Synthesis of hesperetin 7-O-glucoside from flavonoids extracted from
Citrus waste using both free and immobilized α-L-rhamnosidases

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A B S T R A C T

This work was aimed to design alternatives to semi-synthesize hesperetin 7-O-glucoside (HG), a low occurrence natural product, from the flavonoids hesperidin (HES) and neohesperidin (NEO) that can be extracted at large scale from Citrus. To fulfill this objective, the activities of three α-L-rhamnosidases were characterized using both synthetic and natural substrates. Our results indicate that NEO is a good substrate for HG synthesis, mainly because of its solubility in water. Regarding the biocatalysts assessed, all the rhamnosidases exhibited good performances for NEO hydrolysis. Particularly, rhamnosidase from Aspergillus terreus was covalently immobilized onto azide-Dacron magnetized support, activating the support with NaNO₂. Immobilized rhamnosidase presented high stability against both thermal denaturation and washing. Based on the results obtained, two methods to obtain HG from NEO are proposed, the first one involving a soluble commercial enzyme, and the second one using the immobilized biocatalyst. Using the latter approach, HG was obtained almost pure at lab-scale, with an overall yield of 93%.

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1. Introduction

Flavonoids consist of a large group of polyphenolic compounds that are ubiquitously present in plants. Specially, there is a growing interest in the research of the flavonoids extracted from Citrus fruits because of their beneficial properties on human health (for reviews on this topic, see Benavente-García and Castillo, 2008; Mantey et al., 2001; Tripoli et al., 2007; Xiao et al., 2011). Therefore, these compounds and their derivatives are important for pharmaceutical and food industries. Flavanones constitute the major subclass of flavonoids in the Citrus family, hesperidin (HES) being the flavanone of commercial interest. Certain pharmaceutical products contain HES and are used for the treatment of a variety of diseases including venous insufficiency (Lyseng-Williamson and Perry, 2003) and hemorrhoids (Misra and Imlantesu, 2005), among others. The HES isomer neohesperidin (NEO) can be found in bitter oranges (e.g. Citrus aurantium). Both HES and NEO concentrate in the fruit peels, being their concentration in the naturally dropped fruits (3–7 mm diameter) much higher than in the mature fruits (50–70 mm) (Castillo et al., 1992). After juice separation, the solid parts of the fruits are discarded. Hence, the use of these solids parts for the extraction of HES and NEO not only reduce the volume of waste to be disposed of but also increases the profitability of Citrus-related industries.

NEO and HES can be used as sources of the carbohydrate rhamnose, in a process that requires their hydrolysis. Rhamnose is used extensively as a synthesis precursor of diverse organic compounds (Ribeiro, 2011), and as an anti-aging agent in cosmetic and beauty products (Asselineau et al., 2011; Gesztesi et al., 2006; Tran et al., 2011). In addition to rhamnose, the hydrolysis of HES or NEO also renders diverse by-products depending on the features of the procedure used (Fig. 1). One of these by-products is hesperetin 7-O-glucoside (HG). This compound, that has low occurrence in the Nature, is gaining attention because of its recently found biological activities. For example, it has been described that HG inhibits in vitro human intestinal maltase and HMG-CoA reductase –the key enzyme for cholesterol synthesis–, and inhibits the growth of Helicobacter pylori, a causative agent for gastric diseases such as gastritis, duodenal ulcer, and gastric cancer (Lee et al., 2012; Veldhuizen van Zanten and Sherman, 1994). Moreover, it has been shown in rats that the bioavailability of HG is higher than that of HES, and that this compound was effective in the prevention of bone loss (Habauzit et al., 2009). To facilitate the subsequent investigations in this area, it would be desirable to have a method to synthesize HG at a gram scale, yielding a high purity product, and involving an experimental design as simple as possible. As shown in Fig. 1, HG may be obtained from NEO or HES by either acidic or enzymatic hydrolysis.

