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# High pressure-temperature effects on enzymatic activity: Naringin bioconversion

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**Abstract**

Initially exploited in chemistry and physics, high-pressure technology has gained importance in various fields.

Flavonoids, namely naringin and naringenin, from citrus, are functional chemicals with important properties in the fields of health-care, food and agriculture. Naringin, the principal bitter flavonone glycoside and the primary bitter component in grapefruit juice, can be hydrolysed by naringinase into tasteless naringenin.

The temperature of 303 K was ideal for maximizing the activity of the naringinase at 160 MPa when compared to atmospheric pressure. The pressure had a positive effect on the reaction rate, with a value of $-15.0 \pm 1.8\ cm^3\,mol^{-1}$ for the activation volume.

Kinetic parameters, $k_M$ and $V_{max}$ for naringinase were evaluated, the maximum initial rate at 160 MPa ($V_{max} = 2.7\ mM\,min^{-1}$) was higher than that at atmospheric pressure ($V_{max} = 0.06\ mM\,min^{-1}$). This is a promising result for future application of enzymatic hydrolysis of naringin at high pressure, in the citrus juice industry.


*Keywords:* Naringinase; Naringin; High-pressure; Temperature; Kinetics parameters

## 1. Introduction

Initially exploited in chemistry and physics, high pressure technology has gained importance in various fields related to biology, namely, the use of high pressure as an alternative to temperature treatment in the food and pharmaceutical industries, and as a tool to perturb biochemical systems establishing relationships between structure and function. High pressure processing is an innovative technique proposed for the microbiological stabilization of foods instead of heat-treatment, for preservation and sterilization in the food and pharmaceutical industries (Mozhaev, Heremans, Frank, Mansson, & Balny, 1996). High hydrostatic pressure has been found to inactivate microorganisms and to denature several enzymes without greatly affecting low molecular weight food compounds, such as vitamins, pigments, flavouring agents and other compounds related to sensory, -nutritional, - and health-related qualities of the product.

In the field of enzymology, high pressure can be used to modulate both the stability and activity of several enzymes, leading to potential applications (Heremans & Smeller, 1998; Okamoto, Hayashi, Enomoto, Kaminogawa, & Yamauchi, 1991). Pressure can modify the catalytic behaviour of enzymes by changing the rate-limiting step or modulating the selectivity of the enzyme (Okamoto et al., 1991). It was found that pressure could either activate or inhibit enzymatic activities, depending on the proteins and conditions. These results stress the importance of investigating the influence of high pressure on a wide range of enzyme systems. In this work, the influence of high pressure on naringinase activity and stability was investigated. Naringinase has become biotechnologically important,

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due to its role in debittering citrus fruit juices, in the manufacture of rhamnose, and in the preparation of prunin and naringenin. Naringinase contains α-L-rhamnosidase and β-D-glucosidase activities. The available information throws little light on the molecular mechanism of naringinase action. A full understanding of the enzymology of naringinase is essential for its major applications. Puri and Karla (2005) purified an extracelular naringinase from *Aspergillus niger* 1344, a heterodimer of 168 kDa and reported molecular masses of naringinases (from different sources) to range from 70 to 240 kDa. Some of these naringinases comprise two identical subunits, namely the enzyme produced by fermentation of *Penicillium* DSM 6825 with a molecular weight of 60–100 kDa and a specified N-terminal sequence (Puri & Banerjee, 2000). Recently, the gene of α-L-rhamnosidase has been cloned and expressed with marked activity in *Escherichia coli* (Zverlov et al., 2000). The recombinant α-L-rhamnosidase, in naringinase provides an economical and easily available source of this enzyme with important impact in industrial debittering of citrus juices and in the pharmaceutical industry.

