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Enhancement of the antioxidant activity of orange and lime juices by flavonoid enzymatic de-glycosylation

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ABSTRACT

Pure flavonoid glycosides and those present in orange (*Citrus sinensis*) and lime (*Citrus latifolia*) juices were structurally modified by enzymatic de-glycosylation to obtain functional derivatives with higher antioxidant activity. The enzymatic reactions were carried out using commercial rhamnosidases (hesperidinase and naringinase) and β-D-glucosidase. An experimental design and statistical tools for the data analysis were employed for each enzyme to evaluate the independent variables (pH and temperature) that significantly affected the enzyme activity (dependent variable), and the antioxidant activity was evaluated before and after bioconversion using DPPH• and FRAP assays. Aliquots of controls and enzyme-treated samples were taken at different times and analyzed by UPLC–MS. The antioxidant activity of both treated juices was higher than that of the untreated juices, confirming that the bioconversion reaction conditions used were efficient and produced an increment in the antioxidant activity. After the 4 h-hesperidinase reaction 60% of the hesperidin was converted into hesperetin in the orange juice. The antioxidant capacities of the glycosylated standards were also increased by the enzyme treatment. The results presented herein are a step forward in the use of α-L-rhamnosidases to produce functional beverages by way of de-glycosylation reactions of their flavonoid glycosides.

1. Introduction

Hesperidin (6″-*O*-(α-L-rahmnopyranosyl)-D-glucose flavonoid), a member of the flavanone group of flavonoids, is an abundant and inexpensive by-product of citrus cultivation and is widely available in Brazil. This molecule can be isolated in large amounts from the rinds of some citrus species [e.g., *Citrus sinensis* L. (sweet orange) and *Citrus latifolia* (Tahiti lime)] and has been reported to have antioxidant, antiallergenic, anticarcinogenic, antihypotensive, antimicrobial, and vasodilator properties (Garg, Garg, Zaneveld, & Singla, 2001; Hollman & Katan, 1997). Hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone), the aglycone form of hesperidin, is also an important dietary flavanoid with antioxidative, antiinflammatory, hypolipidemic, vasoprotective and anticarcinogenic properties (Hollman & Katan, 1997). The anticarcinogenic activity of hesperetin obtained by the action of α-L-rhamnosidase on hesperidin has been shown in the laboratory animals (Erlund, 2004).

Flavonoids are mainly present in citrus fruits as their glycosyl derivatives. Hesperidin is one such conjugated glycoside consisting of the hesperetin bound at the C-7 position (on ring A) to rutinose ($C_{12}H_{22}O_{10}$), a disaccharide composed of one molecule of rhamnose and one of glucose. One important drawback is the limited bioavailability of many flavonoids, and in fact the sugar moiety has been proposed as the major determinant of the absorption of dietary flavonoids in humans, whereas the rutinoside moiety is poorly absorbed in comparison with the aglycone and glucoside forms (Hollman et al., 1999). The enzymatic de-glycosylation of flavonoids has been reported as a good alternative for increasing their bioavailability (Christensen, 2009; Erlund, Meririnne, Alfthan, & Aro, 2001; Hollman et al., 1999), and increase the antioxidant activity of kaempferol (Park, Rho, Kim, & Chang, 2006) and anti-inflammatory activity of naringin (Amaro et al., 2009).

Conventional chemical methods, such as acid hydrolysis or alkaline cleavage for the preparation of minor flavonoids, inevitably lead to side reactions. In this context, the bioconversion of many compounds by a specific type of glycosyl hydrolase is advantageous due to the selectivity and mildness of the reaction conditions. The enzyme α-L-rhamnosidase [E. C. 3.2.1.40] cleaves terminal α-L-rhamnose specifically from a large number of natural products that include hesperidin, naringin, rutin, quercitrin, terpenyl glycosides and many other natural glycosides containing terminal α-L-rhamnose (Monti, Pisvejcová, Kren, Lama, & Riva, 2004; Yadav, Yadav, Yadav, & Yadav, 2010). Only two commercial

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preparations of α-L-rhamnosidases, naringinase and hesperidinase, are available, and both are from fungal sources. Hesperidinase is obtained from *Aspergillus niger* and *Penicillium* species and naringinase from *Penicillium decumbens*. All these preparations also show significant β-D-glucosidase activity, which catalyzes the hydrolysis of terminal non-reducing residues into β-D-glucosides with the release of glucose (Yadav et al., 2010).

α-L-rhamnosidase has turned out to be a biotechnologically important enzyme due to its applications in a variety of processes such as the debittering of citrus fruit juices (Busto, Meza, Ortega, & Perez-Mateos, 2007), the manufacture of prunin from naringin (Roitner, Schalkhammer, & Pittner, 1984), the enhancement of wine aromas by the enzymatic hydrolysis of terpenyl glycosides containing L-rhamnose (Spagna, Barbagallo, Martino, & Pifferi, 2000), and the de-rhamnosylation of many L-rhamnose-containing steroids such as diosgenin, desglucoruscin and ginsenosides-Rg2, whose de-rhamnosylated products are of clinical importance (Elujoba & Hardman, 1987; Ko, Choi, Suzuki, & Suzuki, 2003; Monti et al., 2004).