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The lack of specificity in the glycosidic bond attacked, the extreme operating conditions, and the multiple procedure steps involved, makes the acidic procedure less attractive. In contrast, α-1-rhamnosidases (EC 3.2.1.40) hydrolyze only the non-reducing terminal α-rhamnose residues and function under mild conditions, making this a preferable approach. Nevertheless, both methodologies are limited by the solubility of the substrate used and, therefore, this property must be determined before any attempt to develop a process for HG obtaining (Peteireit and Saal, 2011).

Overall, immobilized enzymes are preferred over the free enzymes because of their higher stability and their reusability (Ju et al., 2012). There are diverse procedures to immobilize enzymes onto activated polymeric supports. Among others, the covalent field. This kind of immobilization involves the linkage of a solid matrix to any reactive group in the enzyme, and produces a system where the diffusion of the reagents is not a limitation (Cadena et al., 2010). Ferromagnetic materials, such as magnetized Dacron, have been largely utilized for enzyme immobilization since the biocatalyst can be easily separated from the reaction mixture by applying a magnetic field.

In the present study, we optimized the enzymatic hydrolysis of NEO and HES in order to design an efficient methodology to obtain HG under mild experimental conditions, and with an easy downstream processing. For this purpose, the solubility profiles of NEO and HES versus temperature were first determined. Next, three α-1-rhamnosidases (2 commercial and 1 produced in our lab) were characterized according to their activity against synthetic and natural (HES and NEO) substrates. Based on its high performance, the enzyme purified by our group was immobilized onto azide-Dacron magnetized support via the covalent binding method. Finally, and using NEO as the substrate, the yield of HG synthesis at a gram scale was assessed for both the commercial enzymes and the immobilized biocatalyst.

2. Materials and methods

2.1 Materials

HES and NEO were obtained from naturally dropped bitter oranges as described previously (Macoritto et al., 2001; Macoritto et al., 2004). Three enzymatic preparations with rhamnosidase activity were used: Naringinase from Aspergillus niger (NAN) (Amano, Japan), Hesperedinase from A. niger (HAN) (Tanabe Seiyaku Co, Japan) and an α-rhamnosidase from Aspergillus terreus (RAT) (CECT 2663) obtained in our lab as described previously (Soria et al., 1999). Rhamnose, 4-nitrophenyl-α-L-rhamnopyranoside (p-NPR) and 4-nitrophenyl β-D-glucopyranoside (p-NPG) were purchased from Sigma-Aldrich (USA). Commercial standards of hesperetin, hesperidin and neohesperidin were purchased from Extrasynthese (France). Dacron was gently donated by Rodhia do Brasil SA (Brazil). NaCl, FeSO₄, NaNO₂, HCl, dimethylsulfoxide (DMSO) and other reagents used in this work were of analytical grade.

2.2 Solubility of hesperidin and neohesperidin

Solubility profiles of HES and NEO in bidistilled water were determined by preparing HES and NEO suspensions in 16 × 100 mm² glass tubes with screw cap (Hach, USA). Samples were incubated for 2 h at different temperatures (25, 50, 96, 110 or 150 °C) using a heater block (Hach, USA) and with occasional shaking. Immediately after incubation, the suspensions were equilibrated at room temperature and filtered through a 0.45 μm pore cellulose nitrate membrane (Microclar, Argentina). Flavonoid content in the filtrates was determined by HPLC according to the analytical method described in 2.10. In addition, the ratio NEO-solubility/HES-solubility was calculated at each temperature assayed. The assay was performed in duplicates.