A great number of antioxidants are naturally present in citrus juice, being responsible for the potential protective action of citrus juice against certain degenerative diseases (Polydera, Stoforos, & Taoukis, 2005). Flavonoids, namely naringin and naringenin, from citrus, are functional chemicals with important properties in the fields of healthcare, food and agriculture. Naringin, the principal bitter flavonone glycoside and the primary bitter component in grapefruit juice, can be hydrolysed by α-L-rhamnosidase activity of naringinase into rhamnose and prunin, (one third of the bitterness of naringin) which can be further hydrolysed by the β-D-glucosidase component of naringinase, into glucose and tasteless naringenin (Fig. 1). This product of the enzymatic hydrolysis of naringin may be potentially useful as a pharmacological agent, as an anticancer agent, in the treatment or prevention of atherosclerosis, with a number of antiatherogenic effects, such as antioxidant, antithrombotic and vasodilator activities (Chen, Shen, & Lin, 2003). Therefore, the hydrolysis of naringin is still an interesting application. The reduction in bitterness, as a result of the enzymatic process, controlling the quality and improving commercial value of grapefruit and other citrus juices, as well as the maintenance of health properties, increases acceptance by the consumer. Adsorption techniques and enzymatic hydrolysis have been reported to achieve this goal (Prakash, Singhal, & Kulkarni, 2002; Ribeiro, Silveira, & Ferreira-Dias, 2002; Puri & Banerjee, 2000). Due to the loss of acidity, sweetness, flavour and turbidity, as well as poor efficiency in adsorption debittering, enzymatic hydrolysis appears as one promising technique with industrial application (Chien, Sheu, & Shyu, 2001).

The aim of this work was to study the effect of high-pressure on the activity of naringinase. Therefore, the kinetic parameters of naringinase in the hydrolysis of naringin were evaluated at high-pressures under different experimental conditions in model-solutions (Na acetate buffer, 0.02 M, pH 4).

## 2. Materials and methods

### 2.1. Materials

Naringin and naringinase (CAS Number 9068-31-9) were from Sigma Aldrich. The enzyme was kept at 273 K. All other chemicals were of analytical grade and obtained from various sources.

### 2.2. Analytical methods

Reducing sugars were quantified by the 2,4-dinitrosalicylic acid (DNS) method (Miller, 1959). 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 incu-

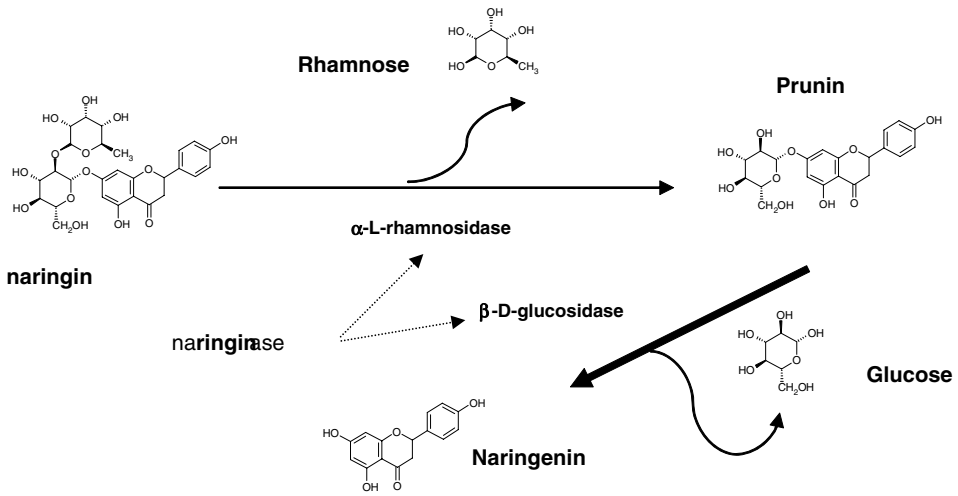


Fig. 1. Hydrolysis of naringin into prunin, rhamnose, naringenin and glucose by naringinase containing α-L-rhamnosidase and β-D-glucosidase activities.

bating the naringin solution at different temperatures and pressures.

Quantification of protein (naringinase) was performed by the Bradford method. Naringin was analyzed by spectrophotometry at 280 nm.

### 2.3. High-pressure apparatus and operation

High-pressure experiments were carried out in a stainless-steel vessel immersed in a thermostatic water bath, according to the sketch in Fig. 2. Pressure measurements were better than ±2 MPa and temperature was maintained constant within ±0.1 K

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. Limited).

Cylindrical glass cells with a volume of 15 $cm^3$ were used in high-pressure experiments (Fig. 2).

