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The Application of Immobilized α-L-Rhamnosidase and L-Rhamnosedehydrogenase in the Analysis of L-Rhamnose and α-L-Rhamnosides

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ABSTRACT

A highly sensitive and specific enzymatic assay for the quantitative determination of α-L-rhamnosides employing naringinase (an enzyme complex consisting of α-L-rhamnosidase and β-D-glucosidase) and L-rhamnose dehydrogenase is described. The test can be carried out in the presence of other glycosides or sugars and deoxysugars. Even compounds having very low solubility can be assayed by this procedure.

Index Entries: Rhamnose, analysis of; glycosides; α-L-rhamnosides; rhamnose dehydrogenase; naringinase; immobilized.

INTRODUCTION

The specific assay of rhamnosides is rather difficult. Most of the available methods are either not specific enough (*1–3*) or require laborious separations (*4,5*). Only one specific assay has been published until now (*6*). In this paper, a specific assay, based on enzymatic techniques is described. It has several advantages.

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a) It is specific and can be carried out in presence of other glycosides, sugars, and deoxysugars.
b) The procedure is simple.
c) The assay works under mild conditions. Therefore, it is possible to recover the aglycones. This might be of interest, if rare and expensive substances are tested.
d) Even hardly soluble compounds can be assayed (*7*).

PRINCIPLE

In the first reaction step α-L-rhamnosides are cleaved quantitatively into their aglycones and the sugar component. The L-rhamnofuranose thus liberated can be oxidized by L-rhamnose dehydrogenase (Fig. 1) to L-rhamnose-1,4-lactone with concomitant reduction of NAD^+. The concentration of NADH formed is proportional to the concentration of α-L-rhamnoside and can be determined by spectrophotometric measurement.

MATERIALS AND METHODS

Materials

Naringinase from *Penicillium* species, consisting of α-L-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase activities (EC 3.2.1.21), NAD^+, NADH, controlled pore glass (100–200 mesh, mean pore diameter 253 Å), trinitrobenzene sulfonic acid, 3-aminopropyl triethoxysilane, naringin ((S)-7-[2-O-(6-Deoxy α-L-mannopyranosyl)– β-D-glucopyranosyl]-oxy-2,3- dihydro-5-hydroxy-2- (4-hydroxyphenyl)- 4H-1-benzopyran-

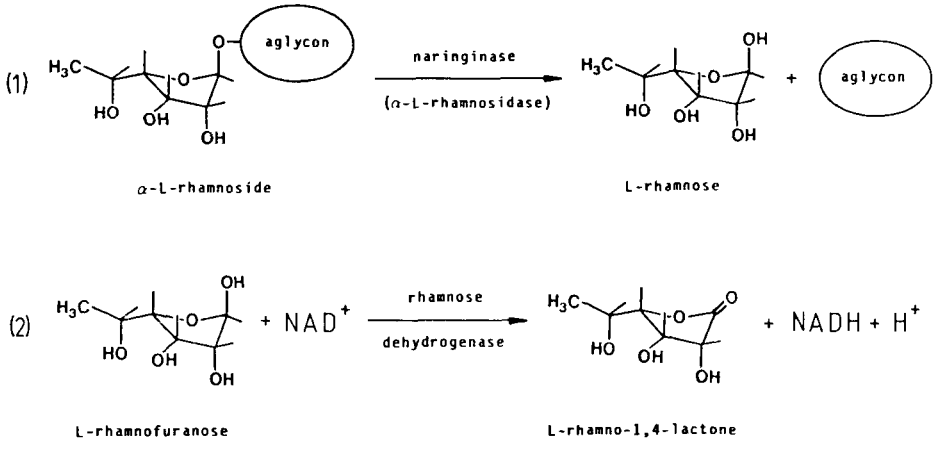

Fig. 1. Reaction steps of the assay.