2.3 Preparation of activated azide-Dacron magnetized support

The azide-Dacron support was obtained based on the procedures described in the literature (Carneiro Leão et al., 1991; Pimentel et al., 2006). Films of Dacron (4 g) were cut in strips and incubated at 40 °C for 48 h with stirring in methanol (100 ml) containing hydradize hydrate (10 ml). After incubation, the hydradize-Dacron powder was washed twice with methanol followed by methanol: water 90:10 (v/v). Previous to the magnetization, a suspension of hydradize-Dacron powder (2 g in 10 ml of water) was sedimented for 8 min and the supernatant was decanted to remove fines four times. The ferromagnetic support was obtained with 2 g of hydradize-Dacron in 100 ml of deionized water and 10 ml of an aqueous solution of FeCl₃·6H₂O (300 mg/ml) and FeCl₂·4H₂O (121 mg/ml) was added dropwise. The mixture was adjusted to pH 10 by adding 28% (v/v) NH₄OH under vigorous stirring, and next incubated at 80 °C for 30 min. The magnetic particles were recovered by applying a 0.6 T magnetic field (Ciba Corning, USA). The magnetic particles were washed with deionized water until attaining a suspension of pH 7, and dried for 16 h at 50 °C. The particles were sieved and only those

Fig. 1. Scheme of the hydrolysis of both hesperidin and neohesperidin catalyzed by acids or by α-rhamnosidases.
that passed through a 250 µm-mesh were used for the subsequent modification. The ferromagnetic hydrazide-Dacron support was converted to ferromagnetic azide-Dacron before the immobilization of the enzyme. For this, 10 mg of support were added with 400 µl of 0.6 M HCl and 50 or 100 µl of 5% (w/v) NaN₃ solution. The system was incubated at 4 °C for 30 min. The sediment was washed successively with 1 ml of water, 1 M NaCl in buffer, water, and buffer. Finally, the support was suspended in the buffer and stored at 4 °C until its use.

2.4 Immobilization of RAT on ferromagnetic azide-Dacron support

To prepare the immobilized catalyst (RAT-I), 1 ml of a 1:10 dilution of the crude RAT preparation (specific activity of 1.46 nkat/µg-protein) was added to 10 mg of the ferromagnetic azide-Dacron support and incubated for 17 h at 4 °C. The RAT-I were recovered by applying a magnetic field, the supernatants were collected, and the protein concentration in the supernatants was determined using 1.75 mM p-NPR as substrate in 20 mM succinic acid/sodium succinate buffer, pH 5.5 (from now on, denominated “the buffer”). For the free enzyme assay, 400 µl of 1.75 mM p-NPR in buffer and 10 µl enzyme solution in buffer of RAT (stock solution diluted with buffer 1:100), HAN (50 mg-powder/ml) or NAN (0.1 mg-powder/ml) were placed in 1.5 ml plastic tubes and incubated at 50 °C. Aliquots containing 50 µl of the samples were withdrawn at 3 min intervals and added to 1.5 ml of 0.5 M NaOH. The absorbance of the solutions was recorded at 410 nm in a CINTRA 101 spectrophotometer (GBC, Australia).

The activity of α-rhamnosidase in the preparations was determined using 1.75 mM p-NPR as substrate in 20 mM succinic acid/sodium succinate buffer, pH 5.5 (from now on, denominated as “the buffer”). For the free enzyme assay, 400 µl of 1.75 mM p-NPR in buffer and 10 µl enzyme solution in buffer of RAT (stock solution diluted with buffer 1:100), HAN (50 mg-powder/ml) or NAN (0.1 mg-powder/ml) were placed in 1.5 ml plastic tubes and incubated at 50 °C. Aliquots containing 50 µl of the samples were withdrawn at 3 min intervals and added to 1.5 ml of 0.5 M NaOH. The absorbance of the solutions was recorded at 410 nm in a CINTRA 101 spectrophotometer (GBC, Australia).

The hydrolytic activities of the free enzymes (NAN, HAN and RAT) against natural substrates (HES and NEO) were determined at 2.6 Evaluation of enzymatic activities using natural substrates

1.1 mol of p-NPR per second. The activity katal (kat) of the enzymes was determined as the amount of enzyme that consumed 1 mol of substrate (NEO or HES) per second.

