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Evaluation of the Effect of High Pressure on Naringin Hydrolysis in Grapefruit Juice with Naringinase Immobilised in Calcium Alginate Beads

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Summary

The reduction of bitterness in citrus juices would increase their acceptance by the consumer. This reduction in grapefruit juices can be achieved as a result of an enzymatic process, with improved commercial value and maintenance of health properties. The use of a cheap, simple and effective immobilisation method combined with high pressure can be a key asset in the debittering of citrus juices. The aim of this study is the debittering of grapefruit juice under high pressure, with naringinase immobilized in calcium alginate beads. Naringinase, an α-rhamnopyranosidase, hydrolyzes naringin (a flavanone glycoside and primary bitter component in grapefruit juice) to naringenin, which is tasteless. High pressure can activate or inhibit enzymatic activities depending on the proteins and conditions. The hydrolysis of naringin was first evaluated in model solution (acetate buffer 0.02 M, pH=4.0) and then in grapefruit juice. In model solution, at 160 MPa and 37 °C, a 50 % increase in the concentration of reducing sugars was obtained when compared to the reaction at atmospheric pressure. The higher naringenin concentration (33 mg/L) was obtained at 54 °C under high pressure of 200 MPa, which corresponds to a naringin reduction of 72 % in model solution, while at atmospheric pressure (0.1 MPa), the naringin reduction was only 35 %. The decrease in naringin content can be directly correlated with the reduction in bitterness. From the concentration of residual naringin, the percentage of reduction in bitterness was evaluated. In grapefruit juice, a debittering of 75 % occurred with a pressure of 160 MPa at 37 °C for 20 minutes.

Key words: high pressure, grapefruit juice, immobilized naringinase, calcium alginate beads, naringin, naringenin

Introduction

In recent years, consumer awareness about the health-promoting ability of bitter flavonoids such as naringin has generated important interest in the processing of fruits, namely grapefruit. One of the major consumer objections to such products is the excessive bitterness. The main objective of the industry is to process fruits at the lowest possible cost, maintaining organoleptic quality and stability of the finished products, with increased

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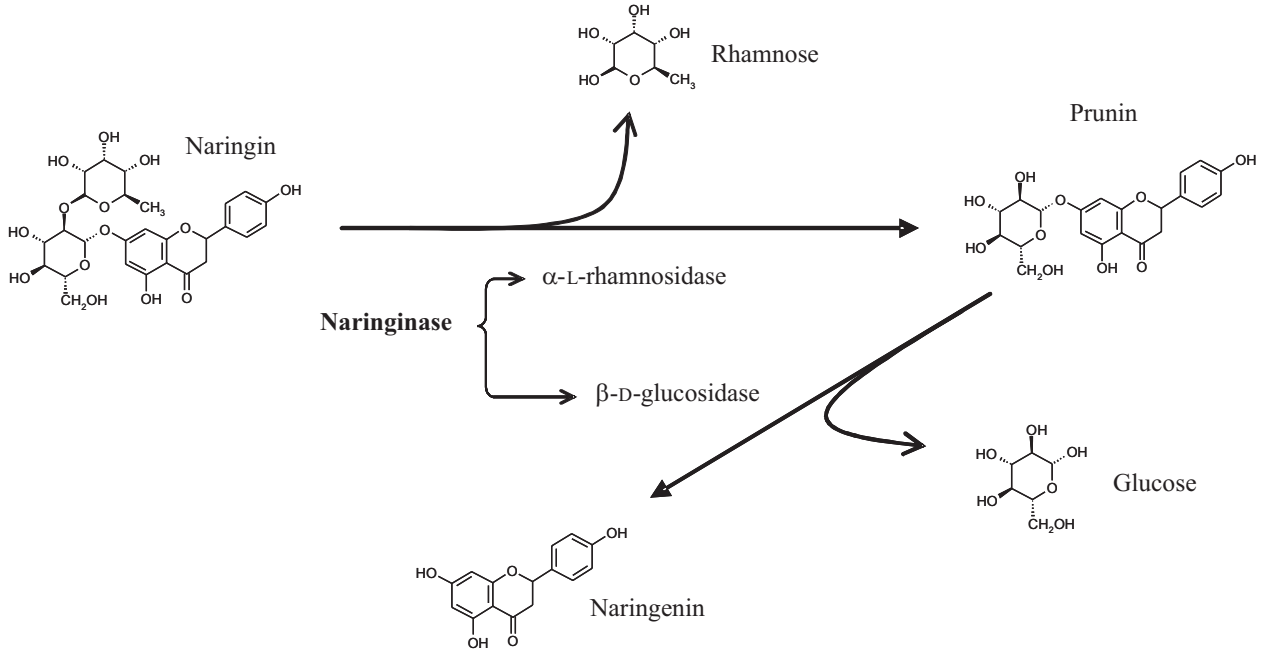
consumer acceptability. Citrus debittering using the enzyme naringinase is a possibility to overcome the bitterness. Naringinase expresses α-L-rhamnosidase and β-D--glucosidase activities. Naringin (4´,5,7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside), the principal bitter flavanone glycoside and the primary bitter component in grapefruit juice, can be hydrolysed by α-L-rhamnosidase into rhamnose and prunin (trihydroxyflavanone-7-glucoside) (Scheme 1). Prunin, with bitterness ratio of 1:3 to naringin can be further hydrolysed by the β-D-glucosidase into glucose and tasteless naringenin (4´,5,7-trihydroxyflavanone).

