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ORIGINAL PAPER

Purification and characterization of a novel alkaline α-L-rhamnosidase produced by *Acrostalagmus luteo albus*

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Abstract Rhamnosidases are enzymes that catalyze the hydrolysis of terminal nonreducing L-rhamnose for the bioconversion of natural or synthetic rhamnosides. They are of great significance in the current biotechnological area, with applications in food and pharmaceutical industrial processes. In this study we isolated and characterized a novel alkaline rhamnosidase from *Acrostalagmus luteo albus*, an alkali-tolerant soil fungus from Argentina. We also present an efficient, simple, and inexpensive method for purifying the *A. luteo albus* rhamnosidase and describe the characteristics of the purified enzyme. In the presence of rhamnose as the sole carbon source, this fungus produces a rhamnosidase with a molecular weight of 109 kDa and a pI value of 4.6, as determined by SDS–PAGE and analytical isoelectric focusing, respectively. This enzyme was purified to homogeneity by chromatographic and electrophoretic techniques. Using *p*-nitrofenil-α-L-rhamnopiranoside as substrate, the enzyme activity showed pH and temperature optima of 8.0 and 55°C, respectively. The enzyme exhibited Michaelis–Menten kinetics, with K_M and V_{max} values of 3.38 mmol l^{-1} and 68.5 mmol l^{-1} min^{-1}, respectively. Neither divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} nor reducing agents such as β-mercaptoethanol and dithiothreitol showed any effect on enzyme activity, whereas this activity was completely inhibited by Zn^{2+} at a concentration of 0.2 mM. This enzyme showed the capacity to hydrolyze some natural rhamnoglucosides such as hesperidin, naringin and quercitrin under alkaline conditions. Based on these results, and mainly due to the high activity of the *A. luteo albus* rhamnosidase under alkaline conditions, this enzyme should be considered a potential new biocatalyst for industrial applications.

Keywords α-Rhamnosidase · Alkaline enzymes · *Acrostalagmus luteo albus* · Rhamnoside hydrolysis

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Introduction

The biotechnological potential of microbial glycolytic enzymes has drawn a great deal of attention from various researchers worldwide, as they are likely to be biological catalysts in a variety of industrial processes. Many microorganisms have been studied for their potential to produce glycosidases; however, little is known about microorganisms that produce α-L-rhamnosidase (Rhase, EC 3.2.1.40) activity. Rhases are exo-type enzymes which remove terminal α-L-rhamnosyl groups at the ends of polysaccharides and glycosides containing L-rhamnose. Rhases are important industrial enzymes of great significance in the current biotechnological area with applications in food [30] and pharmaceutical [18] industrial processes for the bioconversion of natural or synthetic rhamnosides. In particular, several technical applications of fungal Rhases in the food industry, such as the debittering of grapefruit juice by hydrolysis of the bitter flavonoid naringin [28, 29], the elimination of hesperidin crystals in orange juice, and the enhancement of wine aromas by

enzymatic hydrolysis of terpenylglycosides [2], have been investigated. Moreover, the derhamnosylated product quercetin-3-glucoside from the flavonoid rutin increases the antioxidant activity of asparagus juice [32]. This enzyme has also been used in the structural determination of polysaccharides, glycosides, and glycolipids; in the metabolism of gellan [10]; in the production of prunin [23], which possesses anti-inflammatory and antiviral activity against DNA/RNA viruses [12]; and for the derhamnosylation of many L-rhamnose containing steroids such as diosgene desglucoruscin and ginsenosides, whose derhamnosylated products have clinical importance [7, 18, 34]. The glycopeptide antibiotic chloropolysporin C, which exhibits antibacterial activity, is useful in the treatment and prophylaxis of infections and as a growth-promoting agent for animals. It is prepared from the related compound chloropolysporin B [26] by enzymatic hydrolysis using Rhase. Despite this industrial interest, only a few crude Rhase preparations are commercially available so far, specifically the so-called hesperidinase and naringinase of fungal origin. All of these preparations, presently obtained from the genera *Aspergillus* and *Penicillium*, also contain β-D-glucosidase (Gluse) activities that can limit their industrial exploitation. Moreover, they are stable and active at acid pH values, where their natural substrates are slightly soluble. The use of Rhases that are stable and active at alkaline pH values might solve this problem, allowing the hydrolysis of concentrated solutions of substrates under alkaline conditions.