### 2.4. Naringin hydrolysis

#### 2.4.1. High pressure

Naringin bioconversion studies were carried out in standard solutions of naringin (acetate buffer, 0.02 M, pH 4.0), at different pressures, namely, 0.1, 40, 80, 120, 160 and 200 MPa. The high pressure enzymatic reactions were carried out in cylindrical cells. Three cells were simultaneously put inside the high pressure vessel, partially filled with hydraulic oil, and the vessel was sealed by screwing a steel cap. 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 solutions above 273 K and the samples were frozen ($T < 253$ K) until the enzymatic activity assays were carried out. Each data point was obtained in triplicate and required a new experiment because depressurization was necessary at the end of the incubation period under high pressure.

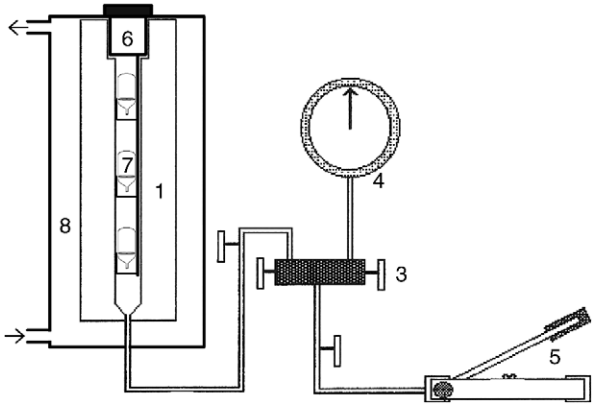


Fig. 2. High pressure apparatus. (1) high pressure vessel; (2) steel pipe; (3) valve; (4) pressure gauge; (5) manual pump; (6) vessel cap; (7) reaction cell; (8) thermostatic bath.

#### 2.4.2. Kinetic parameters

Initial rates of naringin conversion (activity of naringinase) were calculated by linear regression on the first datapoints during the initial 20 min of reaction time (reducing sugars concentration *versus* time). A unit of enzyme was defined as the amount of enzyme which liberated 1 μmol of glucose in 1 min at 303 K and pH 4.0.

The fit of the Michaelis–Menten model [$V = (S\ V_{max})/(S + K_M)$] [$V$–initial rate (mM $min^{-1}$), $S$–substrate concentration (mM), $V_{max}$–maximum initial rate (mM $min^{-1}$), $K_M$–Michaelis–Menten constant (mM)] to experimental data was carried out using a non-linear curve-fit programme in Excel for Windows, version 8.0 SR2, by minimizing the residual sum of squares between the experimental data points and the estimated values by the model. The kinetic parameters were also estimated by linear regression, using the Lineweaver–Burk plot: $[1/V = 1/V_{max} + (K_M/V_{max})1/S]$.

#### 2.4.3. Activation volume

The activation volume, $\Delta V^{\neq}$, is related to the dependence of constant reaction rates, $k$, on pressure, $p$, by $\Delta V^{\neq} = -RT(\partial \ln k/\partial p)_T$, where $R$ is the gas constant and $T$ is the absolute temperature in $K$.

In this study, $\Delta V^{\neq}$ was obtained from the slope, $b$, of the plot, based on the equation $\ln v = a + bp$, where $v$ is the reaction rate in mM $min^{-1}$, $p$ the pressure expressed in MPa and $b$ corresponds to $(-\Delta V^{\neq}/RT)$ (Northrop, 2002).

## 3. Results and discussion

Pressure is able to affect protein structure, at the secondary, tertiary and quaternary levels (Lullien-Pellerin & Balny, 2002). In general, lower pressures induce reversible changes, whilst pressures above 500 MPa can denature proteins, in most cases irreversibly, due to chemical modifications or aggregation. Moderated pressures ($p < 150$ MPa) are able to affect the quaternary structure of a protein due to changes of hydrophobic interactions that maintain this structure of the proteins (Northrop, 2002), also, dissociation of oligomeric proteins is favoured. Significant tertiary structure changes can be observed beyond 200 MPa, whereas reversible unfolding of small and monomeric proteins occurs at higher pressure (400–800 MPa). The secondary structure of the proteins, alone, is normally affected by pressures above 300 MPa. The modification of the secondary structure causes reversible denaturation of the enzyme, depending on the rate of compression and on the extent of structural re-arrangements (Mohana-Borges, Silva, Ruiz-Sanz, & Prat-Gay, 1999). In preliminary work, the effect of pressure, from 0.1 to 200 MPa, on the enzyme, naringinase, was investigated. After having been submitted to a pressure of 200 MPa during 30 min, the concentration of the protein, naringinase, remained constant with

maintenance of its activity. This was an important conclusion of this work.