However, no scientific investigation has been carried out so far, related to hydrolyses of the flavonoid glycosides from orange and lime juices and improving their antioxidant activity. Previous articles reported the use of glycosyl hydrolase as a catalyst in the hydrolysis reaction of flavonoid glycosides in the juices. González-Barrio et al. (2004), used α-L-rhamnosidases from *Aspergillus aculeatus* to produce bioavailable flavonoid glucosides in blackcurrant juice, orange juice and green tea infusion, however, without any report on the effect of this enzymatic treatment on the antioxidant activity of the beverages. Nielsen et al. (2006) proposed a similar concept that the removal of the rhamnose sugar from hesperidin to yield its flavonoid glucoside can improve its bioavailability in orange juice. Lee, Huh, Nam, Moon, and Lee (2012), reported that hesperetin-7-O-glucoside (Hes-7-G) could be produced by the enzymatic conversion of hesperidin in extracts from orange juice and peel by naringinase from *Aspergillus sojae*. Hes-7-G was more potent than hesperidin in the inhibition of human HMG-CoA reductase and 1.7- and 2.4-fold better than hesperidin and hesperetin, respectively, in the inhibition of human intestinal maltase.

The aim of this work was to study the bioconversion of orange (*C. sinensis*) and lime (*C. latifolia*) juices by α-L-rhamnosidases and β-D-glucosidase, alone or in combination, in order to hydrolyze most of the flavonoid glycosides in the juices and obtain derivates with higher antioxidant activity. The effect of pH and temperature on enzyme activity was determined using a statistical experimental design. Two analyses were carried out to determine the total antioxidant activities of the juices, including 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical-scavenging analysis and the metal reduction capacity (FRAP — ferric reducing antioxidant power).

2. Material and methods

2.1. Enzymes and reagents

Hesperidinase from *Penicillium* sp., naringinase from *P. decumbens*, β-D-glucosidase from *A. niger*, DPPH (2,2-diphenyl-1-picril-hydrazyl radical), TPTZ (2,3,5-trifeniltetrazolium chloride) and the standards of hesperidin, hesperetin, quercetin and rutin were purchased from the Sigma-Aldrich Chemical Co. According to the information of Sigma-Aldrich, hesperidinase contains both α-L-rhamnosidase (EC 3.2.1.21) and β-D-glucosidase (3.2.1.21) activities. One unit will liberate 1.0 μmol of reducing sugar (as glucose) from hesperidin per min at pH 3.8 at 40 °C. Naringinase activity was ≥300 units/g solid, with a secondary activity ≥10 units/g solid beta-glucosidase. All the solvents and other reagents were of analytical, spectrometric or chromatographic grade.

2.2. Effect of pH and temperature on enzyme activity

The hydrolytic activity of each enzyme (hesperidinase, naringinase and β-D-glucosidase) was determined using a substrate of standard rutin (quercetin-3-rutinoside), quantifying the reducing sugar formed by the Somogyi method (1945). To determine the optimum pH and temperature, reaction mixtures containing 100 μL of enzyme preparation (0.02 mg/mL) and 4 mL of 1% (m/v) rutin solution in 0.05 M acetate (pH 2.0–4.5) and 0.05 M citrate–phosphate (pH 6.0) buffers were incubated for 20 min at different temperatures (Table 1). A 2^2 central composite design (2^2-CCD) was employed to evaluate the independent variables (pH and temperature) that significantly affected the enzyme activity (dependent variable). One unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of reducing sugars from rutin per minute under the above assay conditions (U/min). Each independent variable was investigated at high (+1) and low (−1) levels. Four center point runs were included in the matrix and a statistical analysis was used to identify the effect of each variable on the response. All the data were processed using Statistica® software, version 5.1.

2.3. Preparation of juices

The juices were prepared by crushing Sweet oranges (*C. sinensis*) and Tahiti limes (*C. latifolia*), purchased from a local supermarket in the city of Bragança Paulista, Brazil. The rinds and seeds were discarded and the juices prepared by placing the material in a blender without the addition of water and blending for 2 minutes. Crude juice was centrifuged at 100 rpm for 15 min, and the supernatant was used as the fresh sample for bioconversion reactions. The juice supernatant were lyophilized for further use. Prepared orange and lime juices had pH values of 3.7 and 2.8 respectively.

2.4. Bioconversion reactions

Bioconversion reactions were carried out in screw-capped glass tubes with shaking (130 rpm) at controlled temperatures for 2, 4, 8, 12 and 16 h using 100 mL of juices (orange or lime). To initiate the hydrolysis of the flavonoid glycosides, 1 mL of enzyme solution (0.02 mg/mL) prepared in 0.1 M acetate buffer was added to the reaction mixture. The optimum temperature and pH values obtained during the preliminary assays were used. The tests with enzyme combinations used equal parts of each up to a final concentration of 0.02 mg/mL. The reactions were stopped by boiling for 20 min, and the samples subsequently freeze-dried and stored at −80 °C prior to extraction and analysis. For the bioconversion of commercial standard hesperidin and rutin, 10 mM of flavonoid glycoside solution in methanol (15% v/v) was mixed with hesperidinase solutions prepared in

Table 1
2^2 Central composite design coded for the study of the influence of pH and temperature on hesperidinase, naringinase and glucosidase activities.

Test set	Variables		Hesperidinase activity (U/min)	Naringinase activity (U/min)	Glucosidase activity (U/min)
	pH	Temperature (°C)			
1	−1 (3.5)	−1 (30)	14.8	12.9	5.9
2	+1 (4.5)	−1 (30)	16.3	14.1	6.4
3	−1(3.5)	+1 (50)	5.4	5.2	8.2
4	+1 (4.5)	+1 (50)	7.5	6.1	9.5
5	−1.41 (2.0)	0 (40)	5.4	5.1	6.1
6	+1.41 (6.0)	0 (40)	10.1	13.4	9.8
7	0 (4.0)	−1.41 (20)	10.6	7.8	4.9
8	0 (4.0)	+1.41 (60)	5.7	7.9	4.6
9	0 (4.0)	0 (40)	43.5	34.7	20.7
10	0 (4.0)	0 (40)	41.5	36.4	19.1
11	0 (4.0)	0 (40)	40.8	38.5	17.8
12	0 (4.0)	0 (40)	40.4	39.6	19.4