Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Fernando SORIA & Guillermo ELLENRIEDER (2002) Thermal Inactivation and Product Inhibition of Aspergillus terreus CECT 2663 α-L-Rhamnosidase and Their Role on Hydrolysis of Naringin Solutions, Bioscience, Biotechnology, and Biochemistry, 66:7, 1442-1449, DOI: 10.1271/bbb.66.1442

To link to this article: http://dx.doi.org/10.1271/bbb.66.1442

Biosci. Biotechnol. Biochem., **66** (7), 1442–1449, 2002

Thermal Inactivation and Product Inhibition of *Aspergillus terreus* CECT 2663 α-L-Rhamnosidase and Their Role on Hydrolysis of Naringin Solutions

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Received October 26, 2001; Accepted March 8, 2002

The kinetics of thermal inactivation of *A. terreus* α-rhamnosidase was studied using the substrate p-nitrophenyl α-L-rhamnoside between 50°C and 70°C. Up to 60°C the inactivation of the purified enzyme was completely reversible, but samples of crude or partially purified enzyme showed partial reversibility. The presence of the product rhamnose, the substrate naringin, and other additives reduced the reversible inactivation, maintaining in some cases full enzyme activity at 60°C. A mechanism for the inactivation process, which permitted the reproduction of experimental results, was proposed. The products rhamnose (inhibition constant, 2.1 mM) and prunin (2.6 mM) competitively inhibited the enzyme reaction. The maximum hydrolysis of supersaturated naringin solution, without enzyme inactivation, was observed at 60°C. Hydrolysis of naringin reached 99% with 1% naringin solution, although the hydrolysis degree of naringin was only 40% due to products inhibition when the initial concentration of flavonoid was 10%. The experimental results fitted an equation based on the integrated Michaelis-Menten's, including competitive inhibition by products satisfactorily.

Key words: α-L-Rhamnosidase; Rhamnose; thermal inactivation; product inhibition

The enzyme α-L-rhamnosidase (Rase, EC 3.2.1.40) was first used in the citrus industry in applications such as debittering of grapefruit juices by hydrolysis of the bitter component naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside)[1–2] and elimination of hesperidin crystals from orange juices.[3] Rase was the main component of the commercial enzyme complexes naringinase and hesperidinase used for these purposes. Later Rase was also used for aroma improvement of wines[4–5] and enzymatic preparation of hydrolysis products of natural glycosides[6]. The manufacture of the sugar rhamnose from naringin is an example of the latter application.[7] For this process it is important that the enzyme be stable at high temperatures where the solubility of the substrate, the stability of a supersaturated solution, and the reaction rate are high.[8] Inhibition of the enzyme by high concentrations of products is also important, because it limits the rate and yields of hydrolysis products.

In previous papers the characterization and purification of an *Aspergillus terreus* α-L-rhamnosidase with high specific activity was described.[9–11] In view of the importance for its applications, we report here the thermal stability and inhibition by products of the enzyme.

Materials and Methods

Materials. Naringin, p-nitrophenyl α-L-rhamnoside (pNPR), and p-nitrophenyl β-D-glucoside (pNPG) were from Sigma (St.Louis, Mo, USA). Glutaraldehyde (50% in water) was from Fluka AG and all other reagents were of analytical grade. Standards for HPLC of naringin and prunin were from Extrasynthese (France).

Prunin (4′,5,7-trihydroxyflavanone 7-glucoside) was prepared by hydrolyzing a 10% supersaturated naringin solution with 7 Uml^{-1} of an *A. niger* naringinase (which was a gift from Tanabe, Japan) at 50°C and pH 4.0 for four hours. After that the system was kept at 4°C for 48 h, and the solid was separated by filtration, dissolved to 10% at 80–90°C, and hydrolyzed again at 50°C with a new portion of enzyme. After a new crystallization at 4°C, the solid was filtered, washed with cold water, and dried at 50°C. The purity of the obtained prunin was 92%,

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Abbreviations: Rase, α-L-rhamnosidase; Glase, β-D-glucosidase; pNPR, p-nitrophenyl rhamnoside; pNPG, p-nitrophenyl glucoside; $[E]_0$, initial enzyme concentration; E, active enzyme; I, irreversibly inactivated enzyme; R, reversibly inactivated enzyme; k_1, k_{-1}, k_2, k_3, rate constants of thermal inactivation; f_1, κ, λ_1, λ_2, constants of integrated equation 3, 4 and 5; K_m^{obs}, Michaelis constant in presence of inhibitor; V_m^{obs}, maximum velocity observed in presence of inhibitor; K_I, inhibition constant; [P], concentration of product; $[S]_0$, initial concentration of substrate; [S], concentration of substrate; a, b, constants of equation 8; X, conversion of enzymatic reaction

measured by HPLC, and it was free from residual naringin.