In order to study the effect of pressure on naringinase activity, the naringin hydrolysis was carried out at different pressures and at atmospheric pressure (0.1 MPa) as a control, to compare high pressure experiments. The results are shown in Fig. 3. In fact, at a pressure of 40 MPa, the initial rate was lower than at atmospheric pressure while, at 80 and 160 MPa, the activity was slightly higher. In Fig. 4, the naringinase relative activity, i.e. [(naringin hydrolysed at different pressure)/(naringin hydrolysed at atmospheric pressure)], is patent. After 15 min, a decrease (about 20%) in naringin hydrolysis was observed at 40 MPa while at 80 and 160 MPa an increase of 5% and 20% was observed, respectively.

The influence of temperature (298–323 K) on the activity of naringinase was studied at 0.1 and 160 MPa (Fig. 5A and B). The bioconversion studies, in acetate buffer, pH 4, were conducted during 90 min. No significant variation in the concentration of reducing sugars was detected after thirty minutes of reaction. The rate curves, Figs. 5A and B, at the various temperatures, respectively, at 0.1 MPa and 160 MPa, exhibit a rapid initial reaction rate in less than 30 min, followed by a relatively steep approach to equilibrium. The biocatalyst at high pressure (160 MPa) presents a higher activity over a wide range of temperature than at 0.1 MPa, with a maximum at 303 K, almost two-fold (Table 1). This means that, if the main goal is to maximize the activity of naringinase at 160 MPa in comparison to atmospheric pressure, the optimum temperature is 303 K. Future experiments were carried out at 160 MPa and 303 K.

The effect of naringin concentration on naringinase activity, at 0.1 and 160 MPa, and 303 K, was evaluated, in order to calculate the kinetics parameters. The parameters estimated, through the non-linear regression method of

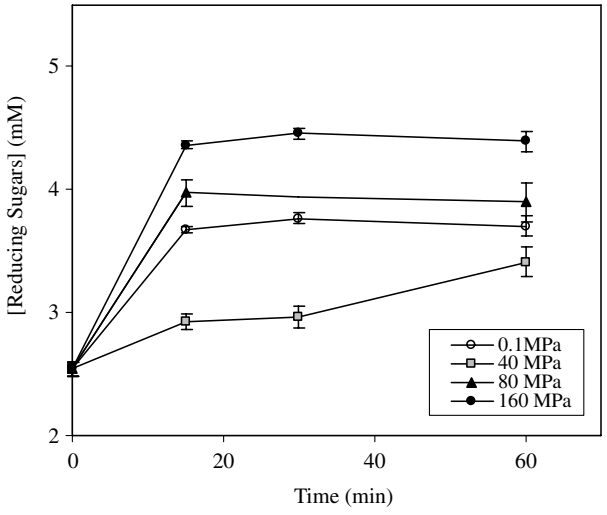


Fig. 3. Pressure effect on naringinase activity. Bioconversion runs were carried out at 303 K, in acetate buffer, pH 4.0; [naringinase] = 250 mg dm$^{-3}$; [naringin] = 1.23 mM.

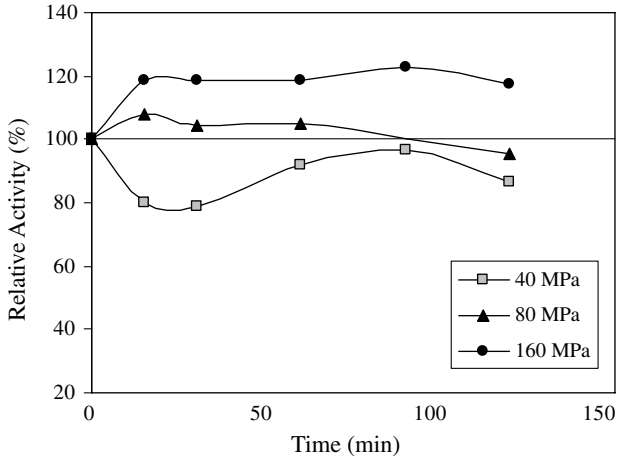


Fig. 4. Relative activity of naringinase at different pressure, at 303 K, in acetate buffer, pH 4.0.