A strain of *Acrostalagmus luteo albus*, a rhizospheric soil microfungus, was isolated from tala (*Celtis tala*) and coronillo (*Scutia buxifolia*) alkaline soil forests in the east central Argentina (Punta Indio and Magdalena) [1]. It was recently reported that this fungus produces a pool of alkali-tolerant hydrolases, including a Rhase with potential biotechnological applications [24].

In the present study we report the production and an efficient and simple method for purifying the *A. luteo albus* Rhase. We also describe the main characteristics required for the industrial application of the purified enzyme, as well as substrate specificity studies towards flavonoid glycosides.

Materials and methods

Medium and growth conditions

Acrostalagmus luteo albus (Link: Fr) Zare, Gams et Schroers (LPS cult # 748) had been previously isolated from calcium carbonate rich soils as described by Cabello and Arambarri [1]. Stock cultures were maintained on PDA tubes under a layer of mineral oil at 4°C. It was grown in minimal medium [5], using L-rhamnose as the carbon and energy source and supplemented with bacto-tryptone (Difco). The culture medium contained (per liter): rhamnose (Sigma, St. Louis, MO, USA) 10 g, tryptone 2.5 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4.7H_2O$ 0.01 g, citric acid 0.01 g, naringin (Sigma) 0.125 g and buffer solution ($K_2HPO_4.2H_2O$ 0.9 g/l; Na_2CO_3 1.0 g/l) 100 ml, pH 9.0. Liquid cultures were inoculated with 10^6 conidia per milliliter and conducted in 1,000-ml Erlenmeyer flasks containing 300 ml of medium, at 30°C, on a rotary shaker at 200 rpm for 14 days. The whole content of each flask was withdrawn, centrifuged (7,000×g, 4°C) to remove suspended solids (fungal biomass), and the supernatant was used for analytical determinations. All these experiments were performed in duplicate and the average of the determinations calculated.

Enzyme activity assays

Enzyme activities were kinetically determined in a spectrophotometer (Beckman DU 640) using *p*-nitrophenyl-α-L-rhamonopyranoside (PNP-Rha) (Sigma) as substrate. The release of *p*-nitrophenolate was evaluated at 405 nm ($\varepsilon = 18{,}500\ M^{-1}\ cm^{-1}$) for 30 min. The reaction mixture consisted of 30 µl of substrate solution (5 mM in buffer), 260 µl of Tris–HCl buffer (20 mM, pH 9.5) and 10 µl of enzyme sample. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per minute at 37°C. All enzymatic measurements were done in duplicate.

Enzyme purification

The culture supernatant was filtered through cheesecloth at 4°C and concentrated under reduced pressure at 37°C. Solid ammonium sulfate was then added to the concentrated extract up to 80% saturation. Precipitated proteins were dissolved in distilled water (8.5 ml) and then applied to a Sephacryl S-100 gel filtration column (XK 16/60, GE, Little Chalfont, UK) equilibrated with MES buffer (20 mM, pH 6.0) (MB), and eluted isocratically with the same buffer at a flow rate of 1.5 ml min^{-1}. Fractions of 3.0 ml were collected, and those exhibiting Rhase activity were pooled, concentrated by lyophilization, resuspended in MB up to 4 ml, and loaded onto a DEAE Sephadex (XK 26/10, GE) anion exchange column. The column was washed with the starting buffer (MB), and the bound proteins were then eluted with a linear gradient of NaCl (0.0–1.0 M) in MB over 10 column volumes of 36 ml at a flow rate of 3 ml min^{-1}. Fractions (1 ml) were collected and analyzed for Rhase activity. Fractions selected for further purification were dialyzed against the same buffer, pooled, concentrated by lyophilization, resuspended in MB

up to 5 ml, and loaded onto a Q Sepharose FF column (HiLoad 16/10, GE) pre-equilibrated with MB, and proteins were eluted on a linear gradient of NaCl (0.3–1.0 M) in MB over ten column volumes at a flow rate of 5 ml min^{-1} on the same chromatographic system. Fractions containing Rhase activity were pooled and dialyzed overnight against MB to give a final volume of 3.7 ml. Finally, this sample was loaded onto a single well 10% SDS–PAGE minigel in a Mighty Small II Unit (Hoefer SE 260, GE). Proteins were visualized by colloidal Coomassie Blue staining and the 109 kDa band excised from the gel and extracted according to Reguera and Leschine [22]. All chromatographic steps were carried out on an Amersham FPLC-U900 system (Amersham GE Healthcare). Protein content was assayed by the Bradford protein method.