Flavonoids naringin and naringenin are functional chemicals with important properties in the fields of healthcare, food, and agriculture (*1,2*). These molecules have a great potential, especially in the food and pharmaceutical industries due to their recognized antioxidant, anti-inflammatory, antiulcer and hypocholesterolemic effects, among others. Both naringin and naringenin have similar medical applications, indicating that the biological activity is related to the aglycon moiety and it is not associated with the sugar residues. Prunin has antiviral activity, whereas naringenin has shown antioxidant, antimutagenic and anti-inflammatory activities, as well as antiproliferative effects and inhibition of aflotoxin B1--induced carcinogenesis, inhibiting the proliferation of breast cancer and delaying mammary tumorigenesis (*1,3*). Naringenin can also function as chemopreventive agent against a neurodegenerative disease such as Alzheimer's disease (*1,4*). Therefore, debittering with naringinase will not reduce the health promoting effects of grapefruit juice.

High pressure may influence the activity of an enzyme-catalyzed reaction in two ways: (*i*) enzyme changes or (*ii*) modification in the reaction mechanism (*5*). High pressure can in some cases be used to modulate the activity, selectivity or stability of several enzymes, leading to potential new applications (*5,6*). Usually, high pressure is not used to increase yields in biocatalytic processes. One of the main reasons is the instability of enzymes at elevated pressures. Studies on high pressure enzymatic reactions traditionally focus on the behaviour of the structure of the enzyme, and on its folding and unfolding (*7,8*). Therefore, the investigation of high pressure as a fundamental system parameter is of interest. Another important application is high pressure processing, an innovative technique proposed for the microbiological stabilization of foods instead of heat treatment, which appears as an interesting alternative to temperature treatment for preservation and sterilization in the food and pharmaceutical industries (*8,9*). High hydrostatic pressure has been found to inactivate microorganisms and denature several enzymes without affecting significantly low molecular mass food compounds, such as vitamins, pigments, flavouring agents and other compounds related to sensory nutritional and health qualities of the product (*8,9*).

Immobilization of biocatalysts has many advantages in large-scale processing, namely biocatalyst reuse, easy separation of biocatalyst from reaction media, continuous mode operation, prevention of contamination of the processed product, higher enzyme concentrations, higher superficial area for reaction, among others. Several authors have used immobilised naringinase in naringin hydrolysis (*10–15*). Considering the advantages of biocatalyst immobilization, the use of a cheap and simple effective immobilisation method combined with the potential of high pressure can provide a key asset if an experimental set-up amenable for scale-up is envisaged. The main goal of this work is to study the effect of high pressure on the enzymatic debittering of grapefruit juice with naringinase immobilized in calcium alginate beads.



Scheme 1. Hydrolysis of naringin into prunin, rhamnose, glucose and naringenin by naringinase containing α-L-rhamnosidase and β-D-glucosidase activities

Materials and Methods

Materials

Naringin, naringenin and naringinase (CAS Number 9068-31-9) were from Sigma Aldrich (USA). The enzyme was kept at 0 °C. All other chemicals were of analytical grade and obtained from various sources. Grapefruits used in this work were bought in a local supermarket.

