambacorta, A.; Trincone, A. Transglycosylation reactions using glycosyl hydrolases from *Thermotoga neapolitana*, a marine hydrogen-producing bacterium. *J. Mol. Catal. B: Enzym.* **2007**, *47*, 21–27.

(11) Tsuruhami, K.; Mori, S.; Amarume, S.; Saruwatari, S.; Murata, T.; Hirakake, J.; Sakata, K.; Usui, T. Isolation and characterization of a β-primeverosidase-like enzyme from *Penicillium multicolor*. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 691–698.

(12) Mazzaferro, L.; Piñuel, L.; Minig, M.; Breccia, J. D. Extracellular monoenzyme deglycosylation system of 7-*O*-linked flavonoid β-rutinosides and its disaccharide transglycosylation activity from *Stilbella fimetaria*. *Arch. Microbiol.* **2010**, *192*, 383–393. Mazzaferro, L.; Piñuel, L.; Minig, M.; Breccia, J. D. Erratum. *Arch. Microbiol.* **2011**, *193*, 461.

(13) Miller, G. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428.

(14) Witham, F. H.; Blaydes, D. F.; Devlin, R. M. *Experiments in Plant Physiology*; Van Nostrand Reinhold Company: New York, 1971; p 245.

(15) Nonami, H.; Fukui, S.; Erra-Balsells, R. β-Carboline alkaloids as matrices for matrix-assisted Ultraviolet Laser Desorption Time-of flight Mass Spectrometry of proteins and sulfated oligosaccharides: A comparative study using phenylcarbonyl compounds, carbazoles and classical matrices. *J. Mass Spectrom.* **1997**, *32*, 287–296.

(16) Mazzaferro, L. S.; Piñuel, L.; Erra-Balsells, R.; Giudicessi, S. L.; Breccia, J. D. Synthesis of 4-methylumberlliferyl-rutinoside: A new fluorogenic substrate to seek for diglycosidases. Submitted for publication.

(17) Yamamoto, S.; Okada, M.; Usui, T.; Sakata, K. Isolation and characterization of a β-primeverosidase-like endo-manner a β-glycosidase from *Aspergillus fumigatus* AP-20. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 801–807.

(18) Ma, S. J.; Mizutani, M.; Hiratake, J.; Hayashi, K.; Yagi, K.; Watanabe, N.; Sakata, K. Substrate specificity of β-primeverosidase, a key enzyme in aroma formation during oolong tea and black tea manufacturing. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2719–2729.