Enzyme preparation and assays. Production of culture filtrates, inactivation of β-glucosidase (Glase) from the culture filtrates at pH 11, and purification of Rase from *Aspergillus terreus* strain CECT 2663, were done as described previously.[10] The enzyme was also freed from Glase by dye affinity chromatography using a column of Red HE-3b/sepharose according to the following procedure: part of the Glase of the enzyme complex was retained in a first step at pH 5.5, then Rase and the rest of the Glase were retained in a second passage at pH 4.7, finally Rase was separated from Glase by eluting the column at pH 5.5 (F. Soria, G. Ellenrieder, M. Graselli, A. N. del Cañizo, and O. Cascone, unpublished results).

For the crosslinking of the protein, samples of 1 ml of the purified enzyme solution (10 Uml^{-1}) were incubated with 10–50 μl of 50% glutaraldehyde at room temperature from 15 up to 60 min, then the excess of reagent was removed by Sephadex G-25 gel filtration.

The activity of Rase was measured using pNPR as described by Romero *et al.*,[12] and 0.05 M succinic acid/sodium succinate pH 5.5 was used as buffer. Glase activity was estimated with pNPG.[13] For both enzymes, one unit of activity (U) was defined as the amount of catalyst that liberates 1 μmol of p-nitrophenol per minute.

Kinetics studies. The kinetics of the thermal inactivation of the Rase were measured as follows: A sample of 0.8 to 26 units (3.5 to 91 μg protein) of Rase in one to three ml of 0.05 M succinic acid/sodium succinate buffer, pH 5.5, was left at the inactivation temperature. Portions of 20 to 100 μl were taken out at different times and the activity measured immediately. The reversibility of the inactivation was tested by incubating the samples at 40°C until no more increase of activity was observed. In order to detect possible interferences in measurements of residual activity in the presence of naringin or its hydrolysis products, blanks, incubated without enzyme under the same conditions of the activity measurements, were tested. Enzyme recovery after hydrolysis was done using Microsep centrifugal concentrators of Filtron (USA).

The measurement of the inhibition constant of prunin was done by dissolving it in buffer at 90°C, then cooling it down to the reaction temperature, then mixing it with pNPR. After the selected hydrolysis time, a sample of 50 μl was added to 1.5 ml of 0.5 M NaOH and the absorbance measured at one-min intervals. Blanks without enzyme were run in parallel in order to correct for the interference produced by prunin.

Hydrolysis of supersaturated naringin solutions were done by dissolving the substrate in 0.05 M succinic acid/Na succinate buffer, pH 5.5, at 80–90°C, cooling down to the reaction temperature and immediately adding the enzyme. To register the reaction course of the hydrolysis of naringin, residual substrate and prunin were measured by high pressure liquid chromatography with a Shimadzu LC-4 A equipment with a C_{18} column and UV detector (280 nm). The carrier was a 32:68 acetonitrile/water mixture.

Triplicate kinetics experiments are shown in the figures. Inactivation was tested in at least two replicates of each purification or culture. The graphics, non-linear regressions, and statistic calculations were done using the PRISM software of GraphPad, USA.

