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Preparation of Prunin with the Help of Immobilized Naringinase Pretreated with Alkaline Buffer

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ABSTRACT

Immobilized naringinase can be converted to a preparation showing only rhamnosidase activity by treatment with 0.1*M* glycine–NaOH buffer, pH 12. A simple method is described to obtain pure prunin in high yield from naringin with the help of this immobilizate.

Index Entries: Naringinase, prunin preparation from; prunin, conversion from naringinase.

INTRODUCTION

Immobilized rhamnosidases can be very useful for structural studies of glycosides since they specifically split rhamnosides and can easily be removed from the reaction mixture. Unfortunately, rhamnosidases are hardly available. Therefore, we converted immobilized naringinase (a seemingly single enzyme protein with two activities, namely α-L-rhamnosidase (E.C.3.2.1.40) and β-D-glucosidase (E.C.3.2.1.21) activities, to an enzyme preparation with mere rhamnosidase activity by simple treatment with alkaline buffer.

The flavonoid prunin obtained from naringin by this enzymatic procedure (Fig. 1) possesses anti-inflammatory activity (*1*), may be used as

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sweetening agent in diabetes therapy (2), and is above all desired for biological testing. Prunin has not yet been readily available in the amounts needed, though the rhamnoglucoside naringin is one of the most readily available flavonoid compounds at present. There are already some procedures published to synthesize prunin in larger amounts: Fox et al. (3) prepared the compound by hydrolysis of naringin with formic acid; unfortunately, time-consuming purification steps are needed to obtain a pure product with this method. Another organic synthesis of prunin and other mono- and diglycosides of flavanones has been published by Bognar et al. (4); with this method, several synthetic steps are necessary. The simple enzymatic preparation described in this paper results in pure prunin with high yield. The enzyme–immobilizate is stable under continuous use for months without loss of activity and can be easily removed from the reaction mixture.

MATERIALS

Naringinase from penicillium species, naringin, naringenin, controlled pore glass (100–200 mesh, mean pore diameter 253 Å), trinitrobenzene sulfonic acid (TNBS), and 2-aminodiphenyl-3-aminopropyl triethoxysilane were from Sigma, St. Louis. TLC plates, silica gel 60F254 with concentrating zone, TLC cellulose plates, organic solvents, glutaraldehyde, ninhydrin, and all buffer substances were from Merck, Darmstadt. Miracloth came from Calbiochem, Luzern.

METHODS

Assay of Naringinase and Its Components, Rhamnosidase and Glucosidase

These assays for activity were carried out following a method of Habelt and Pittner (5). This method was also used for the quantitative determination of naringin, prunin, or naringenin.

Chromatographic Detection of Naringin, Prunin, and Naringenin

These compounds were determined chromatographically on TLC plates silicagel 60F254 with concentrating zone in order to have a semiquantitative control for the reaction and to check the purity of the compounds. The plates were developed in an acetone–chloroform–water mixture (80:20:4.8). The detection of the spots was carried out either under UV at 254 nm or by spraying with vanillin–H_2SO_4 according to Le Rosen et al. (6) and Tyihak et al. (7) or by insertion into a J_2 chamber. R_F (naringin) = 0.17; R_F (prunin) = 0.33; R_F (naringenin) = 0.64.

Chromatographic Detection of Liberated Sugars

These were done on TLC cellulose plates that were developed with acetone–water (85:15). The chromatograms were qualitatively interpreted after treatment with aniline phthalate (*8*): R_F (rhamnose) = 0.7; R_F (glucose) = 0.26.

Determination of Protein Content

The qualitative determination of immobilized protein was carried out on an amino acid analyzer or according to Jacobs (*9*) after acid hydrolysis with 6*M* HCl in a sealed tube for 22 h at 110°C.

Activation of the Carrier for the Immobilization of Naringinase

Preparation of Amino Glass Beads According to the Method of Weetall (10)

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 days in distilled water. The glass beads (20 g) were then 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 filtered, rinsed with water, and dried overnight at 115°C.