2.7 Thermal stability of free and immobilized enzymes

The thermal stability of the free (NAN, HAN, RAT) and the immobilized (RAT-I) enzymes was analyzed. The enzyme dilutions were the same than those used for the determination of the activity against synthetic substrates. Solutions were incubated in buffer at 50 °C, and aliquots containing 20 µl of the samples were withdrawn at several times to quantify the residual α-rhamnosidase activity as described in 2.5.

2.8 Hydrolysis of NEO by RAT-I

The activity of RAT-I was assessed at 50 °C using NEO as substrate. Working with suspensions of a given substrate suppose that a bigger amount of substrate will be hydrolyzed per batch. On this basis, the time-course of NEO hydrolysis by RAT-I performed at 50 °C was determined, the substrate either being completely solubilized (i.e. 4.7 mM) or partially suspended (20 mM, prepared without pre-dissolve at 110 °C). In both situations, the reactions were carried out at 50 °C using 6.7 nkat/ml RAT-I (against p-NPR), with continuous shaking at 200 rpm. Aliquots of the samples were withdrawn at different times to assess NEO hydrolysis.

To quantify the loss of enzyme activity by protein denaturing and/or detachment after washings, 30 min-reaction cycles using 4.7 mM NEO in solution, or 3 h-cycles using 20 mM NEO suspensions, were performed. The lengths of the cycles were set from the time necessary to attain a substrate hydrolysis of approximately 40%. After each cycle, hydrolysis rate was determined by HPLC as described in 2.10. RAT-I was washed to eliminate the residual agents, and the immobilized enzyme was submitted to a next cycle of reaction. Each assay was performed in duplicate.

2.9 Preparation of HG at a gram scale

HG was obtained from NEO either by using the free enzyme system and the immobilized biocatalyst.

The reaction using the free enzyme was performed in two steps. In the first one, 500 ml of 20 mM NEO suspension was poured in an Erlenmeyer flask and allowed to equilibrate at 50 °C. The hydrolysis was initiated by the addition of 2.8 nkat HAN (against p-NPR) per ml of reaction medium, and aliquots of the samples were withdrawn at regular time intervals. The reaction was carried out either at 50 °C for 24 h. Then, the reactor was kept at 4 °C for 6 h. The reaction mixture was filtered by using common filter paper to recover the NEO-HG mixture. It was washed with cool distilled water. In the second step, the solid was suspended in 300 ml of buffer and the reaction was washed to 50 °C for 24 h, after add 2.8 nkat HAN (against p-NPR) per ml of reaction media. Finally, the reactor was kept at 4 °C for 6 h again. The precipitates formed were filtered, washed with distilled water and dried at 50 °C. The yield of HG generation was determined by weighing, and the purity of the final product was determined by HPLC as described in 2.10.

In the second approach, RAT-I was used as the catalyst. Fifty ml of 20 mM NEO solution in buffer were poured in an Erlenmeyer flask and allowed to equilibrate at 50 °C. As described above, NEO hydrolysis was initiated by adding 2.8 nkat/ml RAT-I (against p-NPR), and the reaction was carried out at 50 °C for 24 h and subsequently cooled at 4 °C for 6 h. The sediment was separated
by decanting the supernatant, dissolved in 25 ml of ethanol, and filtered through a 0.45 μm-cellulose membrane to eliminate RAT-I particles. The obtained ethanolic solution was diluted to 50 ml with water and heated at 50 °C under shaking until attaining a final volume of 25 ml. After cooling, HG was recovered from the precipitate and dried at 50 °C. The purity of the product was checked by HPLC as described in 2.10 and the yield of HG generation was determined by weighting.