Enzyme biochemical characterization

Protein molecular mass was estimated using a 10% SDS–PAGE calibrated with low and high molecular weight calibration kits (GE) [15]. Isoelectric point was determined by analytical isoelectric focusing performed on precast IEF polyacrylamide gels containing carrier ampholytes (Pharmalyte) in the 3.5–9.0 pH range and using a pI calibration kit (GE). Samples were focused using a Multiphor II system (GE). Protein bands were stained with Coomassie Brilliant Blue R 250.

Influence of pH and temperature on enzyme activity and stability

Optimal pH and temperature

The influence of pH on Rhase activity was measured by assaying the Rhase activity of the purified enzyme towards PNP-Rha at 37°C at various pH values. The buffers used were 20 mM MES–TrisHCl–glycine adjusted to different pH values of between 5 and 11.

To determine the optimal temperature for Rhase activity, the enzyme activity was assayed at various temperatures between 20 and 70°C at pH 8.0.

Enzyme stability

The thermal stability of Rhase activity was assayed as the residual activity after incubating the purified enzyme with MB at 40, 55 and 60°C without substrate for 10 h.

The pH stability was determined as the residual Rhase activity after the purified enzyme had been incubated for 24 h at 4°C, without substrate, in 20 mM MES–TrisHCl–glycine buffers in the pH range 5–10.

Enzyme kinetics

The kinetic constants K_M and V_{max} of the enzyme were calculated by fitting the activity data at different substrate concentrations ranging from 0.2 to 4 mmol l^{-1} to a linear regression on Lineweaver–Burk double-reciprocal plots.

Influences of metal ions and various compounds on enzyme activity

The effect of a number of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}) and of other chemicals (reducing agents: β-mercaptoethanol, dithiothreitol, and the chelating agent ethylenediaminetetraacetic acid, EDTA) on enzyme activity was tested in the reaction medium. Rhase activity was assayed in the presence of 2 mM of these various substances. Before the assay, the enzyme solution was dialyzed against MB.

Substrate specificity towards rhamnoglucosides

Specificity studies of Rhase towards different rhamnoglucoside substrates (hesperidin, naringin and quercitrin) (analytical grade, Sigma) were performed. Solutions of these three substrates were prepared (3.5 mM in 20 mM Tris–HCl buffer) and adjusted to pH 9.5. Reactions were carried out by mixing 900 µl of each substrate solution and 100 µl of the enzymatic extract. Substrate and sample blanks were performed using distilled water instead of substrate and enzyme solutions, respectively. These reaction mixtures were incubated for 3 h at 37°C. Samples of 300 µl were taken at the beginning and at the end of this assay. After the incubation period, samples were added to an equal volume of 52% acetonitrile in 60 mM H_3PO_4 to finish the reaction. Finally, remaining substrates and reaction products were quantified by HPLC.

HPLC analysis

Rhamnoside hydrolysis studies were performed by HPLC using a Symmetry C18 column (3.9 × 150 mm, Waters, Milford, MA, USA) operating at room temperature, using a solution of acetonitrile/water (0.68:0.32 v/v) as solvent at a flow rate of 1 ml/min, and a UV photodiode array detector (2996, Waters). Naringin, prunine, naringenin, hesperidin, hesperetin, quercitrin and quercetin concentrations were determined according their respective standards (Sigma) [4].

Thin-layer chromatography (TLC) analysis

TLC was carried out on silica gel (60 F254) aluminum sheets (Merck, Whitehouse Station, NJ, USA) using ethyl

acetate/formic acid/acetic acid/water (10:1:1.1:2.7 v/v) as the mobile phase. Visualization of flavonoids was achieved by spraying the sheet with 3% phosphomolybdic acid:10% sulfuric acid solution in ethanol and then heating at 105°C for 5 min.