Analytical methods

Naringin, prunin and naringenin were analyzed by high-performance liquid chromatography (HPLC). The HPLC system (Hitachi, USA) consisted of one high pressure solvent delivery pump (Model L-7100), a variable wavelength UV-VIS detector (Model L-7400), a manual injector with a 20-μL loop (Rheodyne) and an integrator (Model D-2500 Chromato-Integrator). Separation was performed on an RP-18 analytical column, at 280 nm. Linear gradient A (water) and B (acetonitrile) were used. The gradient elution was programmed as follows: 0–15 min, 23 % B; 15–20 min, 70 % B; 20–21 min, 23 % B (*16*).

Reducing sugars were quantified by the 2,4-dinitrosalicylic acid (DNS) method (*17*). Standardization was obtained with different concentrations of an equimolar mixture of D-glucose and D-rhamnose. Any contribution of thermal or pressure hydrolysis was eliminated as no reducing sugars were observed after incubating the naringin solution at different temperatures and pressures.

Quantification of protein (naringinase) was performed by the Bradford method.

High pressure apparatus and operation

High pressure experiments were carried out in a stainless steel vessel immersed in a thermostatic water bath. Pressure and temperature measurements were maintained constant within ±2 MPa and ±0.1 °C, respectively.

The pressurization fluid was hydraulic oil (Enerpac HF 95 Y) and the required pressure was achieved with a 400-MPa manual pump (Enerpac, model P228) and controlled using a pressure gauge (Budenberg Gauge Co. Ltd, UK).

The high pressure enzymatic reactions were carried out on cylindrical cells with a volume of 10 mL. Three cells were put simultaneously inside the high pressure vessel. The pressure was increased steadily in 1 to 3 min, depending on the pressure required, and maintained for different periods of time. Subsequently, the pressure was released within 1 min, the reaction was immediately stopped lowering the temperature of the solutions above 0 °C and the samples were frozen (–18 °C) until naringin, naringenin, reducing sugar and protein assays were carried out. Each data point was obtained in triplicate.

Naringinase immobilization

Alginic acid sodium from brown algae (in a polymeric solution 2 %, *m/V*) was obtained from Fluka.

Entrapment of naringinase in calcium alginate beads was carried out as follows: a certain volume of the naringinase solution, in 0.02 M acetate buffer, pH=4.0, was added to a 4 % sodium alginate solution in order to obtain the desired concentration of naringinase and sodium alginate. This suspension was prepared by a simple mixing step and then it was added to a gently stirred 4 % calcium chloride solution, using a peristaltic pump (Gilson, Miniplus 2) through a 1-mm diameter needle. The gelling was allowed to proceed for 30 min at 4 °C. Beads were separated by filtration, rinsed with acetate buffer (0.02 M, pH=4.0) and used for bioconversion trials.

Naringin hydrolysis

High pressure naringin bioconversion studies were carried out in standard solutions of naringin (0.02 M acetate buffer, pH=4.0) and grapefruit juice with immobilized naringinase in calcium alginate beads (2 mm). The reaction started by adding a given amount of immobilized naringinase in calcium alginate beads to the naringin solution, in 0.02 M acetate buffer, pH=4.0, in a proportion (by volume) of 4 (reaction media) to 1 (immobilized enzyme). Bioconversion trials in acetate buffer (naringin concentration of 500 mg/L) were performed, with a naringinase concentration of 500 mg/$L_{reaction\ media}$: (*i*) under different pressures (0.1, 120, 160 and 200 MPa), (*ii*) at different temperatures (15, 20, 37, 54 and 61 °C).

Bioconversion experiments in grapefruit juice were carried out at 160 MPa and 37 °C, with naringinase (500 mg/L_{juice}) immobilized in calcium alginate (2 %) beads (2 mm). Before use in the experiments, grapefruit juice was centrifuged at 8000 rpm for 15 min and used in bioconversion runs.

Results and Discussion

In the present work, the debittering of grapefruit juice was carried out with naringinase immobilized in calcium alginate (2 %) beads. The hydrolysis of naringin was first evaluated in model solution (acetate buffer 0.02 M, pH=4.0) and then in grapefruit juice. In the model solution, the naringin conversion was determined at different pressures (120, 160 and 200 MPa) and temperatures (37, 54 and 61 °C) for 30 min. Naringin concentration of 500 mg/L and naringinase concentration of 500 mg/$L_{aqueous\ medium}$ were used. The naringin conversion [(naringin$_{initial}$ – naringin$_{time=t}$)/naringin$_{initial}$], obtained at pressures of 160 and 200 MPa at 37, 54 and 61 °C, increased during the first 20 min of the reaction time (Fig. 1). Controls were maintained for each set of enzyme studies.