2.10 HPLC procedures

The identification, purity and concentration of flavonoids in the final preparations were assessed by HPLC from the comparison of their retention times and the absorbance at 280 nm with those obtained using preparations of commercial standards. Aliquots containing 20 μl of the preparations were eluted isocratically with acetonitrile:water (32:68) at 0.7 ml/min flow rate, using a GraceSmart RP 18 column (Grace, USA), with a 118 UV/vis Detector (Gilson, France) fixed at 280 nm and a CR-8A integrator (Shimadzu, Japan). α-L-Rhamnose was determined by isocratic elution with water at 80 °C and a flow rate of 0.5 ml/min, using a Rezex RAM Carbohydrate column (Phenomenex, USA) and an IR detector Smartline 2300/2400 (Knauer, Germany).

3. Results and discussion

3.1 HES and NEO water solubility profiles

An important parameter that limits the feasibility of an enzymatic hydrolysis is the solubility of the substrate in the reaction media. Thus, the solubility profiles of both HES and NEO were determined (Fig. 2). It is worth to note, when a saturated solution of flavonoid is prepared at high temperature and the obtained solution is subsequently equilibrated at a lower temperature, a supersaturated solution is formed with no apparent precipitate formation. Thus, this procedure allows determining the solubility of flavonoids at temperatures over the normal boiling point of water.

Obtained results indicate similar solubility profiles for both isomers in the range of temperature assessed, although they differed in their absolute solubility. NEO was 9 times more soluble than HES at 25 °C and this ratio rose up to 32 at 50 °C.

3.2 RAT immobilization on a ferromagnetic azide-Dacron support

Dacron is a synthetic polymer that lacks functional groups on the surface capable of attaching biologically active molecules. However, the surface of Dacron can be functionalized by treating it with hydrazine, which renders hydrazide-Dacron. The NH₂ groups in the polymer can be subsequently diazotized upon treatment with NaN₂ in HCl to form azide groups. In turn, these functional groups will react with the NH₂-groups of the proteins through an amide bonding.

In this work the α-L-rhamnosidase from A. terreus (RAT) was immobilized covalently onto ferromagnetic azide-Dacron. The relationships between the initial amount of protein used for the immobilization procedure and the amount of protein fixed to the matrix, as well as the retained activities upon enzyme immobilization are shown in Fig. 3. Azide-Dacron showed remarkable immobilization capacity. Supporting that, the retaining capacity of the activated polymer was calculated in 36 μg/mg when offered with 50 μg-protein per mg-support, representing an immobilization capacity of 72%. In addition, when the enzyme activity was tested for the preparation obtained offering 15 μg-protein per mg-support, the specific activity was 1.05 nkat/μg-protein for azide-Dacron (15.1 nkat/mg-support). In a previous study, the same RAT was immobilized covalently onto ferromagnetic hydrazide-Dacron activated via the cross-linking with glutaraldehyde. In that study, the specific activity measured in the same conditions was estimated in 0.53 nkat/μg-protein (Soria et al., 2012), thus indicating that the current method constitutes an improved approach for RAT immobilization with high enzymatic capacity retained.

Covalent immobilization can induce conformational changes in a protein, thereby modifying its enzymatic activity. Based on the current results, it is possible to speculate that the treatment of the hydrazide-Dacron support with NaN₂/HCl generates a higher amount of groups with better conformational properties than the obtained after its reaction with glutaraldehyde. In addition, the binding of RAT to the azide-Dacron support may not involve aminoacids in the vicinity of the active site, which could favor the retaining the enzymatic activity.

3.3 Thermal stability of the enzyme preparations

The thermal stability of the free enzymes (NAN, HAN and RAT), and the immobilized preparation (RAT-I) were studied at 50 °C for 85 h. After incubation, the retained activities were 90%, 80%, 70% and 45% (SD = ± 5%) for HAN, NAN, RAT-I and RAT, respectively. The thermal deactivation profile obtained fitted to a first-order decay process with half-lives of 523, 226, 136 and 79.5 h for HAN, NAN, RAT-I and RAT, respectively.