Results and discussion

Kinetics of the thermal inactivation of purified α-rhamnosidase

The knowledge of the mechanism of thermal inactivation of enzymes can be of importance in the selection of the most convenient experimental conditions and the definition of strategies for thermostabilization.[14–15] In order to obtain information of utility for applications of the enzyme, the kinetics of the thermal inactivation of Rase was studied at pH 5.5 between 50°C and 70°C. To detect and avoid possible interferences of contaminant proteins, purified Rase[10] was used, and the results were compared with that of the crude and partially purified preparations of the enzyme. The values of residual activity are plotted as a function of time in Fig. 1(A). It was observed that the activity was completely recovered when the inactivated enzyme at 50°C or 60°C was incubated at 40°C for about five h. The recovery of the activity was only partial for treatments at 65°C and it was not observed at 70°C (Fig. 1(A)). From these results it could be assumed that at least three different states of the enzyme were present during the process: active or ''native'' enzyme (E), deactivated enzyme that can be reversibly recovered (R), and irreversibly inactivated enzyme (I). Assuming that only these three species were present, their concentrations were calculated from the data of Fig. 1(A) and plotted as a function of time for the inactivation at 65°C in Fig. 1(B). The existence of the enzyme state R is postulated only taking into account the reversibility of the thermal inactivation. If E and R differ appreciably in size, form, or number of ionic charges exposed to the solvent, their separation by chromatography or electrophoresis could be proposed. However, traditional chromatographic and electrophoretic methods are not fast processes compared with the rate of transformation of R to E (Table 3), so this hypothetic separation could be ineffective. Experiments at pH 4.0 and 60°C showed that the residual activity was 20% after 30 min. This indicated that the thermal stability of the enzyme was appreciably lower than that observed at pH 5.5. These values are

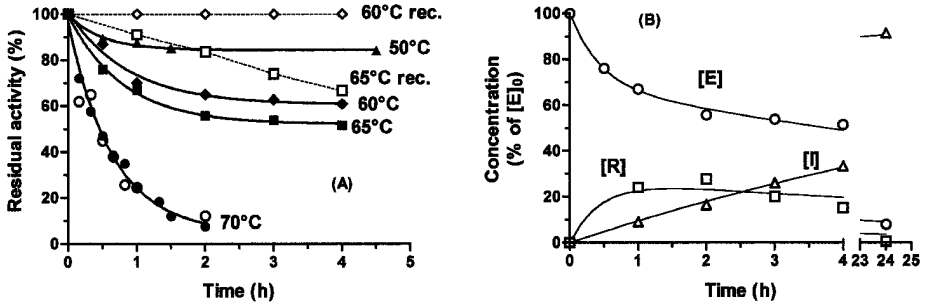

Fig. 1. Thermal Inactivation of Purified α-Rhamnosidase at Different Temperatures.
Initial activity: 5.24 Uml^{-1}; Buffer: 0.05 M succinic acid/Na succinate pH 5.5 A) Effects of temperature. Residual activity immediately after the inactivating treatment (RA): Full symbols and solid lines. Activity after a recovering treatment at 40°C (RAR): Open symbols and dotted lines. B) Time course of the concentration of the enzyme species. [E] active enzyme ([E] = RA); [R] reversibly deactivated enzyme ([R] = RAR-R); [I] irreversibly inactivated enzyme ([I] = $[E]_0$-RAR). Solid lines were obtained by non-linear regression using equations 3, 4, and 5. Temperature 65°C.

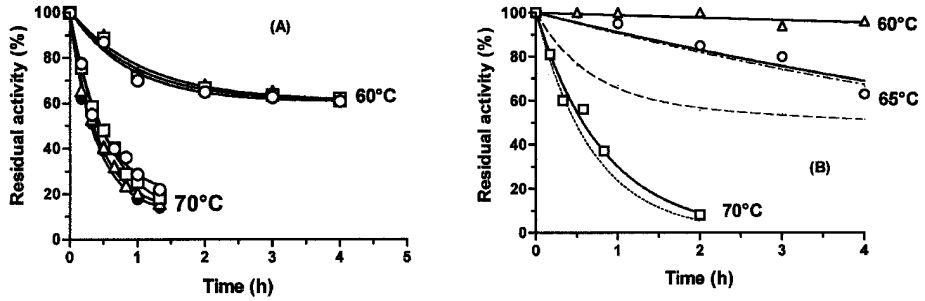

Fig. 2. Effects of Enzyme Level and Presence of Substrate on Thermal Inactivation.
A) Effects of enzyme concentration. Initial activity: ○ 0.8 Uml^{-1}; □ 5.24 Uml^{-1}; △ 21 Uml^{-1}; ● 3.3 Uml^{-1} + 2 mg ml^{-1} BSA (bovine serum albumin). B) Inactivation in presence of 4% naringin. □: 70°C with 4% naringin; ·····: 70°C without naringin; ○: 65°C with 4% naringin; ----: 65°C without naringin; – – – 65°C without naringin, after incubation at 40°C; △: 60°C with 4% naringin.

near of that published by Gallego *et al.*,[11] which were done at pH 4.0.