As mentioned in the introduction, naringin (4´,5,7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside) is hydrolysed by α-L-rhamnosidase activity of naringinase into rhamnose and prunin (trihydroxyflavonone-7-glucoside) (Scheme 1), which is further hydrolysed by the β-D-glucosidase activity into glucose and tasteless naringenin (4´,5,7-trihydroxyflavanone). In this work, besides naringin, we quantified the reducing sugars (rhamnose and glucose) and naringenin formation. Prunin was identified, but unable to quantify as we do not have standards.

In naringin hydrolysis, the concentration of reducing sugars formed at 160 MPa and 37 °C increased 50 %, in comparison with the concentration obtained in the

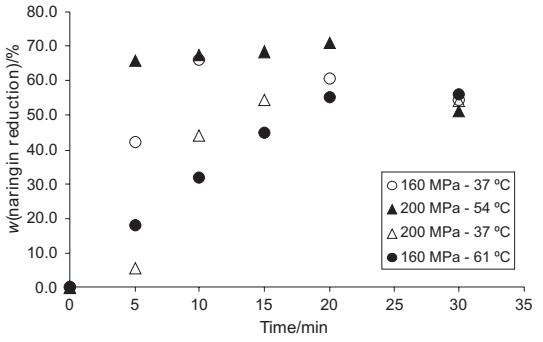


Fig. 1. Effect of reaction time on naringin reduction with naringinase immobilized in calcium alginate beads (500 mg/L of naringin, in acetate buffer, pH=4.0), at 160 and 200 MPa and 37, 61 and 54 °C

hydrolysis carried out at atmospheric pressure (Fig. 2). A naringin reduction of 35 % was obtained at atmospheric pressure (0.1 MPa).

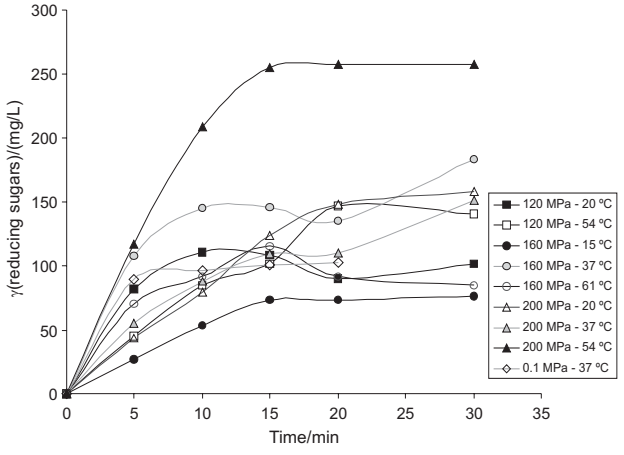


Fig. 2. Effect of reaction time on the formation of reducing sugars (rhamnose and glucose) formation at different pressures (0.1 to 200 MPa) and temperatures (15 to 61 °C). Bioconversion runs were carried out with immobilized mass of naringinase calcium alginate beads per volume of reaction media (500 mg/L) and 500 mg/L of naringin in acetate buffer, pH=4.0

When naringin hydrolysis was carried out at the pressures of 120, 160 and 200 MPa, and different temperatures, the reducing sugar formation increased with the increase of temperature from 15 to 54 °C. The maximum reducing sugar concentration was obtained at 200 MPa and 54 °C (257.5 mg/L), which is almost twofold of that at 37 °C (110 mg/L) (Fig. 2). The concentration of naringenin increased with pressure from 120 to 200 MPa and temperature from 15 to 61 °C.

In naringin hydrolysis, in model solution (0.02 M acetate buffer, pH=4.0), with naringinase immobilised in calcium alginate beads, the highest naringin conversion (72 %) was obtained at 200 MPa and 54 °C during 20 min, with the formation of 33 mg/L of naringenin and 257.5 mg/L of reducing sugars (Fig. 3).