Results and discussion

Enzyme purification

An enzyme showing Rhase activity was purified to homogeneity from the culture filtrate of *A. luteo albus* grown on L-rhamnose as the sole carbon source using gel filtration and ion exchange chromatographies and preparative electrophoresis. A summary of the purification procedure is presented in Table 1.

The original culture filtrate showed both Rhase and Gluse enzyme activities in addition to water-soluble dark brown substances that were produced during cultivation. Using gel filtration chromatography it was possible to achieve a colorless fraction. The main contaminating proteins did not bind to the DEAE Sepharose FF column, and Rhase activity was eluted as a broad peak at 0.37–0.40 M NaCl. After the subsequent purification step using Q-Sepharose FF column, Rhase activity was eluted as a single peak at 0.4–0.45 M NaCl yielding 9% of the original Rhase activity with a 13-fold increase in specific activity. Finally, preparative electrophoresis yielded a single electrophoretic homogeneous fraction containing Rhase activity free of Gluse activity (Fig. 1). This result demonstrates that, in our case, the Rhase and Gluse enzyme activities correspond to different proteins.

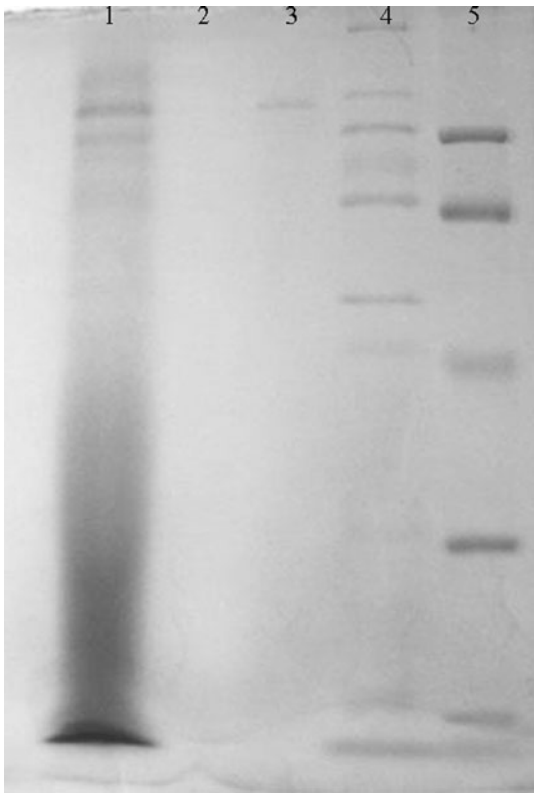

Fig. 1 SDS-PAGE with Coomassie staining of the purification steps of *A. luteo albus* rhamnosidase. *Lane 1*: concentrated supernatant, after $(NH_4)_2SO_4$ precipitation. *Lane 2*: after Sephacryl S-100 chromatography. *Lane 3*: after Q Sepharose chromatography. *Lane 4*: high molecular weight marker. *Lane 5*: low molecular weight marker

Biochemical characterization

SDS–PAGE revealed an apparent molecular weight of 109 kDa for *A. luteo albus* Rhase. Values ranging from 90 to 96 kDa have been described for *Clostridium stercorarium* [35], *Aspergillus terreus* [9] and two *A. niger* Rhases [11, 23], whereas molecular mass values of 70, 72 and 75 kDa were reported for the two *N*-glycosylated Rhases from *A. aculeatus* [16] and another *A. niger* Rhase [17], respectively.

Analytical IEF data showed the Rhase activity to be an acidic protein with a pI of approximately 4.6, thus falling within the pI range 4.5–5.2 described for some *Aspergillus* Rhases [9, 19].