The effect of enzyme concentration on inactivation at 60°C and 70°C is shown in Fig. 2(A). At 70°C only a small decrease was observed with this inactivated enzyme, although the enzyme level was increased from 0.8 Uml^{-1} to 26 Uml^{-1} (3.5 to 91.3 μg protein ml^{-1}). Even the addition of two mg ml^{-1} of bovine serum albumin (BSA) had no appreciable influence on the inactivation. A similar effect was observed at 60°C. This behaviour indicates that neither aggregation nor dissociation are important steps in the inactivation mechanism. This result is different from that found for the α-rhamnosidase of *Penicillium decumbens*,[16] which is stabilized at high enzyme concentration and also by inert proteins.

The activity of the Rase was measured in the presence of naringin and prunin. These flavonoids can be transformed to chalcones in the basic medium used for p-nitrophenol measurement in the test of activity,[12] being a possible source of interference due to the production of color at 400 nm.[17] However, after dilution, this interference was only of the order of 5% of the p-nitrophenol absorbance and was easily corrected by subtracting the value of the control. Activity after the hydrolysis in presence of naringin was also measured using membrane-centrifuge concentrators, which separate the enzyme from the main part of low molecular weight substrate and products. These results of both methods were similar. The thermal inactivation of purified Rase in the presence of 4% initial naringin is plotted at different temperatures as a function of time in Fig. 2(B). These curves are of importance because they represent the operational stability of the enzyme during the process of production of rhamnose and prunin. It can be seen that the reversible deactivation at 60°C was practically eliminated. A loss of activity (which was not restored by incubation at 40°C) was observed at 65°C, it was appreciably smaller than that of the reversible inactivation of the enzyme alone, but approximately similar to its corresponding irreversible process. At 70°C, the inactivation was near that of the enzyme alone. These results suggest that the protective effect of this substrate is due to an inhibition of the reversible inactivation, but the irreversible process is not affected by its presence. Nearly the same amount of [I] was found in inactivations of the free enzyme as in presence of naringin.

Other additives, like rhamnose, and polyalcohols such as sorbitol and glycerol, also reduced the reversible thermal inactivation at 60°C (Table 1). The

stabilization effect caused by the bifunctional reagent glutaraldehyde is also shown in Table 1. It reduced the reversible deactivation without any effect on the irreversible one. The crosslinking produced by this substance prevents normal unfolding of the enzyme,[18] however in this case the effect is somewhat lower than that of the additives.

Table 1. Effects of Additives and Cross-linking on the Thermal Stability of Purified α-Rhamnosidase

Additive or treatment	T (°C)	Treatment	Residual activity (%) Time (h) 1	2	3
None	60	Inactivation[a]	70 ± 1	65 ± 0	63 ± 2
	60	I + Recovery[b]	99 ± 2	100 ± 1	99 ± 4
	65	Inactivation	67 ± .3	56 ± 4	54 ± 1
	65	I + Recovery	91 ± 3	84 ± .5	74 ± .3
Rhamnose (5 g^{-1})	60	Inactivation	99 ± 4	98 ± 3	96 ± 5
Sorbitol (0.1 M)	60	Inactivation	87 ± 5	95 ± 3	93 ± 2
	65	Inactivation		75 ± 2	
	65	I + Recovery		76 ± 3	
Glycerol (25%)	60	Inactivation	90 ± 5	95 ± 4	88 ± 1
GA cross-linking	60	Inactivation	90 ± 5	89 ± .4	82 ± 1
	60	I + Recovery	89 ± 3	87 ± 1	82 ± 3
	65	Inactivation	77 ± 1	59 ± 0	45 ± .3
	65	I + Recovery	77 ± 1	58 ± 2	44 ± 1

Values are the mean of two experiments ± standard deviation.
[a] Residual activity immediately after incubation.
[b] Inactivation follow by incubation at 40°C, 10 hours.
GA: glutaraldehyde.