4-one [10236-47-2]), naringenin ((S)-2,3-Dihydro-5,7- dihydroxy-2-(4-hydroxyphenyl)- 4H-1-benzopyran-4-one [480-41-1]), rhamnose, cyanogen bromide, 4-nitrophenol, pyridine- 4-aldehyde, dithiothreitol, glutathione (reduced form), convallatoxin (3-[(6-Deoxy α-L-mannopyranosyl) oxy]5,14-dihydroxy-19-oxo-(3 β,5β)-card-20(22)- enolide [508-75-8]) and strophanthidin (3,5,14-Trihydroxy-19-oxo- (3β,5β)-card-20(22)- enolide [39006-76-3]) were obtained from Sigma, St. Louis; silica gel plates 60F254, ninhydrin, EDTA, organic solvents, all buffer substances and inorganic compounds were purchased from Merck, Darmstadt; frangulin (3-[(6-Deoxy- α-L-mannopyranosyl) oxy]-1,8- dihydroxy-6- methyl-9,10- anthracenedione [521-62-0]) was obtained from Roth, Karlsruhe; periplorhamnoside (3-[(6-Deoxy- α-L-mannopyranosyl) oxy]-5,15- dihydroxy- (3β,5β)-card-20(22)- enolide [1064-16-0]), scilliphaeosid (3-[6-Deoxy- α-L-mannopyranosyl) oxy]-12,14- dihydroxy (3β,12β)- bufa-4,20,22-trienolide [21256-71-3]) and proscillaridin A (3-[(6-Deoxy- α-L-mannopyranosyl) oxy]-14-hydroxy-(3β)- bufa-4,20,22-trienolide [466-06-8]) were gifts from the Pharmacognostic Institute of the University of Vienna; scilliglaucosidin α-L-rhamnoside (3-[(6-Deoxy- α-L-mannopyranosyl) oxy]-14-hydroxy-19- oxo-(3β)-bufa-4,20,22-trienolide [25955-03-7]) was a gift of Laevosan, Linz. All reagents were of analytical grade or the best grade available. *Pullularia pullulans* (synonyms: *Aureobasidium pull., Dematium pull.*) was from Deutsche Sammlung von Mikroorganismen, Goettingen.

METHODS

Spectrophotometric measurements were carried out on a Beckman-25 spectrophotometer. Induction and isolation of L-rhamnose dehydrogenase in *Pullularia pull.* was carried out by slightly modifying a procedure described by Rigo et al. (*8*): The fungus was grown at 30°C in a liquid medium containing (per L): $(NH_4)_2SO_4$, 2 g; NH_4NO_3, 1 g; KH_2PO_4, 1.25 g; Na_2HPO_4, 0.184 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 10.6 mg; $ZnCl_2$, 4.16 mg; $MnCl_2 \cdot 4H_2O$, 3.6 mg; KCl, 0.74 mg; NaCl, 0.25 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.03 mg; $CaCl_2 \cdot 2H_2O$, 0.132 g; $CuSO_4 \cdot 5H_2O$, 7.6 mg; yeast extract, 1.0 g; L-rhamnose, 5 g. (The sugar has to be sterilized in a separate flask and added to the solution of inorganic ions before use). After 3 d, the cells could be harvested by centrifugation with yields of 8 g/L (wet wt). After washing the cells with a 0.15 M KCl solution, cell-free extracts were prepared by grinding in a mechanical homogenizer with glass beads in 0.05 M Tris/HCl buffer, pH 9.0, 1 m*M* with respect to EDTA. The suspension was freed from cellular particles by centrifugation and the supernatant fluid was used as a source of the enzyme. The partial purification of L-rhamnose dehydrogenase was accom-

plished according to (*8*), but for the assay described in this paper it is sufficient to isolate the enzyme using fractional ammonium sulfate precipitation, between 40–65% saturation.

Immobilization of the Enzymes

Preparation of Amino Glass Beads

According to the method of Weetall (*9*), controlled pore glass beads were treated for 1 h with 3% HNO_3 at 90°C, rinsed with water until the filtrate was neutral, and kept for several d in distilled water. The glass beads (20 g) then were mixed with 100 mL 10% 3-aminopropyltriethoxy silane and the pH was quickly adjusted to 3.5 with 6 M HCl. The suspension was gently agitated at 75°C for 2 h on a shaker. The coated glass beads were then filtered, rinsed with water, and dried overnight at 115°C.