Increasing interest in applying enzymes in industrial processes has driven the search for biocatalysts with new or improved properties [13, 33]. Due to the unique capacity of enzymes to catalyze reactions with high velocity and unmet specificity under a variety of conditions, the development

Table 1 Summary of the *A. luteo albus* α-L-rhamnosidase purification procedure

Purification step	Volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)
Concentrated extract	56	38.7	1421	36.7	100
Precipitation and filtration	8.5	37.4	1331	35.5	94
S-100	4.0	9.6	758	79.2	53
DEAE Sepharose	5.0	1.2	418	363.9	29
Q Sepharose	3.7	0.3	128	496.8	9
Preparative electrophoresis	2.0	0.12	63	526.5	4.5

of new biocatalytic processes is feasible in principle and potentially profitable. The use of biotransformations in industry will increase and it has been claimed that a doubling of the number of industrially established biocatalytic processes every decade is probable [21, 27, 31]. Unfortunately, naturally available enzymes are usually not optimally suited for industrial applications. This incompatibility often relates to the stability of the enzymes under process conditions. Although it is sometimes beneficial to adapt industrial processes to mild and environmentally benign conditions favored by the enzyme, the use of more extreme conditions is often desirable. For example, the use of high process temperatures may be beneficial with respect to factors such as substrate and product solubility, viscosity, process speed and microbial contamination. Regardless of process conditions, the stability of the biocatalyst is often an important economic factor.

The stability of an enzyme is affected by many factors, such as temperature, pH, oxidative stress, solvent, binding of metal ions or cofactors, and the presence of surfactants [6]. The optimum temperature for the purified Rhase at pH 9.0 was found to be 55°C (Fig. 2a). Characterized fungal Rhases are optimally active at temperatures between 40 and 80°C [9, 11, 16, 17, 20, 35].

The dependence of the enzymatic activity of the isolated enzyme on pH is shown in Fig. 2b. The *A. luteo albus* Rhase exhibits optimal activity at pH 8.0 towards PNP-Rha as substrate, which is quite different from those of *Aspergillus* Rhases [17, 20, 23]. Most of the fungal Rhases described in the literature have maximal activity at acid pH values when using PNP-Rha as substrate. At pH 9.5, *A. luteo albus* Rhase retained more than 67% of its maximum activity, and 62% of its maximum activity at pH 11.

A broad pH optimum is preferable for applications of Rhase in the food and pharmaceutical industries. The activity of Rhase under alkaline conditions where flavonoids such as naringin and hesperidin are highly soluble is remarkable. Therefore, its potential application for the production of L-rhamnose and the corresponding glycosylated flavanone should be considered.

The thermal stability of Rhase activity was assayed as the residual activity after incubating the purified enzyme with MB at 40, 55 and 60°C without substrate. The enzyme was stable up to 40°C for 4 h, whereas it retained 95% of its original activity after 10 h of incubation, even when no protective agent was added. At its optimum temperature (55°C), the enzyme retained 81% of its initial activity after 4 h of incubation. Temperatures higher than 55°C resulted in a rapid loss of enzyme activity (Fig. 3a). Slightly lower thermal stabilities have been reported for fungal Rhases [14, 25].

pH stability was determined as the residual Rhase activity after the purified enzyme had been incubated for