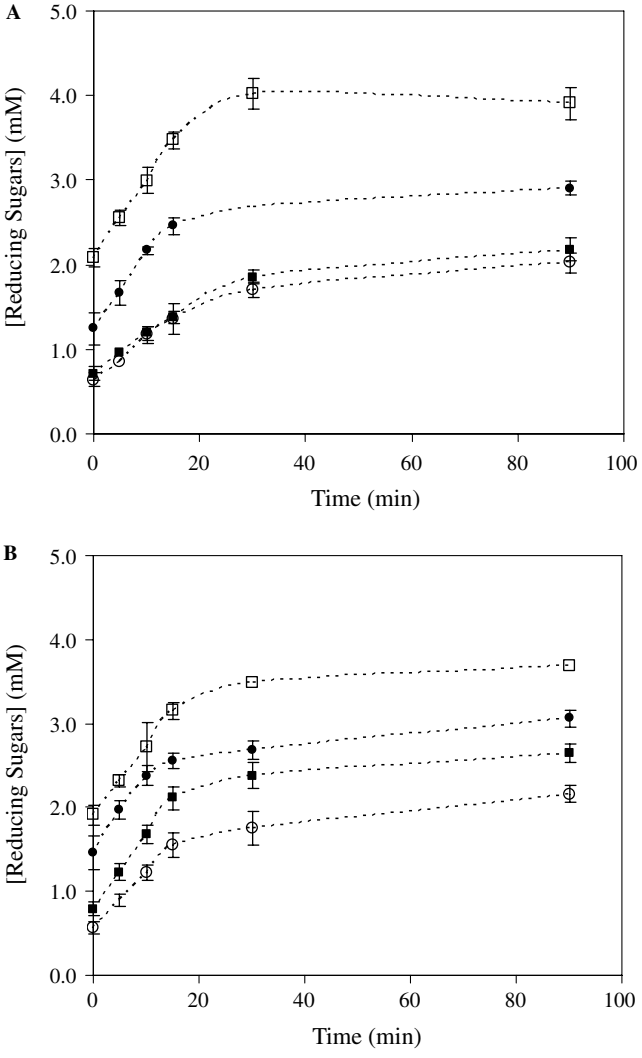


Fig. 5. Temperature effect on naringinase activity at 0.1 MPa (**A**) and 160 MPa (**B**); [naringinase] = 250 mg dm$^{-3}$ and [naringin] = 1.23 mM. (○ 298.15 K; ■ 303.15 K; ● 308.15 K; □ 313.15 K). Initial rates of naringin conversion (activity of naringinase) were calculated by linear regression on the first data-points.

Table 1
Initial rate of the hydrolysis of naringin (1.23 mM) by naringinase (250 mg dm$^{-3}$) at atmospheric pressure and at 160 MPa, pH 4.0

| Temperature (K) | Initial rate/ mM min$^{-1}$ 10$^{-4}$ | | $V$ (160 MPa)/$V$ (0.1 MPa) |
|---|---|---|---|
| | 160 MPa | 0.1 MPa | |
| 298 | 606 ± 2 | 484 ± 40 | 1.25 |
| 303 | 882 ± 2 | 455 ± 25 | **1.94** |
| 313 | 914 ± 67 | 828 ± 58 | 1.10 |
| 323 | 831 ± 8 | 722 ± 12 | 1.15 |

the Michaelis–Menten model, yield a curve that well describes the experimental data over the range of naringin concentrations. The Lineaweaver–Burk linearization leads to poorly accurate assessments, at high pressure, 160 MPa (Table 2).

The estimated parameters, by the non-linear regression method, were, at 0.1 MPa, $V_{max} = 0.063$ mM min$^{-1}$ and $K_M = 0.55$ mM, (Table 2). These parameters describe saturation enzyme kinetics within the studied concentration range, e.g. at conditions for which the reaction rate is above one half of the maximum theoretical reaction rate.

At 160 MPa, the estimated kinetics parameters were, $V_{max} = 2.68$ mM min$^{-1}$ and $K_M = 43.3$ mM (Table 2). These results demonstrate that, at high pressure, 160 MPa, most experiments were carried out below $K_M$. At these substrate concentrations, the initial reaction rate is approximately proportional to the substrate concentration, corresponding to first-order kinetics. However it is not feasible to work at higher naringin concentrations, due to the naringin solubility limit, 1.5 mM, in acetate buffer, at 303 K. The higher maximum reaction rate (3-fold, with the higher naringin concentration tested, due to the naringin solubility limit), at high pressure, is a good and encouraging result for a future application of enzymatic hydrolysis of naringin at high pressure in the citrus juice industry, with the main goal of debittering, and maintaining the juice organoleptic characteristics.