The decrease in naringin content can be directly correlated with the reduction in bitterness. From the concentration of residual naringin, the percentage of the reduction of bitterness was evaluated.

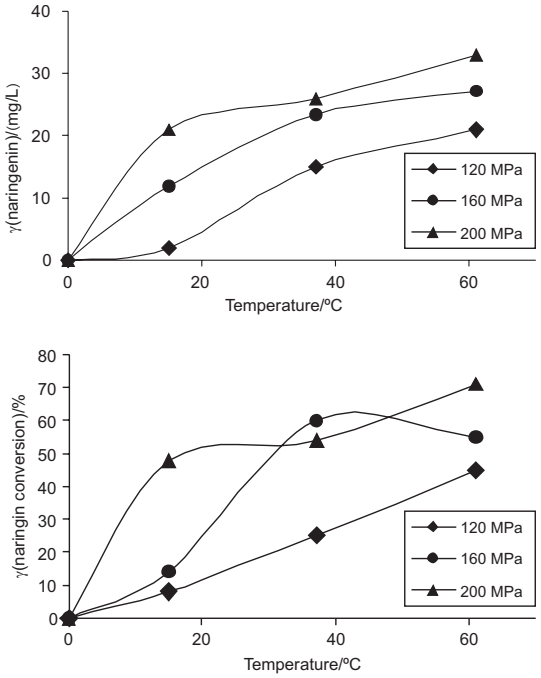


Fig. 3. Effect of temperature on naringenin formation and naringin conversion at 120, 160 and 200 MPa. Bioconversion runs were carried out with immobilized mass of naringinase calcium alginate beads per volume of reaction media (500 mg/L) and 500 mg/L of naringin in acetate buffer 0.02 M, pH=4.0

The enzymatic reaction with naringinase immobilized in calcium alginate beads (500 mg/L_{juice}) was carried out in grapefruit juice at 0.1 and 160 MPa and 37 °C. The initial naringin concentration in grapefruit juice was 515 mg/L. After 20 minutes of enzymatic reaction, a 75 % reduction in naringin concentration was observed, with the formation of naringenin (60 mg/L). The formation of reducing sugars under high pressure (160 MPa) corresponds to a 3-fold increase when compared to the formation at atmospheric pressure (Fig. 4).

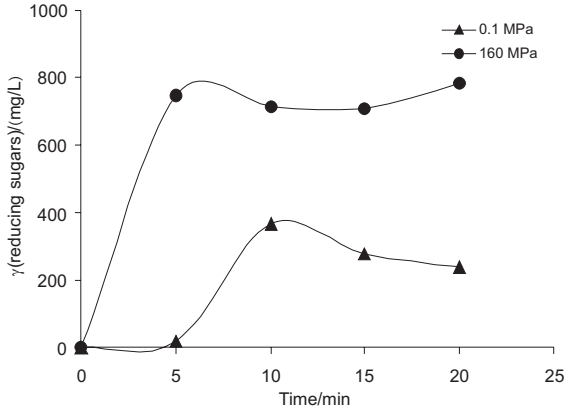


Fig. 4. Effect of reaction time on the formation of reducing sugars (rhamnose and glucose) formation at 0.1 and 160 MPa and 37 °C. Bioconversion runs were carried out in grapefruit juice with immobilized mass of naringinase calcium alginate beads per volume of reaction media (500 mg/L)

Some bitterness in grapefruit juice is acceptable to consumers, as it contributes to the characteristic taste and flavour. In our research a reduction in naringin of 75 % was obtained with immobilized naringinase in calcium alginate beads, in naringin hydrolysis at 160 MPa and 37 °C, which makes the juice acceptable to consumers. In order to optimize this technique of debittering as a sterilisation/pasteurization process, microbiological studies are being carried out in which grapefruit juice is submitted to different high pressures and temperatures in different intervals.

Conclusions

In grapefruit juice a debittering of about of 75 % occurred under the pressure of 160 MPa at 37 °C during 20 min after naringin hydrolysis by naringinase immobilized in calcium alginate beads. These are promising results for a future application of enzymatic hydrolysis of naringin under high pressure, with immobilized naringinase in calcium alginate beads, in grapefruit juice industry with the main goal of debittering while maintaining organoleptic characteristics of the juice. Future experiments will optimize this technique for the removal of the bitter taste and as high pressure processing for pasteurization and sterilization applications.

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