Thermal inactivation of crude and partially purified α-rhamnosidase

The residual activity after treatments at 60°C of several crude and partially purified samples are shown in Table 2. It can be observed that the inactivation of Rase in crude culture filtrates depends on the carbon source used in its production. Cultivations using rhamnose as the carbon source showed an inactivation of the same order as that of the purified enzyme. Gallego-Custodio *et al.*[9] found comparable results. However, when naringin was used as the carbon source, the enzyme was more stable, and was scarcely inactivated at 60°C. This difference could be attributed to the rest of naringin in the culture filtrates. However, one of these samples maintained its stability after it was treated at pH 11 for selective inactivation of β-glucosidase, precipitated with $(NH_4)_2SO_4$, redissolved and then dialyzed. These steps certainly removed the rest of the naringin. This difference in thermal stability was caused perhaps by different glycosylation of the enzymes during the production step.[19] The recovery of activity at 40°C of the non-purified Rases produced with rhamnose as carbon source, was however only partial. In samples taken during the different steps of the purification, it was only after the last gel filtration chromatography that the inactivation at 60°C became fully reversible. In spite of the partial irreversibility of the inactivation, the presence of naringin prevented the loss of activity of the crude enzyme at 60°C. Probably the transformation of the state R to I at 60°C was favored by impurities, but naringin inhibited the transformation of E to R. Due to this effect of the substrate, crude preparations of Rase could be nearly as effective for the hydrolysis at 60°C as the purified enzyme.

Table 2. Thermal Inactivation at 60°C of α-Rhamnosidase Preparations of Different Purity

Sample	Treatment	Residual activity (%) Time (h) 1	2	3	4
1. Culture filtrate carbon source: rhamnose	Inactivation[a]	66 ± 4	67 ± 3	61 ± 3	59 ± 2
	I + Recovery[b]	77 ± 3	80 ± 2	75 ± 1	70 ± 2
2. Culture filtrate carbon source: naringin	Inactivation	98 ± 3	100 ± 3	98 ± 2	104 ± 5
3. Partially purified sample carbon source: naringin[c]	Inactivation	96 ± 1	95 ± 1	90 ± 1	96 ± 0
	I + Recovery	99 ± 3	95 ± 1	93 ± 3	100 ± 4
4. Partially purified sample carbon source: rhamnose[d]	Inactivation	70 ± 2	63 ± 2	62 ± 2	62 ± 1
	I + Recovery	70 ± 0	68 ± .3	65 ± .4	
5. Purified enzyme carbon source: rhamnose	Inactivation	70 ± .3	65 ± 0	63 ± 2	61 ± .4
	I + Recovery	99 ± 2	100 ± 1	108 ± 2	99 ± 4

Values are the mean of two experiments ± standard deviation.
[a] Residual activity immediately after incubation at 60°C.
[b] Incubation at 60°C follow by incubation at 40°C, 10 hours.
[c] Purification includes: treatment pH 11, $(NH_4)_2SO_4$ precipitation, and dialysis.
[d] Purification includes: $(NH_4)_2SO_4$ precipitation, dialysis, and DEAE sepharose cromatography.

Table 3. Kinetic Constants of the Thermal Inactivation of Purified α-Rhamnosidase

Constant	Temp.(°C)	Value	Evaluation procedure
k_1	50	0.32 ± .04	Data fit by non-linear regression using the equation of a reversible first order process.
k_1	60	0.44 ± .06	
k_{-1}	50	1.7 ± 0.3	
k_{-1}	60	0.7 ± 0.1	
k_2	65	0.099 ± .002	Fit of the data of Fig. 1(B) by non-linear regression using equation 5.
	70	1.4 ± 0.5	
k_2	65	0.09 ± 0.01	Fit of the data of inactivation in the presence of naringin (Fig. 2(B)) by non-linear regression
	70	1.2 ± 0.6	using the equation of a first order decay.
f_1	65	0.31 ± 0.03	Data fit by non-linear regression using equations 3 and 4.
λ_1	65	2.3 ± 0.5	
λ_2	65	0.084 ± 0.009	
κ	65	0.40 ± 0.02	

Mechanism of thermal inactivation

Assuming that only the enzyme species E, R, and I participate in the inactivation process of the purified enzyme, the following mechanism is proposed:

1) $E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R$

2) $R \xrightarrow{k_2} I$

3) $E \xrightarrow{k_3} I$

The step 3 was introduced considering that naringin reduced the concentration [R] without modification of the rate of production of I, so, the species I could be produced directly from E. The first two steps resemble the classical mechanism of inactivation including reversible unfolding followed by an irreversible inactivation,[15] however R is not an intermediate between E and I, and actual unfolded states between E or R and I could be present. In a lapse of 4 hours the irreversible inactivation became appreciable only at 65°C (Fig. 1(A)), but at lower temperatures the process was reversible and only the first two steps could be observed. Therefore the kinetic constants k_1 and k_{-1} were evaluated at 50°C and at 60°C using the rate equation of a reversible first order process, (Table 3). At 65°C and at 70°C all steps must be taken into account. The results obtained in the presence of substrate can be summarized according to the following: if during inactivation of the enzyme alone $[E]_0 = [E] + [R] + [I]$ and in the presence of substrate $[E']_0 = [E'] + [R'] + [I']$, experimentally it was taken that $[E]_0 = [E']_0$ and it was observed that $[R'] = 0$ (no reversible inactivation in the presence of substrate). As the rate of production of I was independent of the presence of substrate, $d[I]/dt = d[I']/dt$ and $[I] = [I']$. As a consequence of these equations $[E'] = [E] + [R]$, and considering the steps (2) and (3) of the proposed mechanism $d[I]/dt = k_2[R] + k_3[E] = d[I']/dt = k_3'[E'] = k_3'([E] + [R])$, assuming that the substrate does not modify the rate coefficient of step (3), $k_3' = k_3$ (as it was observed at 70 °C) $k_2[R] + k_3[E] = k_3([E] + [R])$. This last equation indicates that $k_2 = k_3$, a relation that permits us to simplify the differential equations corresponding to the proposed inactivation mechanism. Taking into account that $k_2 = k_3$, from the reactions scheme, the following system of independent first-order differential equations can be easily derived:

$$\begin{cases} \dfrac{d[E]}{dt} = -(k_1 + k_2)[E] + k_{-1}[R] & (1) \\ \dfrac{d[R]}{dt} = k_1[E] - (k_{-1} + k_2)[R] & (2) \end{cases}$$

the resolution of this system leads to:

$$[E] = [E]_0(f_1 e^{-\lambda_1 t} + (1 - f_1)e^{-\lambda_2 t}) \quad (3)$$

$$[R] = [E]_0(1 - f_1)\kappa(e^{-\lambda_2 t} - e^{-\lambda_1 t}) \quad (4)$$

$$[I] = [E]_0(1 - e^{-k_2 t}) \quad (5)$$

where f_1, κ, λ_1 and λ_2 are constants that depend on the kinetics constants k_1, k_{-1}, and k_2. The curves of Fig. 1(B) at 65°C and 70°C were fitted by non-linear regression using these eq. (3) to (5). In the three cases the run test of the residues indicated that the deviation from the models were not significant. The kinetic constants are shown in Table 3.

In the presence of naringin, step 1 is presumably inhibited, with R being absent in the system, equation 5 is yet valid and [E] can be evaluated considering that: $[E]_0 = [E] + [I]$. The decay of E became a first-order process with a rate constant k_2.

This is a simplified mechanism for the inactivation of purified Rase, and probably additional steps could be included, however the derived equations fitted the experimental results.

Inhibition by products

Measurements of the enzyme activity done in the presence of rhamnose or prunin showed that these products inhibited the hydrolysis. For each level of

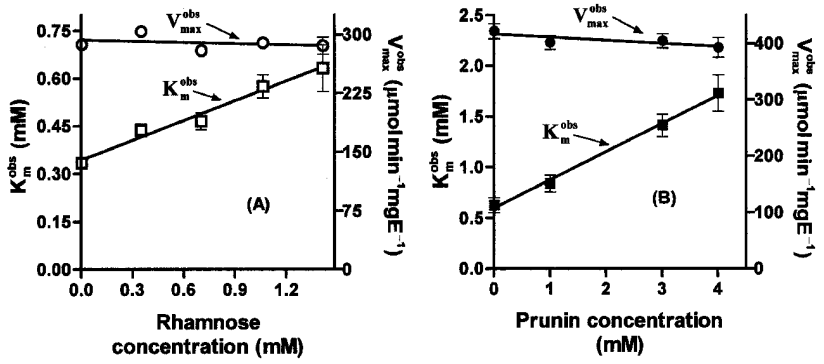

Fig. 3. Inhibition of α-Rhamnosidase by Products. "Observed" Michaelis Parameters as a Function of Inhibitor Concentration.
A) Rhamnose; buffer: 0.05 M Na succinate pH 5.5. Temperature: 50°C. B) Prunin; buffer: 0.05 M Na succinate pH 5.5. Temperature: 60°C.