The behaviour of proteins under high pressure is governed by Le Chatelier's principle (Northrop, 2002). Depending on the volume changes measured for the major interactions in proteins, electrostatic and hydrophobic interactions are the main targets of pressure (Balny, 2004).

The pressure had a positive effect on reaction rates, indicating that the catalytic process involves a negative activation volume $\Delta V^{\neq}$. This was confirmed with the value of $-15.0 \pm 1.8$ cm$^3$ mol$^{-1}$ for the $\Delta V^{\neq}$, calculated from the slope of $\ln v$ versus $p$ (Fig. 6). This experimental value

Table 2
Kinetic parameters, $V_{max}$ and $K_M$, in acetate buffer, pH 4.0, at 303 K

| Pressure (MPa) | $V_{max}$ (mM/min) | | $K_M$ (mM) | |
|---|---|---|---|---|
| | Lineweaver–Burk plot | Non-linear regression | Lineweaver–Burk plot | Non-linear regression |
| 1 | 0.065 | 0.063 | 0.597 | 0.55 |
| 160 | (a) | 2.68 | (a) | 43.3 |

(a) Unable to calculate.

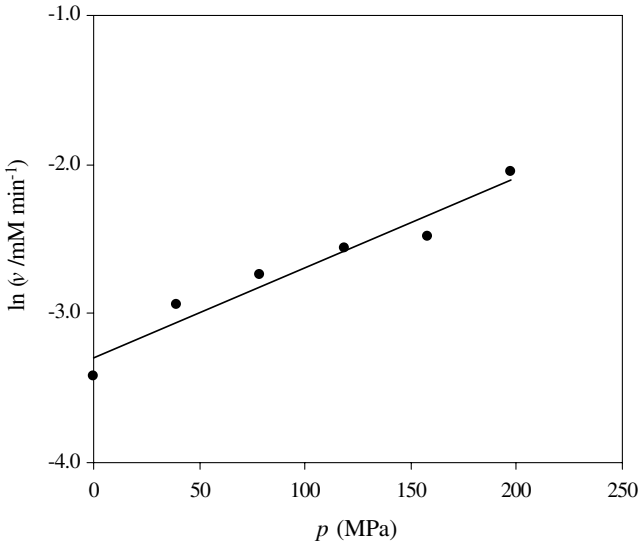


Fig. 6. Effect of pressure on naringinase activity, at 303 K, pH 4.0, in acetate buffer, [naringinase] = 250 mg dm$^{-3}$ and [naringin] = 1.23 mM. Activation volume $\Delta V^{\neq}$ was calculated from the slope ($-\Delta V^{\neq}/RT$).

can be compared to that of $-52$ cm$^3$ mol$^{-1}$ found for β-galactosidase (pressure range 50–150 MPa) (Cavaille-Lefebvre & Combes, 1998), with $-60$ cm$^3$ mol$^{-1}$ found for invertase in the pressure range 500–650 MPa (Cavaille & Combes, 1995) and $-113$ cm$^3$ mol$^{-1}$ for bovine liver glutamate dehydrogenase (pressure range of 180–280 MPa) (Fukushima, Matsumoto, Okawawuchi, Inoue, & Shimozawa, 1986). These authors associated this volume change with conformational changes and hydration modifications and it can represent the hyperbaric stability of the enzyme (Cavaille & Combes, 1995). According to Cavaille-Lefebvre and Combes (1998) for $\Delta V^{\neq}$ values obtained in the same pressure range, the more that $\Delta V^{\neq}$ is negative, the more is the catalytic reaction sensitive to high pressure.