Activation with Glutaraldehyde (11)

Silane-coated glass beads (5 g) were moistened with a small amount of water and treated *in vacuo* until the pores of the glass beads were completely degassed. Glutaraldehyde activation was performed by shaking the glass beads with 100 mL 10% aqueous glutaraldehyde solution for 4 h at room temperature. The glass beads were then filtered and washed with ice-cold water until the filtrate showed no reaction with 2,4-dinitrophenyl hydrazine. A negative TNBS test (*11,12*) indicated the completion of the reaction.

RESULTS

Preparation of Immobilized Naringinase

Naringinase lyophilizate (500 mg) dissolved in 200 mL of 0.15*M* phosphate buffer, pH 7, was mixed in a 500 mL Erlenmeyer flask with 3 g wet glutaraldehyde-activated amino glass beads prepared according to the procedure given in the Methods section. For coupling of the naringinase to the carrier, the stoppered flask was kept under shaking at 30°C for 22 h. The filtered immobilizate was washed with buffer, followed by distilled water, until no protein could be found in the filtrate

with ninhydrin reagent. The immobilized naringinase thus obtained contained 3.4 mg protein/g wet carrier (9.7 mg protein/g dry carrier). Aliquots (20 mg) converted 1.7 μmol of naringin to prunin and naringenin, respectively (Fig. 1), within 30 min at 37°C when assayed according to Habelt and Pittner (*5*).

Conversion of the Naringinase Immobilizate to a Preparation with Rhamnosidase Activity Only

The immobilized naringinase was treated overnight on a shaker with an excess of 0.1*M* glycine/NaOH buffer, pH 12, at room temperature until glucosidase activity could no longer be observed. The tests for activity must be made after washing with water until all glycine/NaOH buffer was removed (*see* Methods). The rhamnosidase activity thus obtained was at the utmost 10% lower than the activity of the rhamnosidase in the untreated naringinase immobilizate. The rhamnosidase thus obtained could be used continuously for the preparation of prunin for months without a loss in activity when 37°C was not exceeded.

Enzymatic Preparation of Prunin

In preliminary experiments the substrate naringin was used in solution, which led to large volumes of the reaction mixture since the solubility of naringin is rather low. (To dissolve 500 mg of naringin, 1200 mL of buffer solution are necessary.) Since the reprocessing of such amounts of solution is laborious and time-consuming, a procedure was worked out allowing the use of solid naringinase dispersed in the reaction mixture.

In a plastic vessel with a screw cap (6 × 3 cm) several holes with a diameter of 1 cm were drilled and tightly covered with Miracloth, as

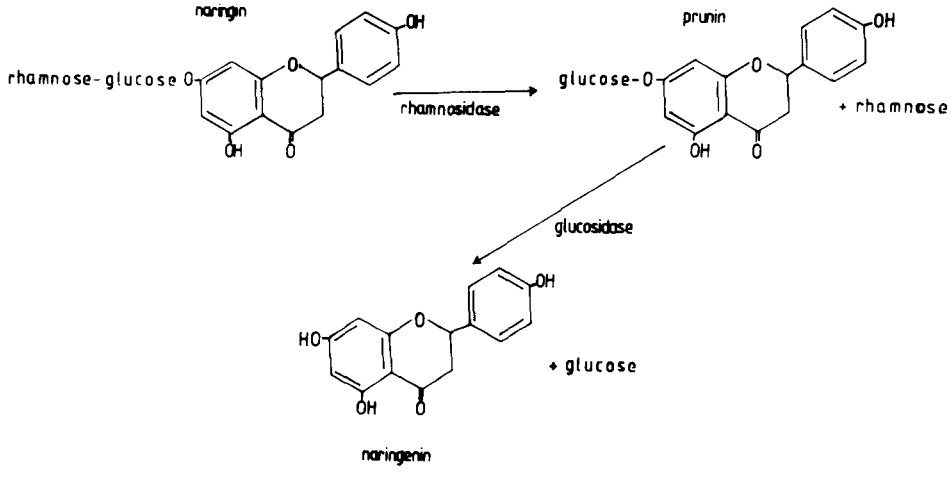

Fig. 1. Degradation of naringin by the action of naringinase.