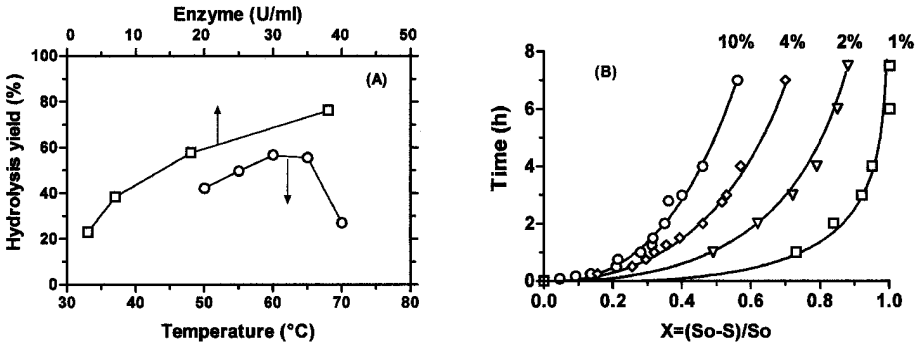

Fig 4. Hydrolysis of Supersaturated Naringin Solutions.
A) Effects of temperature and enzyme level on yield after 4 hours of hydrolysis. □ : 4% naringin, 60°C; ○ : 10% naringin, 7 U/ml B) Effects of initial substrate concentration at the necessary time to reach different conversions. Symbols: experimental points; lines: theoretical curves obtained with eq. 8 and 9.

inhibitor the plott of initial rate versus pNPR concentration was "Michaelian" as demonstrated by run tests. The values of the "observed" Michaelis constant (K_m^{obs} and V_m^{obs}) were calculated by non-linear regressions and plotted as a function of inhibitor concentration in Figs. 3(A) and 3(B), respectively. The linear dependence of K_m^{obs} indicated that both inhibitors acted competitively.[20] According to the equations for enzyme inhibition,[21] the values of V_m^{obs} must be constant for competitive inhibition. They showed a very low decrease, which may be due to the experimental deviation. From the equation: $K_m^{obs} = K_m + K_m/K_I[I]$ the inhibition constants were calculated, being 1.65 mM for rhamnose at 50°C and 2.15 mM for prunin at 60°C.

Other α-rhamnosidases also show competitive inhibition by rhamnose, with K_i ranging from 0.3 to 4.2 mM.[12,22-23] Glucose inhibits the enzyme in a less proportion, it was found that in a concentration of 100 mM the inhibition is about 20%, similar to that reported by other authors.[9] The enzyme from *A. niger*, is more inhibited by glucose, this effect being about 50% for a concentration of 29%.[5,24]

Hydrolysis of supersaturated naringin solutions

Using enzymes to produce rhamnose from naringin, the low solubility of this flavonoid produces some problems associated with the manipulation of suspensions. They can be avoided by hydrolyzing a supersaturated solution. As the solubility of naringin increases abruptly above 70°C, concentrated solutions can be prepared at 80°C–90°C, which after a fast cooling to 50°C–60°C form homogeneous supersaturated solutions. The supersaturated solutions are stable during several hours, and the enzymatic reaction can be run in this time interval. This procedure increases the reaction rate and yield.[8] Special attention was given to the process taking place within the first 4–6 hours of reaction because in this time the supersaturated naringin solutions were stable and the conversions were appreciable. Using purified *Aspergillus terreus* α-rhamnosidase as the catalyst, the hydrolysis yield depended on the enzyme activity and temperature, as shown in Fig. 4(A). It can be seen that the optimum temperature for the hydrolysis was 60°C, which was higher than that of the enzymes from *P. decumbens* and *A. niger* previously assayed.[8] The results on the kinetics of thermal inactivation are in accordance with this behaviour and in-

dicated that during the hydrolysis the enzyme was not inactivated. Considering the dependence of yield on enzyme activity the value of 7 U/ml was selected for subsequent runs because the rate of increase of yield over this value became lower and did not justify the spending for the expensive enzyme. The influence of the initial naringin concentration over the necessary time to reach different hydrolysis conversions is shown in Fig. 4(B). It can be seen that, in a lapse of seven hours, only the 1% naringin solution was totally hydrolyzed, and when the initial substrate concentration was increased, the conversion became lower. It was shown previously, using a commercial enzyme, that this limitation of the hydrolysis is caused by inhibition by the product rhamnose,[8] the concentration of rhamnose and prunin after a fixed time increased when the initial naringin was increased and consequently the yield decreased. To fit the experimental data of yields as a function of time, the Michaelis-Menten equation with competitive inhibition by the product was chosen as a model. It can be easily demonstrated that replacing the concentration of inhibitor [I] by that of product $[P] = [S]_0 - [S]$ in that equation, the following expression is obtained:

$$-\frac{d[S]}{dt} = \frac{K_I V_m [S]}{K_m(K_I + [S]_0) + (K_I - K_m)[S]} \quad (6)$$

