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# Simple Enzyme Reactors Suitable for the Byproduct-Free Preparation of the Aglycones of Naturally Occurring Glycosides Under Mild Conditions

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## ABSTRACT

Naringinase from Penicillium species—containing α-L-rhamnosidase and β-D-glucosidase activity—immobilized on silicate carriers can be used as a mild and effective means to cleave naturally occurring glycosides into their aglycones and sugar components. The procedure was tested with flavonoid-, anthraquinone-, and steroid-glycosides. Since the solubility of such compounds is limited in aqueous solutions, a simple batch procedure has been developed to convert solid substrates suspended in only small amounts of buffer to the desired products with high yields.

**Index Entries:** Immobilized naringinase; glycosides; aglycones, preparation of; glycosides, cleavage of.

## INTRODUCTION

In many fields (e.g., natural products chemistry, pharmacognosy), when dealing with the elucidation of chemical structures of naturally occurring compounds, it is of great interest to obtain the aglycones of glycosides with high yield under mild conditions to avoid artifacts and unwanted by-products. Since acid hydrolysis destroys many glycosides,

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it is desirable to avoid this treatment by the employment of immobilized glycosidases, working under very mild conditions. However, we have to deal with additional problems, since the solubility of many glycosides in aqueous solutions is rather low, leading to high volumes of solutions and laborious and time-consuming reprocessing procedures.

To avoid these problems we introduced an enzyme reactor that converts solid substrate, suspended in only small amounts of buffer, to the desired product. The conversion of naringin to prunin with the aid of immobilized rhamnosidase was quoted as an illustration in a previous paper (*1*). In this paper we describe the cleavage of flavonoide-, anthraquinone-, and steroid- rhamnoside on a laboratory scale in a simple batch reactor, with the aid of immobilized naringinase, an enzyme, consisting of α-L-rhamnosidase- (EC 3.2.1.40) and β-D-glucosidase activities (EC 3.2.1.21).

## MATERIALS

Controlled pore glass (100–200 mesh, mean pore diameter 253 Å), trinitrobenzene sulfonic acid, 4-nitrophenyl-glucoside, 3-aminopropyl triethoxy silane, naringinase from Penicillium species, naringin, naringenin, rhamnose, cyanogen bromide, 4-nitrophenol, pyridine-4-aldehyde, triethanolamine, convallatoxin, and strophanthidin were obtained from Sigma, St. Louis, MO; silicagel plates 60F254, urea, ninhydrin, $ZnCl_2$, $AlCl_3 \cdot 6H_2O$, NaCl, $CaCl_2$, $NH_3$, acetic anhydride, sodium citrate, picric acid, organic solvents, all buffer substances, and inorganic acids were purchased from Merck, Darmstadt; Dowex 1 × 4 ion exchanger was obtained from Fluka, Buchs; quercitrin and frangulin A and B were obtained from Roth, Karlsruhe; periplorhamnoside, scilliphäosid, convallosid, and proscillaridin A were gifts from the Pharmacognostic Institute of the University of Vienna; Scilliglaucosidin-α-L-rhamnoside was a gift of Laevosan, Linz; and gulsenit was a gift of Magindag, Vienna.

## METHODS

### *Preparation of Pyridino Carriers*

Gulsenit or controlled pore glass was coated with 3-aminopropyl triethoxy silane, according to the method of Weetall (*2*). The silicate-carrier particles were treated for 1 h with excess 3% $HNO_3$ at 90°C, rinsed with water until the filtrate was neutral, and kept for several days in distilled water. The particles (20 g) were then mixed with 10 mL 10% 3-aminopropyl triethoxy 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 silane-coated amino carriers were filtered, rinsed with water, dried over night at 115°C, and treated further according to a procedure described in a previous paper (*3*).

Silane-coated amino glass beads, or amino gulsenit (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 kept at room temperature for 1 h under constant shaking. The derivatized particles were collected by filtration, washed excessively with distilled water, and dried in a desiccator over NaOH. The completion of the reaction was indicated by a negative test with trinitrobenzene sulfonic acid.

### Determination of Protein Content

The quantitative determination of immobilized protein was carried out on an amino acid analyzer, or according to Jacobs (*4*), after acid hydrolysis with 6*M* HCl in a sealed tube for 22 h at 110°C.

### Enzyme Assay with Artificial Substrates

A substrate solution containing 28.4 mg 4-nitrophenyl rhamnoside or 31.5 mg 4-nitrophenyl glucoside in 100 mL 1*M* acetate buffer, pH 4, was prepared. A mixture of 3 mL substrate solution and 1 mL buffer, containing 0.2 mg soluble naringinase or 20 mg enzyme immobilisate, was incubated under shaking at 37°C for 15 min, together with a blank, without enzyme. Aliquots (1 mL) of the incubation mixture were mixed with 5 mL of a 50% aqueous triethanolamine solution, and the absorbance was measured spectrophotometrically at 405 nm. A calibration curve with 4-nitrophenol (25–350 nmol/mL) was used for quantitative estimation.