Coupling of the Enzymes

Naringinase was coupled using two different methods (*10*). 1) Silane-coated amino glass beads (10 g) were suspended in 250 mL of a 2.5% solution of pyridine-aldehyde in 0.25 M phosphate buffer, pH 7. The mixture was degassed in vacuo and kept at room temperature for 1 h under constant shaking. The derivatized particles were collected by filtration, washed extensively with distilled water, and dried in a desiccator over NaOH. The pyridino glass beads thus obtained were suspended in 20 mL dry dioxane and treated with 3 mL cyanogen bromide solution (1 g BrCN/mL absolute dioxane) following a procedure previously described (*10,12*). After 5 min of activation 80 mL of 0.2 M borate buffer, pH 9, were added and the mixture stirred at room temperature for an additional 20 min. The pH was kept constant with 2 M NaOH by manual titration. The product was washed with 0.1 M acetate buffer, pH 4, on a sintered glass funnel until the filtrate gave no color reaction with pyridine. Then the activated carrier was incubated with 200–300 μkat naringinase dissolved in 30 mL 0.1 M acetate buffer, pH 4, or with 10–20 μkat rhamnose dehydrogenase in 20 mL 50 m*M* Tris buffer, pH 9 (1 m*M* with respect to EDTA and dithiothreitol) in a stoppered Erlenmeyer flask. (1 kat = 1 mol product formed/min). The suspension was kept, with shaking, for 24 h at room temperature. The filtered enzyme immobilizates thus obtained were washed with the respective incubation buffer, followed by 0.1 M NaCl solution until no more protein could be found in the filtrate with the aid of ninhydrin. The preparations can be stored as wet suspensions under sterile conditions at 4°C for prolonged periods

without loss of activity. In the case of rhamnose dehydrogenase all coupling procedures should be carried out under nitrogen. 2) Silane-coated glass beads (10 g) were moistened with a small amount of water and treated *in vacuo* until the pores of the glass beads were completely degassed. Next, the glass beads were incubated with 150 mL 10% aqueous glutaraldehyde for 4 h at room temperature with constant shaking. The glass beads were then filtered and washed with ice cold water until the filtrate showed no reaction with 2,4-dinitrophenyl hydrazine. Now the enzymes could be coupled to the activated carrier in the same manner as described in paragraph 1 of this chapter with the exception that in this case the enzymes have to be dissolved in 0.15 M phosphate buffer, pH 7.

To reduce unreacted aldehyde groups, the immobilized enzymes were finally treated with sodium borohydride.

Coupled Assay for the Determination of α-L-Rhamnosides

α-L-Rhamnosides (0.05–10 mg)—even relatively insoluble ones–were suspended in 3 mL 1 M acetate buffer, pH 4.0. The samples were incubated under shaking at 37°C for 24 h with 20 mg of immobilized naringinase or rhamnosidase. To be sure that the incubation time was long enough to split the rhamnosides under investigation quantitatively, aliquots were subjected to thin layer chromatography on silica gel plates (60F254 Merck). If necessary, the incubation time must be prolonged until the reaction is complete. After sedimentation of the glass beads, aliquots of 200 μL were taken from the supernatant fluid and diluted with water to 300 μL after neutralization with 46 μL 1 M NaOH. To aliquots of 150 μL neutralized sample, 50 μL 10 m*M* NAD^+ solution, 50 μL 0.05 M Tris/HCl buffer pH 9.0, 1 m*M* with respect to dithiothreitol and EDTA and 100 μL stabilized rhamnose dehydrogenase (*see* above) were added and incubated for 30 min at room temperature. Finally, the samples were diluted with water to 1 mL and the formation of NADH measured at 340 nm on the spectrophotometer as a function of time. A blank consisting of 200 μL buffer, 50 μL NAD^+ solution and 100 μL stabilized rhamnose dehydrogenase diluted to 1 mL was used. This blank, containing all components except the rhamnoside, corrects also for the change in absorbance due to the reduction of NAD^+ by the activity of other hydrogenases, which might be present in the assay mixture.

When the assay was carried out with immobilized rhamnose dehydrogenase, the procedure had to be altered in the following manner: to aliquots of 150 μL neutralized sample, 50 μL NAD^+ solution, 1 mL 50 m*M* Tris buffer (1 m*M* with respect to EDTA and dithiothreitol) and 50 mg immobilized rhamnose dehydrogenase were added and incubated under vigorous shaking for 120 min at room temperature. After rapid

centrifugation, the supernatant was measured spectrophotometrically at 340 nm. A blank lacking neutralized sample was used.

RESULTS

Enzymes Needed for the Assay

Rhamnosidase

Since it is rather difficult to obtain rhamnosidases, the commercially available enzyme complex naringinase, consisting of an α-L-rhamnosidase (EC 3.2.1.40) and a β-D-glucosidase (EC 3.2.1.21) was used. For the assay, the enzyme had to be used in its immobilized form, because otherwise its individual absorbance would interfere with the assay. The activity of the immobilized enzyme varied between 200–300 nkat/g dry carrier when tested with nitrophenylrhamnoside as an artificial substrate. The optimal pH was 4.0.