## 4. Conclusions

The temperature of 303 K is ideal for maximizing the activity of the naringinase at 160 MPa when compared to atmospheric pressure. The maximum activity of naringinase at 160 MPa is higher than at atmospheric pressure. The pressure had a positive effect on reaction rates, with a value of $-15.0 \pm 1.8$ cm$^3$ mol$^{-1}$ for the activation volume. Michaelis–Menten kinetics describe the best fit for the experimental data, where the use of a non-linear regression method proved more accurate in the estimation of the kinetic parameters than did the Lineaweaver–Burk linearization. A higher maximum reaction rate of 2.68 mM min$^{-1}$ (3-fold with the higher naringin concentration tested, due to the naringin solubility limit) was obtained, at high-pressure (160 MPa). These preliminary results are very encouraging for future implementation of the industrial process, of enzymatic hydrolysis of naringin at high pressure, in the citrus juice industry with the main goal of debittering, with the maintenance of the juice organoleptic characteristics. In

citrus juice, namely grapefruit juice, studies should be carried out at high pressure, at different temperatures and several reaction times, in order to optimize this technique for removal of the bitter taste and as a sterilisation/pasteurization process.

## References


Balny, C. (2004). Pressure effects on weak interactions in biological systems. *Journal of Physics : Condensed Matter, 16*, 1245–1253.

Cavaille, D., & Combes, D. (1995). Effect of temperature and pressure on yeast invertase stability: a kinetic and conformational study. *Journal of Biotechnology, 43*, 221–228.

Cavaille-Lefebvre, D., & Combes, D. (1998). Irreversible high pressure inactivation of β-galactosidase from *Kluyveromyces lactis*: Comparison with thermal inactivation. *Journal of Biotechnology, 61*, 85–93.

Chen, Y., Shen, S., & Lin, H. (2003). Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. *Biochemical Pharmacology, 66*, 1139–1150.

Chien, P., Sheu, F., & Shyu, Y. (2001). Monitoring the enzymatic debittering in grapefruit juice by high performance liquid chromatography. *Journal of Food and Drug Analysis, 9*, 115–120.

Fukushima, K., Matsumoto, K., Okawawuchi, M., Inoue, T., & Shimozawa, R. (1986). Kinetics of pressure-induced inactivation of bovine liver glutamate dehydrogenase. *Biochemistry and Biophysics Acta, 872*, 42–49.

Heremans, K., & Smeller, L. (1998). Protein structure and dynamics at high pressure. *Biochemistry and Biophysics Acta, 1386*, 353–370.

Lullien-Pellerin, V., & Balny, C. (2002). High-pressure as a tool to study some proteins'properties: conformational modification, activity and oligomeric dissociation. *Innovative Food Science and Emerging Technology, 3*, 209–221.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of rreducing sugar. *Analytical Chemistry, 31*, 426–428.

Mohana-Borges, R., Silva, J., Ruiz-Sanz, J., & Prat-Gay, G. (1999). Folding of a pressure-denatured model protein. *Proceedings of the National Academy of Sciences, 96*, 7888–7893.

Mozhaev, V. V., Heremans, K., Frank, J., Mansson, P., & Balny, C. (1996). High pressure effects on protein structure and function. *Trends in Biotechnology, 12*, 81–91.

Northrop, D. (2002). Effects of high pressure on enzymatic activity. *Biochemistry and Biophysics Acta, 1595*, 71–79.

Okamoto, M., Hayashi, R., Enomoto, A., Kaminogawa, S., & Yamauchi, K. (1991). *Agricultural and Biological Chemistry, 55*, 493–499.

Polydera, A. C., Stoforos, N. G., & Taoukis, P. S. (2005). of high hydrostatic treatment on post processing antioxidant activity of fresh Navel orange juice. *Food Chemistry, 91*, 495–503.

Prakash, S., Singhal, R. S., & Kulkarni, P. R. (2002). Enzymatic debittering of Indian grapefruit (*Citrus paradasis*) juice. *Journal of Science of Food and Agriculture, 82*, 394–397.

Puri, M., & Banerjee, U. (2000). Production, purification, and characterization of the debittering enzyme naringinase. *Biotechnology Advances, 18*, 207–217.

Puri, M., & Karla, S. (2005). Purification, and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. *World Journal of Microbiology and Biotecnology, 21*, 753–758.

Ribeiro, M. H. L., Silveira, D., & Ferreira-Dias, S. (2002). Selective adsorption of limonin and naringin from orange juice to natural and synthetic adsorbents. *European Food Research and Technology, 215*, 462–471.

Zverlov, V. et al. (2000). The thermostable alpha-L-rhamnosidase RamA of *Clostridium stercorarium*: biochemical characterization and primary structure of a bacterial alpha-L-rhamnoside hydrolase, a new type of inverting glycoside hydrolase. *Molecular Microbiology, 35*, 173–179.