K_m and V_m are the Michaelis constants and K_I the product inhibition constant. Inverting this equation to $dt/d[S]$, it can be integrated as usual, yielding t as function of the conversion X

$$t = -\frac{K_m(K_I + [S]_0)}{V_m K_I}\ln(1 - X) + \frac{[S]_0(K_I - K_m)}{V_m K_I} X \quad (7)$$

$$t = aX - b\ln(1 - X) \quad (8)$$

$$\text{with } X = \frac{[S]_0 - [S]}{[S]_0}$$

It can be easily demonstrated that an equation similar to eq. 8 is valid also for an enzyme inhibited competitively by two products. Equation 8 was used to fit the experimental data of Fig. 4(B) and the values of the constants "a" and "b" were obtained by non-linear regression. The fit was good, and run tests indicated that the deviations from the model were not significant. The dependence of "a" and "b" on $[S]_0$ slightly deviated from the linearity, and could be better reproduced by the equations

$$a = 2.33 - 4.77[S]_0 + 0.167[S]_0^2;$$
$$b = -2.73 + 4.88[S]_0 - 0.179[S]_0^2 \quad (9)$$

where $[S]_0$ is expressed as a %. Using equations 8 and 9, the solid lines of Fig. 4(B) were drawn, and it can be seen that the experimental results fitted well. These equations can also be used for predictions of hydrolysis conversions for other substrate concentrations.

In order to compare the purified enzyme with other cheaper preparations of the same source, hydrolysis of 10% naringin at 60°C was done using the following enzyme preparations: 1) a crude *A. terreus* culture filtrate (containing Glase), 2) a culture filtrate after Glase inactivation at pH 11,[10] and 3) Rase purified by dye affinity chromatography. In case 1), the aglycone naringenin was formed as a consequence of the partial hydrolysis of prunin. It can be seen in Table 4 than the level of prunin in the hydrolysis with the purified enzyme was near to that of the cases 2) and 3). The reduction of concentration of naringin was, however, comparable for the 4 preparations.

Table 4. Hydrolysis of 10% Naringin Solutions by Crude and Partially Purified α-Rhamnosidase

enzyme	conversion 4 (h)	prunin (gl^{-1})
1. culture filtrate	0.39 ± 2	9 ± .4
2. culture filtrate treated at pH 11[a]	0.41 ± 1	30 ± 1
3. dye affinity purified enzyme[b]	0.44 ± 2	32 ± 2
4. purified enzyme	0.46 ± .4	33 ± 1

Values are the mean of two experiments ± standard deviation.
[a] This treatment inactivates β-glucosidase.
[b] This enzyme preparation (α-rhamnosidase 7 Uml^{-1}) was free from β-glucosidase.

The effects of inhibition by products depends on the source of enzyme, the *A. niger* Rase[8] showed a smaller inhibition than that of *A. terreus*. In a process for rhamnose and prunin production by enzymatic hydrolysis of naringin, a low inhibition is desirable in order to obtain products in concentrated solutions and reduce the cost of water elimination in the recovering step. Although rhamnose and prunin are expensive chemicals and the relative incidence of cost of this step on the final product price is not very high, it is necessary to take into account these aspects.

It can be concluded that *Aspergillus terreus* α-rhamnosidase is a very convenient catalyst for several applications because of its high thermal stability (especially in the presence of substrate). Its high specific activity and good production level are also advantageous characteristics. These properties of *A. terreus* Rase are also convenient for the rhamnose and prunin production from naringin. Although inhibition by products limits the rhamnose concentration to be obtained with maximum yield in only one step, alternatives could be considered on the production scale to overcome this fact. Recycling of the enzyme and non-hydrolyzed naringin and the two-step procedure must be analyzed. Furthermore the concentration of rhamnose solutions could have not a great relative incidence in the cost of the total process due to the high price of this sugar.

Acknowledgments

The authors would like to thank Universidad Nacional de Salta, CONICET, and Agencia Nacional de Promoción Cientifica y Tecnol. of Argentina for

the financial support that made this work possible.

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