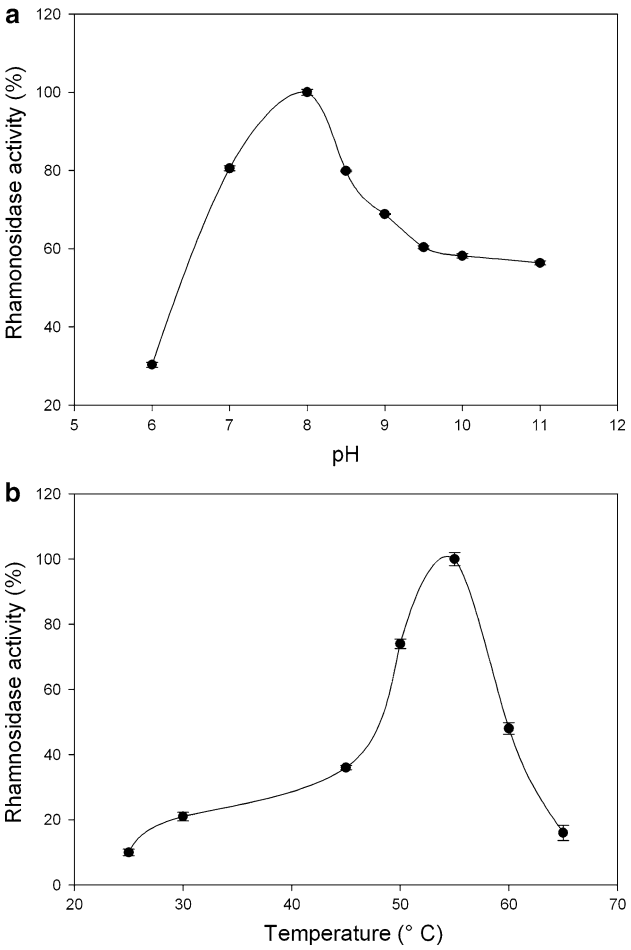

Fig. 2a–b Influences of temperature (**a**) and pH (**b**) on purified rhamnosidase activity from *A. luteo albus* towards PNP-α-L-rhamnopyranoside. For both assays, the standard deviation (SD; *vertical bars*) of the mean was calculated for three replicates

24 h at 4°C, without substrate, in the pH range 5–10. The isolated Rhase proved to be very stable over pH values of 7.5–9.0, retaining 95% of its initial activity (Fig. 3b).

Kinetic parameters

The Michaelis constant K_M and the V_{MAX} value of the *A. luteo albus* Rhase using PNP-Rha as substrate were found to be 3.38 mmol l^{-1} and 68.5 mmol l^{-1} min^{-1}, respectively. Lower K_M values ranging from 1.52 to 2.9 mmol l^{-1} have been described for *Penicillium sp.* [25] and some *Aspergillus* [2, 14, 16, 17] Rhases.

End-product inhibition by L-rhamnose was also studied. The reaction was competitively inhibited by L-rhamnose, with a K_I value of 4.7 mmol l^{-1}. Inhibition studies have only been described for the Rhase activities from *Penicillium* sp and *Aspergillus*, with K_I values ranging from 1.2 to 4.2 mmol l^{-1} [16, 25]. The impact of a competitive inhibitor is to alter K_M such that the enzyme would appear

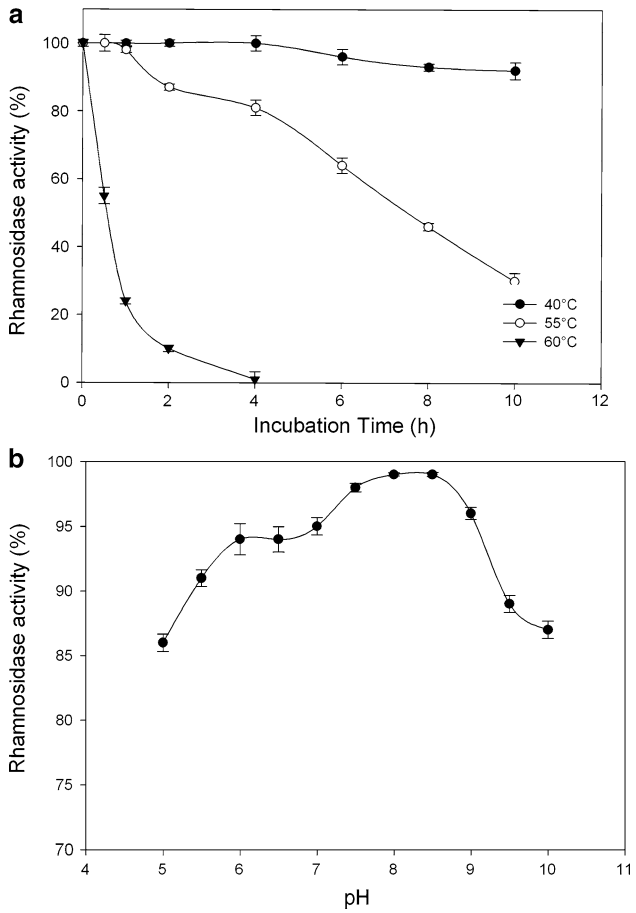

Fig. 3a–b Thermal (**a**) and pH (**b**) stabilities of purified rhamnosidase from *A. luteo albus*. For both assays, the SD (*vertical bars*) of the mean was calculated for three replicates