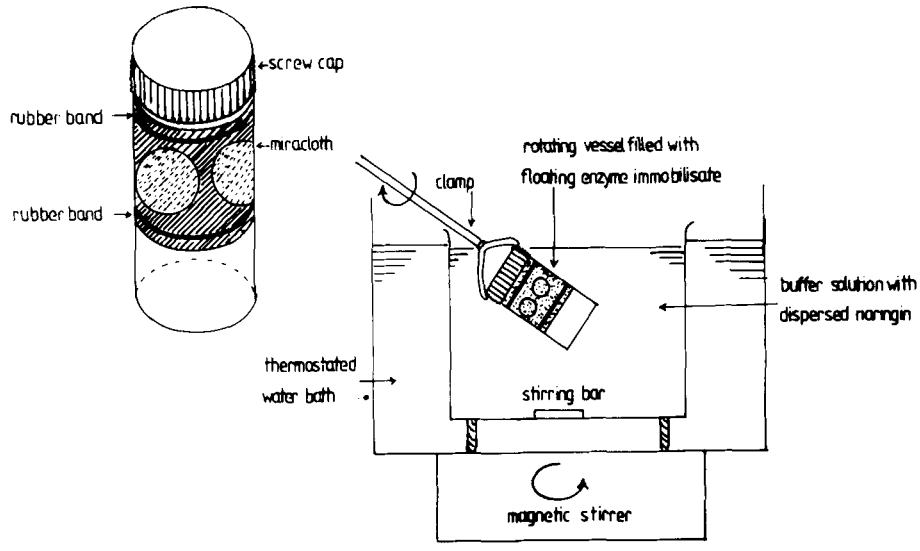

Fig. 2. Experimental arrangement for the preparation of prunin.

shown in Fig. 2. The covered holes are permeable to buffer and dissolved substrate or product, but prevent the passage of immobilizate or undissolved naringin particles. The vessel thus prepared was filled with 2 g of rhamnosidase immobilizate and fastened in a clamp that was connected to a stirrer. This inclined rotating device was dipped into a 1000 mL beaker containing 5 g of naringin suspended in 700 mL of water and 200 mL of 0.2*M* acetate buffer, pH 4. The glass beads with the immobilized enzyme were kept afloat by the rotation of the vessel, and the naringin-containing buffer solution was stirred with a magnetic stirrer. The conversion to prunin was carried out at 37°C in a thermostated water bath. The formation of the product was tested with thin layer chromatography (*see* Methods). After 24 h, 95% of the naringin was converted to prunin. After 48 h, the yield was 98%.

After this incubation time, the aqueous reaction mixture was removed from the reactor and precipitated prunin was collected by filtration and dried in a vacuum desiccator. The solution was extracted with 200 mL of ethyl acetate in small portions and the combined organic fractions were evaporated after drying with Na_2SO_4 to about 5 mL. Prunin was precipitated by the addition of about 15 mL of chloroform. The prunin fraction obtained by filtration was also purified further by dissolving in ethyl acetate and precipitation with chloroform. If the product thus obtained is still contaminated with traces of naringin, this purification step can be repeated, followed by recrystallization from aqueous methanol. Quantities of 2 g of pure prunin could be thus obtained. Attempts to increase the yield may result in a slightly less pure product.

A variant of the described procedure was to avoid the suspension of the substrate in the buffer solution by incubating the solid naringin entrapped in a dialysis sac. The disadvantage of this variant was the pro-

longed incubation time (about 4 d) necessary to form the wanted product. The prunin thus obtained had a melting point of 225–226°C, which is identical with the data given in the literature (*3*). Chromatography on silica gel TLC plates 60F254 with acetone–chloroform–water (80:20:4.8) (*see* Methods) showed the presence of only one spot (R_F = 0.33) according to the migration length of prunin. After enzymatic hydrolysis of the substance with immobilized naringinase, thin layer chromatography on cellulose plates with acetone–water (85:15) showed the presence of glucose (R_F = 0.26), but no rhamnose (R_F = 0.7). The aglycon thus obtained was identical with a pure authentic sample of naringenin (recrystallized product of Sigma). Mixed chromatograms of the prepared aglycon with naringenin showed no separation (R_F for both was 0.64). The aglycon isolated by extraction with ethyl acetate and recrystallized from aqueous ethanol had a melting point of 251°C in accordance with the melting point of naringenin (*13*). No lowering occurred on the addition of a sample of recrystallized, authentic naringenin.

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