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Grapefruit debittering by simultaneous limonin adsorption and naringin hydrolysis

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Citrus juices constitute one of the main fruit juices consumed around the world, playing a major role in the entire food industry as well. Even though citrus juices are usually slightly bitter in taste, consumers are very sensitive to bitterness and this has been a long-standing problem for industry that reduces the quality and commercial value of the products. There are two types of bitter compounds in citrus juices, flavonoids and limonoids. Naringin is the main flavonoid responsible for the “immediate” bitterness, while limonin is the main limonoid responsible for the “delayed” bitterness. The efficient removal of these compounds by their adsorption on resins without affecting the quality of the juice is complex, since chemical properties of limonin and naringin are different, resulting normally in a higher selectivity to limonin adsorption. Hydrolysis of naringin by the enzyme naringinase represents one of the best solutions for reducing the concentration of this bitter compound; however, its application at commercial scale is limited mainly by the cost and availability of the enzyme, and by the fact that its application does not affect the content of limonin of the juice.
The aim of the present investigation was the debittering of grapefruit juice by a simultaneous limonin adsorption and naringin hydrolysis using naringinase immobilized in heterofunctional agarose supports.
The supports butyl-glyoxyl agarose (BGA) and octyl-glyoxyl agarose (OGA) were prepared by simple periodate oxidation of the commercial butyl-agarose and octyl-agarose. Naringinase from *Aspergillus aculeatus*/*Aspergillus niger* (Novozyme®) was covalently immobilized in both supports through their glyoxyl groups. Biocatalysts were applied in grapefruit juice previously filtered and incubated at 30º C. The samples were analyzed to evaluate limonin, naringin, prunin, and naringenin concentrations via HPLC-DAD.
After 5 h of reaction, the use of naringinase immobilized in BGA and OGA resulted in a reduction of 36 and 33% of naringin and 57 and 21% of limonin, respectively. Naringin hydrolysis was confirmed by an increase of prunin concentration, no naringenin was detected. Enzyme-free supports were incubated in grapefruit juice showing that only limonin was adsorbed, proving that a simultaneous limonin adsorption and naringin hydrolysis was achieved when naringinase immobilized in heterofunctional supports were utilized.

1. Introduction

Taste is one of the sensory quality attributes that, together with color and flavor, determines food acceptance. Bitterness in citrus juices has been a long-standing problem that reduces the quality and commercial value of the product (Shaw *et al*., 2000; Mongkolkul *et al*., 2006).

There are two types of bitter compounds in citrus juices, limonoids and flavonoids. Limonoids are highly oxygenated triterpenes, classed as tetranorterpenoids. The main limonoid present in citrus juices is limonin, which is responsible for a “delayed” bitterness developed after juice extraction (Fellers, 1989; Puri *et al*., 1996). The intact fruit barely contains limonin; however, its non-bitter precursor, limonate-A-ring lactone (LARL) is present in cell cytoplasm in membranous sacs. When these sacs are ruptured during juice processing, the acidic pH of the juice gradually catalyzes closure of the ring of LARL forming the limonin (Puri

et al., 1996). The threshold for limonin in orange juice is affected by the pH, corresponding to 6.5 ppm at pH 3.8 (Guadagni *et al*., 1973). On the other hand, flavonoids are a large group of very different compounds sharing the common feature of phenol moieties. They are mainly present in citrus fruits as their glycosyl derivatives. The forms lacking sugar moieties (aglycone) occur less frequently owing to their lipophilic nature and hence their low solubility in water (Gattuso *et al*., 2007). Hesperetin and naringenin are the most common flavanones in fruits, and they are usually conjugated to a glucose-rhamnose disaccharide at the 7-position, typically rutinose or neohesperidose. Flavanone aglycones and rutinosides are tasteless, whereas flavanone neohesperidose conjugates such as naringin from grapefruit (*Citrus paradasi*) and neohesperidin from bitter orange (*Citrus aurantium*) are intensely bitter (Tomás-Barberán and Clifford, 2000). Narinigin is found in the membranes and albedo of the fruits and is extracted into the juice, giving it an “immediate” bitterness when their levels exceed 20 ppm (Fisher and Wheaton, 1976).

Bitterness due to flavonoids and limonoids poses a major problem for the citrus industry and without proper debittering technology, the profitable citrus industry cannot flourish (Singh *et al*., 2003). Due to the importance of reducing or removing bitterness in citrus juices below the threshold level for consumers acceptability, several physicochemical and biochemical strategies have been developed. Several investigations concerning strategies to reduce the bitterness of citrus juices have been carried out, including physicochemical and biochemical methodologies including: (i) removal of bitter compounds by their adsorption on different resins, (ii) chemical modification of pH, (iii) encapsulation of bitter compounds, (iv) hydrolysis of naringin by enzymes, and (v) microbial debittering.