The large thermal stability measured for RAT-I contrasts with that measured for the free enzyme, and can be attributed to the formation of covalent bonds between the protein and the support. As a consequence, the conformational flexibility of the enzyme might be reduced and the molecular distortion caused by heating, diminished.
Table 1
Enzymatic activities of free enzymes.

<table>
<thead>
<tr>
<th>Enzyme preparation(s)</th>
<th>Protein concentration</th>
<th>Substrate</th>
<th>p-NPR (nkat/µg-protein)</th>
<th>p-NPG (nkat/µg-protein)</th>
<th>HES</th>
<th>NEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringinase from A. niger (NAN)</td>
<td>560 (µg-protein/mg-powder)</td>
<td>0.54</td>
<td>&lt;1.7 x 10^-4</td>
<td>125 nkat/mg-powder</td>
<td>98.5 nkat/mg-powder</td>
<td></td>
</tr>
<tr>
<td>α-Rhamnosidase from A. terreus (RAT)</td>
<td>1810 µg-protein/ml-solution</td>
<td>1.36</td>
<td></td>
<td>109 nkat/ml-solution</td>
<td>291 nkat/ml-solution</td>
<td></td>
</tr>
<tr>
<td>Hesperedinase from A. niger (HAN)</td>
<td>0.48 µg-protein/mg-powder</td>
<td>0.73</td>
<td></td>
<td>0.133 nkat/mg-powder</td>
<td>0.12 nkat/mg-powder</td>
<td></td>
</tr>
</tbody>
</table>

Enzymes activities are expressed in katal (amount of enzyme that hydrolyzes 1 mol of substrate per second). µg-protein: micrograms of protein. Results are shown as the mean of two independent assays with the standard deviations being below 5%.

3.4 Enzymatic activity

All the free enzyme preparations showed high α-rhamnosidase activities and very low β-glucosidase activities (Table 1). RAT had the highest specific activity against p-NPR (nkat/µg-protein). Among the commercial preparations, HAN showed the lowest protein concentration and the higher α-rhamnosidase specific activity (Table 1).

In relation to activity against natural substrates, the chromatograms obtained after NEO and HES hydrolysates separation in a C18 column and UV detection showed a unique peak with a retention time of 7.8 min that was assigned to hesperetin 7-O-glucoside (HG). Hesperetin (retention time 14.7 min) was not detected in the samples. Moreover, rhamnose but not glucose was detected by HPLC using a carbohydrate column and IR detection.

All the enzymatic preparations had the ability to release rhamnose bound to β-D-glucoside moiety through α-1,2 bonding in NEO, and through α-1,6 bonding in HES. The performance of our RAT preparation to hydrolyze NEO was comparable with those of the commercial preparations. To notice, this was the only enzyme preparation that had a higher specific activity in hydrolyzing NEO than HES (Table 1).

3.5 Hydrolysis of NEO by RAT-I

Free enzymes have the disadvantage that they cannot be reused, thus increasing the cost of the process. Aimed to improve the economic factor for the potential large-scale production of HG, we tested the enzymatic activity of RAT immobilized on an inert material with ferromagnetic properties.

The conversion profile obtained when NEO was completely dissolved (4.7 mM) or partially suspended (20 mM, prepared without pre-dissolve at 110 °C), catalyzing the reaction with RAT-I, are shown in Fig. 4A. After 2 h of incubation, the substrate conversion was 80% when using 4.7 mM NEO, whereas after 8 h of incubation 70% conversion was achieved when using 20 mM NEO. This finding indicates that the immobilized catalyst had a good efficiency to conduct the reaction in both conditions, since the solid particles of NEO were dissolved gradually, providing substrate continuously to the media. The possibility that RAT-I may be reused several times was also evaluated, using 4.7 mM NEO as substrate and incubation cycles of 30 min each. During the first 10 cycles of reaction an average NEO to HG conversion of 36 ± 4% was achieved (Fig. 4B), thus indicating that there was not significant loss of the activity either by washing or by enzyme denaturation. When RAT-I reusing capability was evaluated using a NEO suspension (20 mM), the enzymatic activity decreased systematically after each cycle (Fig. 4C). This might be caused by the high concentration of HG achieved in the media, beyond its solubility limit. Probably, the finest RAT-I particles serve as a nucleus for HG precipitation. It could cause the systematic loss of biocatalyst when the product is removed from one cycle to the next.