Rhamnose Dehydrogenase

The activity of the soluble enzyme preparations that had an absolute requirement for NAD^+ varied between 0.2–4 μkat/mL. The immobilized enzyme had an activity of 400–500 nkat/g dry carrier. The optimal pH was 9.0, the k_M values for L-rhamnose and NAD^+ were 0.2 m*M* and 0.02 m*M*, respectively. These results were in accordance with data published by Rigo et al. (*8*). The enzyme was highly specific for its substrate rhamnose. None of the following compounds tested under assay conditions could act as a substrate (nor did they act as inhibitors) when added to the standard incubation mixture at concentrations up to 10 μmoles/mL test solution: D-digitoxose; D-galacturonic acid; D-mannose; L-mannose; D-fucose; L-xylose; D-xylose; D-glucose; D-galactose; D-arabinose; L-arabinose; L-gulonic acid; D-melobiose; 2-deoxy-D-glucose; sorbose; D-ribose; 2-keto-L-gulose; D-lyxose; D-glucuronic acid.

A calibration curve of rhamnose dehydrogenase activity with L-rhamnose is shown in Fig. 2.

Assay of α-L-Rhamnosides

The determination of α-L-rhamnosides was carried out according to the procedure lined out in detail in the Methods section, using immobilized rhamnosidase coupled to rhamnose dehydrogenase. The assay can be applied to amounts of rhamnosides varying between 0.1–5 mg suspended per mL of incubation buffer. The standard curves given in Fig. 3 show a linear relationship of the α-L-rhamnoside proscillaridin A and the NADH formed after cleavage with immobilized and solubilized naringinase. Test runs with known amounts of various rhamnosides showed recoveries between 94–105%.

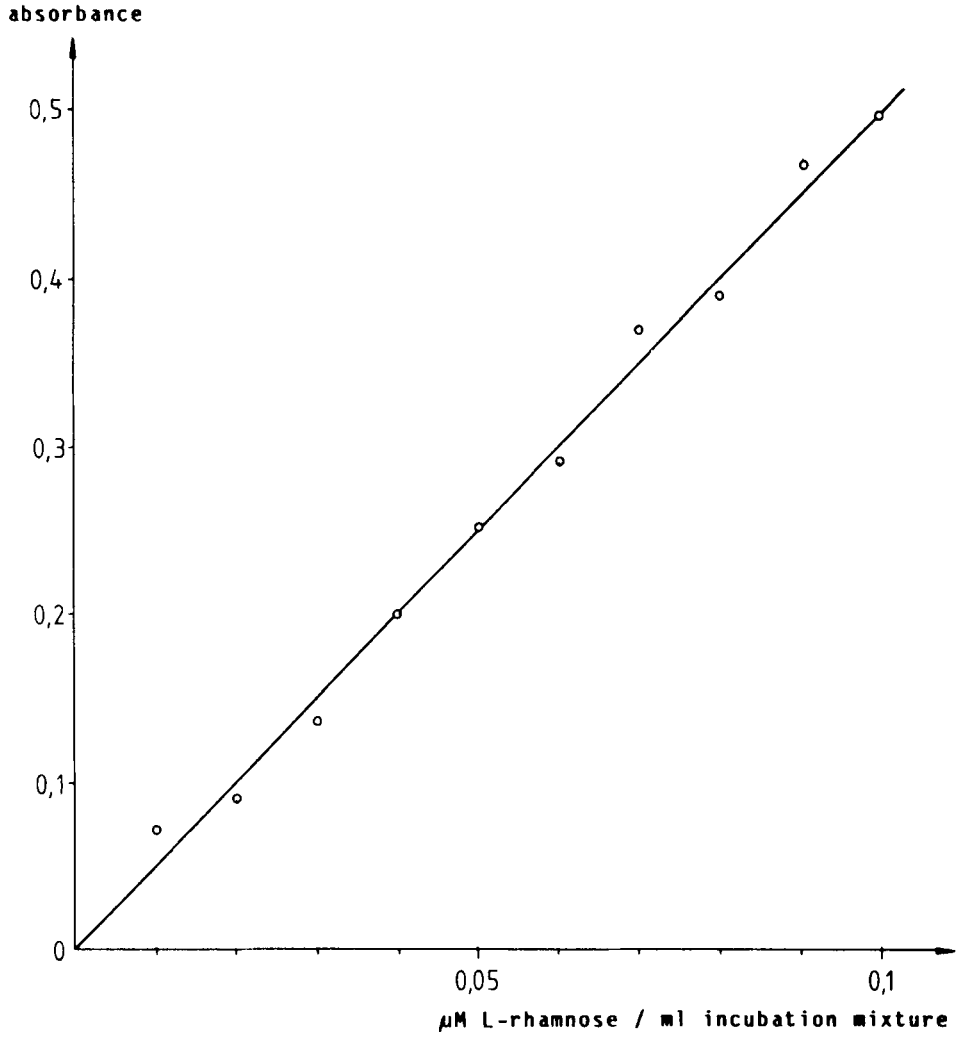

Fig. 2. Calibration curve with L-rhamnose.