Using adsorptive and/or ion-exchange resins are the preferred methods for the removal of bitter compounds due to easy handling and possibility of regeneration for long-term use. Several natural or synthetic hydrophobic and hydrophilic adsorbents have been tested. Current commercial debittering units (first installed in the U.S. in 1988) use styrene-divinylbenzene copolymer as the hydrophobic adsorbing resin (Shaw *et al*., 2000). The use of neutral adsorbents resins has shown a preferential adsorption of limonin over naringin, probably due to the greater hydrophobicity of the former, reporting the adsorption up to 65% of naringin and 85-95% of limonin present in citrus juice (Johnson and Chandler, 1982; Hernandez *et al.,* 1992; Ribeiro *et al.,* 2002). In the case of ion-exchange resins, the use of weakly basic anion exchange resins has been effectively used for reducing the concentration of bitter compounds and may also have an efficient function in the adjustment of taste equilibrium of the products since it also reduces the acidity of the juice (Couture and Rouseff, 1992; Kola *et al*., 2010). This is an important factor considering in increased demand for products free of sugar products.

Due to the several drawbacks associated with physicochemical treatments, the enzymatic conversion of bitter compounds has been investigated as an alternative process. Even though in the case of limonin it has been reported the use of the enzyme limonoate dehydrogenase for the oxidation of LARL to 17-dehydrolimonoate, a non-bitter derivate, their application at the acidic pH of fruit juices has been difficult due to its alkaline optimum pH (Puri *et al*., 2002). Due to this reason, the reduction of juice bitterness using enzymes is mainly based in the conversion of the naringin by the enzyme naringinase. Naringinase (EC 3.2.1.40) is a hydrolytic enzyme containing both ±-L-rhamnosidase and ²-D-glucosidase activities, which are located on two separate polypeptides (Puri, 2012). Firstly, ±-L-rhamnosidase hydrolyzes naringin into rhamnose and prunin (4,5,7-trihydroxy flavanone-7-glucoside), the prunin is then simultaneously converted into glucose and naringenin (4,5,7-trihydroxy flavanone) by the ²-D-glucosidase activity (Yusof *et al*. 1990). Prunin is 33% as bitter as naringin and its further hydrolysis reduces even more the bitter taste (Puri *et al*., 1996). Despite many attractive features of the enzymatic process some reasons for their limited application at present are the cost and availability of commercial enzymes, limonin content is not at all affected by naringinase treatment and the availability of neutral ion exchange resin technology for debittering and deacidifying the grapefruit juice at the same time (Puri and Chand Banerjee, 2000).

Physicochemical and biochemical approaches have been widely investigated highlighting the removal of limonin by adsorption in resins and the hydrolysis of naringin catalyzed by the enzyme naringinase. A single strategy that effectively combines both principles would offer the technical and economic advantage of reducing the number of operations required to obtain a citrus juice with a concentration of both bitter compounds under their threshold. In this investigation an attractive method for grapefruit juice debittering is proposed immobilizing naringinase in heterofunctional supports in order to favor a simultaneous adsorption of limonin.

2. Material and methods

2.1 Materials

Naringinase from *Aspergillus aculeatus*/*Aspergillus niger* Novozyme NZ 33 (Novozymes, Denmark), *p*-nitrophenyl-±-L-rhamnopyranoside (Rha-pNP), *p*-nitrophenyl-² -D-glucoside (Glc-pNP), Naringin (e95% HPLC), Limonin (>90% HPLC) were purchased from Sigma-Aldrich. Octyl Sepharose and Butyl Sepharose (GE Healthcar, EEUU), sodium metaperiodate, was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Grapefruit was purchased from the local market and handmade grapefruit juice was used for debittering test.

2.2 Analytical methods

Substrates and products were analyzed quantitatively by HPLC analysis (JASCO-DAD HPLC). Naringin, pruning, naringenin, and limonin were visualized and detected under UV light (210-280nm). Separation was performed on a C-18 (15 cm x 0,4 cm) analytical column, at a 0.5 mL min^{-1} flow rate, and at 35ºC (column oven temperature). The injection volume was 20 µL and components were separated using a gradient of acetonitrile in water.

2.3 Supports preparation

The supports were prepared by simple periodate oxidation of commercial butyl-agarose and octyl-agarose. The preparation of butyl-glyoxyl agarose (BGA) and octyl-glyoxyl agarose (OGA) was carried out as following: 10 g of commercial supports was washed and filtered five times with distilled water, then they were suspended in 50 mL of sodium periodate (10 mM) and gently stirred for 2 h at 25ºC, and finally then the supports were filtered and washed with distilled water. The non-consumed periodate was measured by titration of the filtrate with KI in saturated bicarbonate solution.

2.4 Naringinase immobilization

The immobilization of the enzyme on BGA and OGA was performed as follow: 1g of supports and 10 mL enzyme solution (3.5 mL naringinase and 6.5 mL bicarbonate buffer 100 mM, pH 10) were added and mixed in a closed flask under gentle agitation for 24 h at 24ºC. After that, the immobilized naringinase was separated using a paper filter and next washed with phosphate buffer (0.5 mM, pH 7.0) and distilled water.