3.6 Synthesis of HG at a lab-scale

The NEO to HG conversion profiles were obtained for the production at a lab-scale of HG (Fig. 5A). Via the first methodology presented, using 20 mM NEO as substrate and free HAN as catalyst, 86% conversion was achieved after 24 h of incubation. After the second reaction step, the conversion reached 100%. After cooling and filtering, the greenish white solid obtained was washed with distilled water and dried at 50 °C. The amount of HG obtained was 4.3 g, which represents an overall yield of 93% of the theoretical mass (4.6 g). The purity of obtained HG preparations was
investigated (Fig. 6). HPLC chromatograms of substrate and product – both dissolved in DMSO – were obtained, being hesperetin and NEO not present in the final product. Moreover, the solution obtained during the first hydrolysis step with HAN had a concentration of α-L-rhamnose of 3.5 g/l in buffer, and it could be used to attain products rich in α-L-rhamnose, for cosmetic and other pharmaceutical preparations, due their harmlessness.

The second methodology involved the use of 20 mM NEO as substrate and RAT-I as catalyst. With this method, 0.41 g of solid was obtained, with HG content near to 80% (Fig. 5B) which represented an overall yield of 70%. In this case, most of the biocatalyst was separated easily from the reaction medium by applying a magnetic field. However, a minor amount of this remained occluded by HG. For that, the recovery of the flavonoids was performed as is described in Section 2.9. Another possibility to circumvent HG precipitation over the biocatalyst could be to control the size of the particles of Dacron, prior to the immobilization of enzyme, using large particles instead of the small ones.

According Grohmann et al. (2000), to obtain HG by acid hydrolysis of HES, incubation temperatures higher than 120°C are required, and mixtures of hesperidin, hesperetin 7-O-glucoside and the aglycone hesperetin are obtained. In addition, the reaction media must be treated with strong bases and afterward with organic solvents to separate the different chemical byproducts. As a consequence, the yields of HG generation ranged 20–42% (Grohmann et al., 2000) which is markedly lower than the obtained using both methodologies described in the current work.

HES was also assessed as a source of HG, despite its low solubility. For this, HES was dissolved at 150°C (4.3 mM) and the free enzymes were tested as biocatalysts at 50 °C. The obtained results indicated that the 3 enzymes were able to perform the conversion (data not shown), but the process was strongly limited by the precipitation of HES within the first hour of reaction. Therefore, the enzymatic hydrolysis of HES was not a good option to obtain HG at gram scale. However, it is noted that HES has been used at low concentrations (0.1–1.0 mM) to obtain HG via the enzymatic procedure, rendering mixtures of HES, HG and hesperetin, which had to be further purified by preparative chromatography (Lee et al., 2012).

Altogether, our results indicate that it is possible to obtain HG at gram scale with purity close to 100% and a total yield of 93% without having to perform long purification steps, and without using strong bases, organic solvents or preparative chromatography.

4. Conclusion

NEO is a good substrate for obtaining HG, mainly due its high water solubility. The four enzymatic preparation studied showed hydrolytic activity on both α-1,2 and α-1,6 terminal rhamnose residues. The immobilization of RAT by covalent bond onto azide-Dacron magnetized support was improved by activating the support with NaNO2 instead glutaraldehyde. The biocatalyst prepared shown good thermal stability and enzyme did not detach from the matrix upon washing. The two methods proposed to obtain HG at a laboratory-scale were easy to carry out and it is expected that these could be scaled for the production of HG at a larger scale.

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