Stabilization of Soluble Rhamnose Dehydrogenase

Rhamnose dehydrogenase is rather sensitive under working conditions to oxidation by air and to proteolytic degradation. D,L-Dithiothreitol and gluatathione, reduced form (1 m*M*), were necessary to stabilize or reactivate the enzyme. Therefore, dithiothreitol should be added continuously during all purification steps and under assay conditions. Inhibitors of proteinases such as *N*-tosyl-L-lysine-chloromet hyl ketone (TLCK) and *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), 1 m*M* were used to stabilize the enzyme activity in crude cell extracts of *Pullularia pull.* (Fig. 4), which could be used as well as the purified enzyme for the assay of rhamnosides. To get further stabilization and to provide a means to reuse the enzyme, rhamnose dehydrogenase was covalently immobilized on controlled pore glass as described in the section Materials and

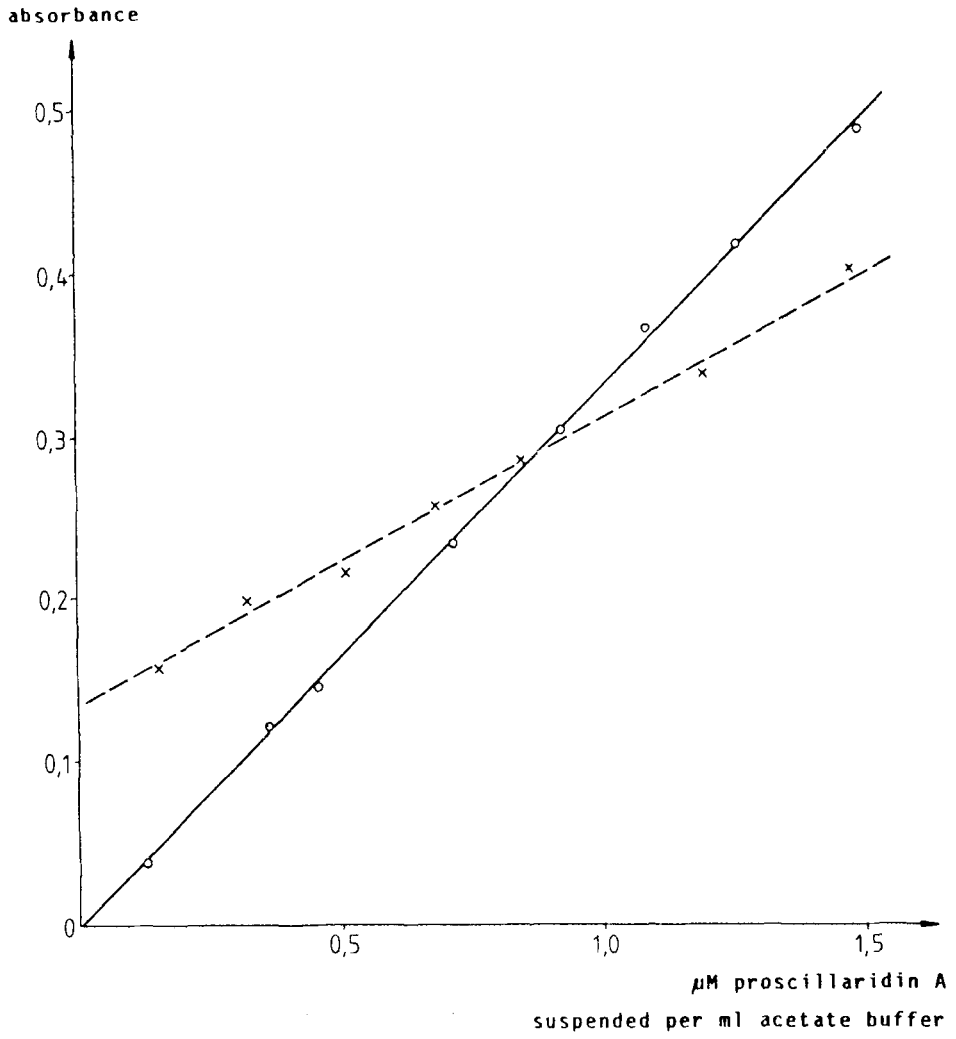

Fig. 3. Calibration curves of the assay of the α-L-rhamnoside proscillaridiin A using L-rhamnose dehydrogenase from *Pullularia pullulans*; absorbance at 340 nm as a function of the proscillaridin A concentration in incubation solutions with solubilized (---) and immobilized (——) naringinase.