2.5 Debittering of grapefruit juice

Debittering of grapefruit juice using naringinase immobilized in BGA and OGA was evaluated mixing 1 g of biocatalyst per 5 mL of juice previously filtered using a Whatman filter paper. The reaction was carried out in a flask covered from light, at 30ºC and stirred at 150 rpm. Samples of supernatant (200 µL) were withdrawn periodically, centrifuged and filtered with 0.45 µm pore diameter. Concentration of naringin, prunin, naringenin, and limonin was measured by HPLC.

Adsorption of limonin in BGA and OGA free of enzyme was also evaluated using the same conditions presented above but replacing the mass of biocatalysts for the supports free of enzyme.

3. Results and discussion

The biocatalysts immobilized in BGA and OGA were applied in grapefruit juice debittering showing in Figure 1 the kinetic of each reaction. In both cases, no naringenin was detected, indicating that until 5 h of reaction the prunin obtained after naringin hydrolysis was not further hydrolyzed by the ² -D-glucosidase activity of the immobilized enzyme. Naringin concentration decrease along reaction time and 33 and 36% of reduction was obtained after 5 h of reaction of BGA and OGA biocatalysts, respectively. On the other hand, prunin concentration increase along reaction as a consequence of the ±-L-rhamnosidase activity of both immobilized biocatalysts. In the case of limonin, its concentration decrease along reaction time and 57 and 21% of reduction was obtained after 5 h of reaction of BGA and OGA biocatalysts, respectively.

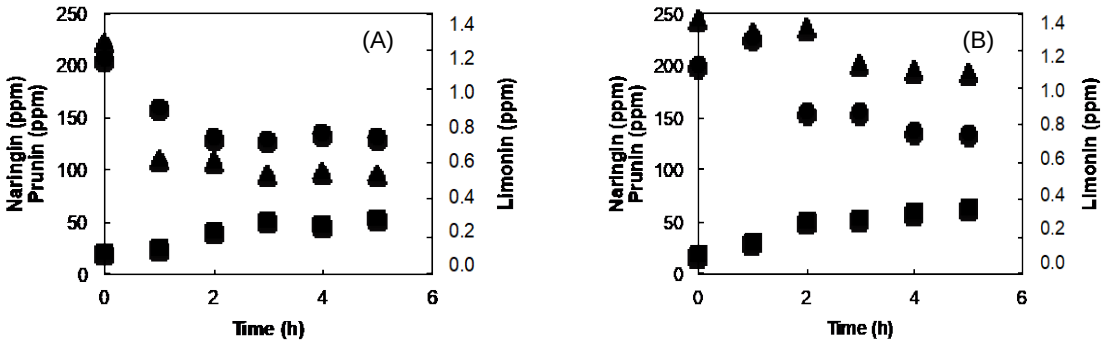

Figure 1. Kinetic of grapefruit debittering by naringinase immobilized in (A) BGA and (B) OGA.(▲) Naringin, (■) prunin, (•) limonin.

Enzyme-free supports were mixed with grapefruit juice showing in Figure 2 the variation of limonin and naringin concentration along 5 h of incubation. As may be observe, in both cases only limonin decrease indicating that the reduction of naringin in Figure 1 is caused only by the action of immobilized enzyme.

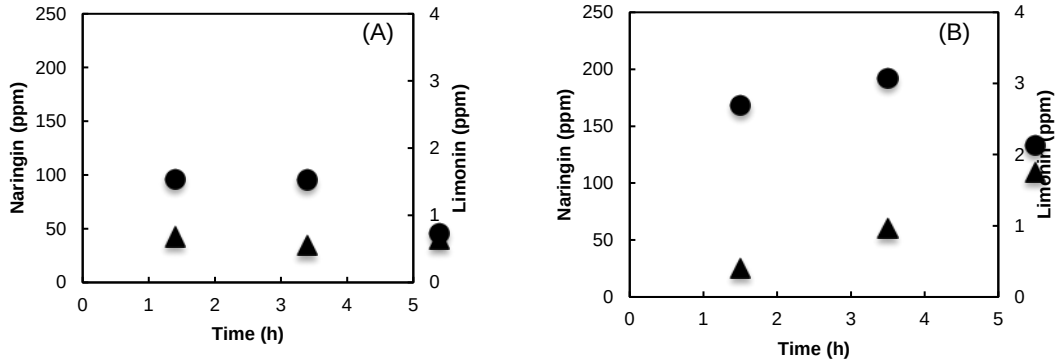

Figure 2. Variation of (▲) naringin, and (•) limonin along 5 h of incubation of supports (A) BGA and (B) OGA (free of enzyme) in grapefruit juice

4. Conclusions

Simultaneous limonin adsorption and naringin hydrolysis was achieved when naringinase immobilized in heterofunctional supports were utilized; therefore, reduction of grapefruit juice bitterness using a simple process that joins physical and biochemical approaches was possible. Improvement of immobilized biocatalyst properties (activity and stability) is being carried out in order to improve their performance in grapefruit juice debittering, including the evaluation of biocatalysts reuse.

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