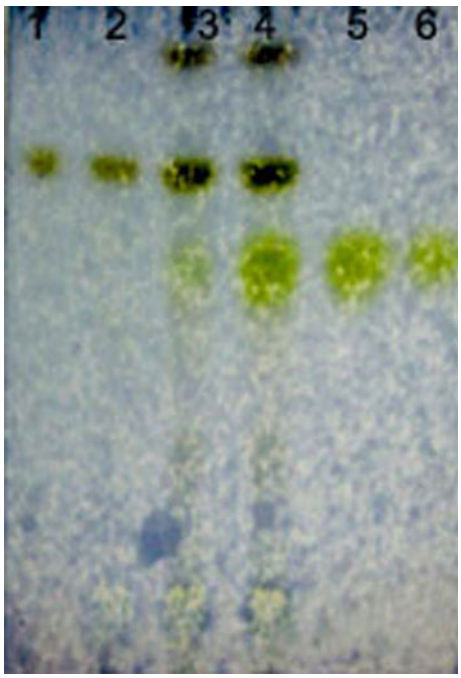

Fig. 4 TLC analysis of the products obtained after the hydrolysis of naringin with *A. luteo albus* rhamnosidase. *Lane 1*: naringin standard (10.5 μg). *Lane 2*: hydrolyzed naringin ($t = 0$ h). *Lane 3*: hydrolyzed naringin ($t = 3$ h). *Lane 4*: hydrolyzed naringin ($t = 3$ h) with rhamnose internal standard (11.25 μg). *Lane 5*: rhamnose standard (11.25 μg). *Lane 6*: rhamnose standard (5.62 μg). The reaction mixture containing 900 μl of 3.5 mM naringin in Tris HCl buffer (20 mM, pH 9.5) and 100 μl of the enzymatic extract was incubated for 3 h at 37°C

to have a lower affinity for the substrate. This makes sense, since the inhibitor is binding to the same site as the substrate. So, as is the case with high K_M, it is necessary to have more substrate to achieve a higher reaction rate, since the substrate can outcompete for the binding sites. These results suggest that a continuous enzymatic process should be developed with the enzyme immobilized onto a solid support in order to avoid competitive inhibition due to product accumulation.

Effects of various reagents on Rhase activity

Rhases tend to undergo changes in their physical and chemical properties in the presence of salts and other substances affecting their catalytic properties. Therefore, it was necessary to test the effects of a number of representative cations and (reducing and chelating) agents in the reaction medium on Rhase activity. Before the assay, the enzyme solution was dialyzed against MB in order to remove all interfering substances. Purified Rhase activity was not affected by β-mercaptoethanol and dithiothreitol. This result suggests there is no critical role of the cysteine residue(s) in the catalysis and/or substrate binding site(s).

On the other hand, cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} did not show any significant effect on Rhase activity under the assay conditions, whereas the enzyme activity was strongly inhibited in the presence of 0.2 mM Zn^{2+}.

The chelating agent EDTA also inhibited the enzyme activity, indicating an important role of free cations in the catalysis process and/or in the maintenance of the native three-dimensional structure of the enzyme.

Substrate specificity towards rhamnoglucosides

The enzyme was active towards naringin (Fig. 4), in which the L-rhamnose residue is α-1,2 linked to the β-D-glucoside; towards hesperidin (Fig. 5), which has an α-1,6 linkage to the β-D-glucoside; and also towards quercitrin (Fig. 6), where the L-rhamnose residue is linked directly to the aglycon. Based these results, it could be inferred that this enzyme is able to hydrolyze α-1,2 and α-1,6 linkages to β-D-glucosides in addition to direct linkages from the L-rhamnose residue to the aglycon. Finally, initial and final