Methods. Reactivation of the immobilizates with dithiothreitol before reuse is highly recommended.

REMARKS

The enzymatic assay described in this article can be applied to all rhamnosides (even relatively insoluble ones) which can be accepted as substrates by naringinase. The test is specific for α-L-rhamnosides, which can be determined even in the presence of other glycosides, sugars, or deoxysugars. The procedure was tested with flavonoid-, anthraquinone-, and steroid rhamnosides (*7,11*). Furthermore, pectins, heteropolysaccha-

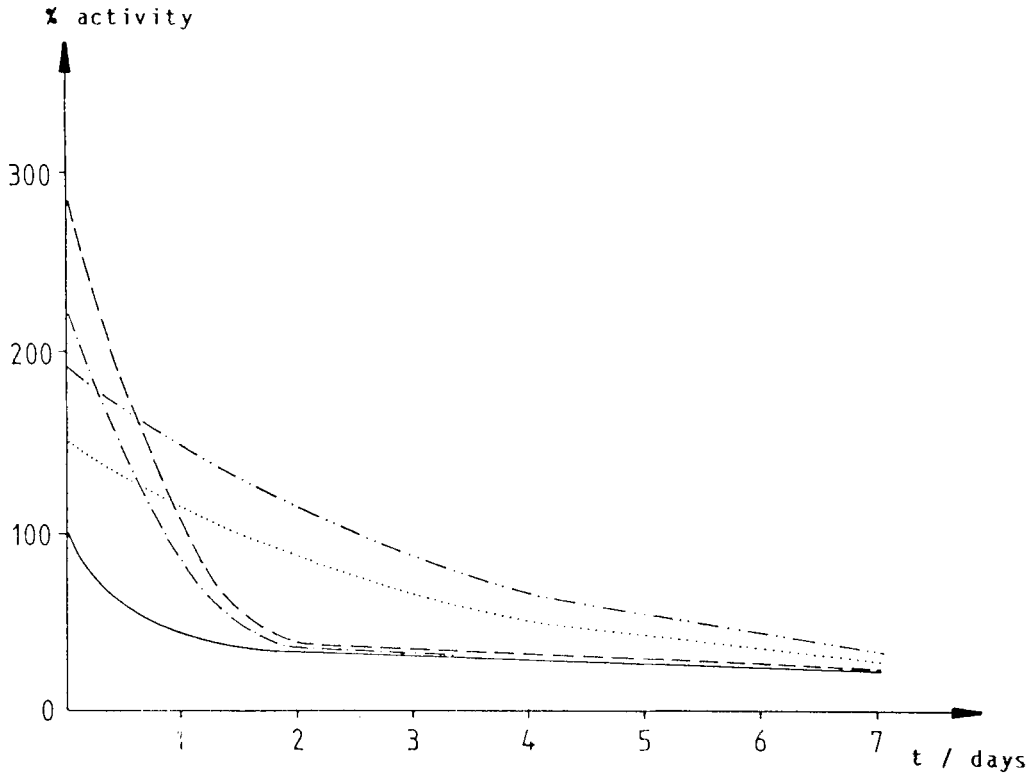

Fig. 4. Activity of L-rhamnose dehydrogenase as a function of time. (——) unstabilized rhamnose dehydrogenase; (- - - -) after stabilization with dithiothreitol; (·—·) with glutathione, reduced form; (··—··) with dithiothreitol and TPCK; (·····) with glutatione and TPCK; 100 % activity ≙ 1.5 μkat/mL, resembling the activity of unstabilized, soluble rhamnose dehydrogenase in the crude extract immediately after grinding the cells (see Methods section).

rides, and other compounds containing rhamnose may be quantitatively assayed if it is possible to liberate rhamnose from these compounds by chemical or enzymatic cleavage. Investigations employing pectic enzymes to split rhamnose from pectins or related compounds are in progress and will be published later. The immobilized enzymes needed for the assay could be reused after washing with buffer without loss in activity and were stable for two yr when stored in buffer at 4°C under sterile conditions.

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