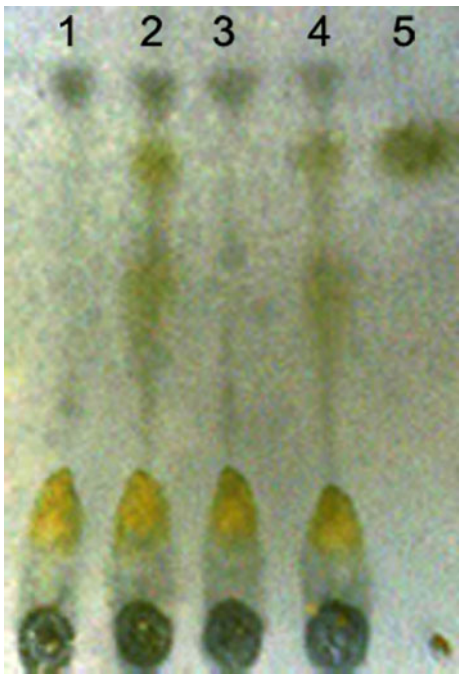

Fig. 5 TLC analysis of the products obtained after the hydrolysis of hesperidin with *A. luteo albus* rhamnosidase: *Lane 1*: hesperidin standard (10.5 μg). *Lane 2*: hydrolyzed hesperidin with rhamnose internal standard (11.25 μg) ($t = 0$ h). *Lane 3*: hydrolyzed hesperidin ($t = 3$ h). *Lane 4*: hydrolyzed hesperidin with rhamnose internal standard (11.25 μg) ($t = 3$ h). *Lane 5*: rhamnose standard (11.25 μg). The reaction mixture containing 900 μl of 3.5 mM hesperidin in Tris HCl buffer (20 mM, pH 9.5) and 100 μl of the enzymatic extract was incubated for 3 h at 37°C

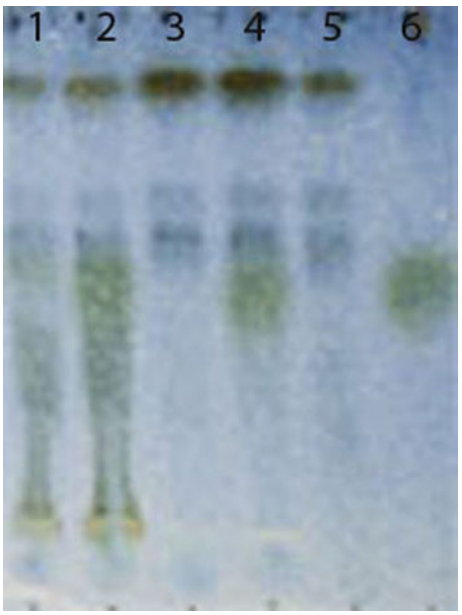

Fig. 6 TLC analysis of the products obtained after the hydrolysis of quercitrin with *A. luteo albus* rhamnosidase. *Lane 1*: hydrolyzed quercitrin. *Lane 2*: hydrolyzed quercitrin with rhamnose internal standard (5.62 μg). *Lane 3*: quercitrin standard (10.5 μg). *Lane 4*: quercitrin standard (10.5 μg) with rhamnose internal standard (5.62 μg). *Lane 5*: quercitrin standard (5.62 μg). *Lane 6*: rhamnose standard (5.62 μg). The reaction mixture containing 900 μl of 3.5 mM quercitrin in Tris HCl buffer (20 mM, pH 9.5) and 100 μl of the enzymatic extract was incubated for 3 h at 37°C

amounts of substrates and reaction products were quantified by HPLC, and hydrolysis percentages were calculated as follows: hesperidin 9.44%, naringin 29.6%, and quercitrin 90.5%. It can be concluded that under the standard assay conditions, the enzyme is much more active towards quercitrin, indicating a higher specificity for direct linkages from the L-rhamnose residue to the aglycon.

Aspergillus Rhase activities showing different substrate specificities have been described, but only one Rhase from *A. niger* [17] and the two Rhases purified from *A. aculeatus* [16] were reported to be active towards all three of these rhamnoglucosides.

The *A. luteo albus* Rhase described in this report displays a broad substrate specificity that allows its application to rhamnoside hydrolysis in industrial processes under alkaline conditions.

Although Rhases have several biotechnological applications, a limited number of these microbial enzymes have been characterized. As far as the authors are aware, there are only a few fungal extracellular Rhases, free of Gluse activity, that are active at high pH values.

Only a few reports have addressed the fermentative production of Rhase on a large scale and its subsequent application to rhamnose production. Therefore, the development of new commercially viable processes is desirable for the bulk production of this enzyme by fermentation. Fungal enzyme expression can be increased up to tenfold by optimizing the fermentation conditions [3, 8]. Thus, the cloning of the corresponding gene in order to overproduce this enzyme is now in progress.

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