### Assay of Glycosides and Their Aglycones

For the separation, identification, and semiquantitative estimation of all compounds, thin-layer chromatography on silica gel plates (60F254, Merck) was used. The various conditions are listed in Table 1.

For the quantitative determination of steroid compounds, HPLC on a Perkin-Elmer (Series 3) apparatus with Rheodyne injector was used. A column (250 × 4 mm) filled with reversed-phase material, Polygosil 60-7/C18 (7 μ*M* of Macherey-Nagel), was employed as a separating device. The separation of the glycosides and their aglycones was carried out by a gradient of 23–70% acetonitril within 30 min. The separated compounds were assayed in a flow cell at 300 nm.

Naringin, naringenin, and prunin were extracted from the reaction mixtures with ethyl acetate and assayed spectrophotometrically, following a procedure previously published (*5*).

Convallosid, convallatoxin, and strophanthidin were assayed quantitatively by modifying a procedure according to Wichtl (*6,7*). The compounds were extracted from the incubation mixture with chloroform–ethanol (2:1) and, after evaporation of the solvent, rediluted in chloroform–methanol (1:1). The samples thus obtained were sub-

TABLE 1
Thin-Layer Chromatography of the Studied Compounds on TLC Plates Silica Gel 60F254

| Compound | | Extractive agent | Development | Detection of the spots | $R_f$ | |
|---|---|---|---|---|---|---|
| Glycoside | Aglycone | | | | | |
| Naringin<br>Prunin | Naringenin | Chloroform | Acetone/$CHCl_3$/water (80:20:4.8) | UV at 254 nm; spraying with reagent A[a], heating at 105°; $j_2$ chamber | 0.20<br>0.30 | 0.70 |
| Quercitrin | Quercetin | Ethylacetate | Acetone/$CHCL_3$ (80:20) | UV at 254 nm; $J_2$ chamber individual color | 0.26 | 0.62 |
| Frangulin A<br>Frangulin B | Frangula emodin | Ethylacetate | $CHCl_3$/ethanol (10:1) | UV at 254 nm; individual color | 0.34<br>0.30 | 0.65 |
| Proscillaridin A | Scillarenin | Chloroform | $CHCl_3$/methanol/water (70:22:3.9) | Spraying with $H_2SO_4$ | 0.59 | 0.69 |
| Scilliphäosid | Scilliphäosidin | | | | 0.61 | 0.79 |
| Scilliglaucosidin α-L-rhamnoside | Scilliglaucosidin | | | | 0.49 | 0.66 |
| Periplorhamnoside | Periplogenin | | | | 0.57 | 0.74 |
| Convallatoxin | Strophanthidin | | | | 0.61 | 0.75 |

[a]Reagent A: 0.1 g vanillin + 4 mL ethanol + 16 mL $H_2SO_4$ conc.

jected to descending paper chromatography (paper: Schleicher & Schüll 20436 Mgl) on papers pretreated with a mixture of formamide–acetone (1:4) for 8 min. The chromatograms were developed with chloroform–tetrahydrofuran–formamide (50:50:6.5) for 6–7 h and dried for 1 h at 120°C. The detection of the standard spots was carried out by spraying with Baljet reagent (95 mL 1% aqueous picric acid and 5 mL 10% NaOH). The corresponding cross sections of the sample lanes were cut out and eluted with methanol. The various sample batches thus obtained (3 mL) were incubated with the same volume of Baljet reagent and measured spectrophotometrically after 18 min at 494 nm on a Beckman DU8 spectrophotometer.

All other compounds were separated via thin-layer chromatography (*see* Table 1). The separated compounds were eluted and assayed spectrophotometrically.

Flavonoides (except naringin, prunin, and naringenin) were assayed with a modified procedure according to Christ and Müller (*8*). The samples, eluted with ethyl acetate (5 mL), were mixed with 250 μL of a 0.5% aqueous sodium citrate solution and 1 mL of a reagent containing 2 g $AlCl_3 \cdot 6H_2O$ in 100 mL methanol–acetic acid (95:5). After 45 min the absorbance was measured at 425 nm. The anthraquinone compounds were assayed using the Bornträger reaction (*9*). The spots on the thin-layer plates with individual colors were eluted with 2 mL ethyl-acetate, which was extracted with 5 mL of 2*M* aqueous NaOH solution containing 18% concentrated $NH_3$. After 30 min the samples were measured spectrophotometrically at 530 nm.

## Preparation of 4-Nitrophenyl Rhamnoside

The synthesis and purification of 4-nitrophenyl rhamnoside was carried out following a procedure of Westphal and Feier (*10*), with several modifications. Water-free L-rhamnose (10 g) was heated under reflux with 5 g dry sodium acetate in 90 mL acetic anhydride for 90 min. After cooling, the clear solution was poured into 500 mL ice-cold water. After 3 h the aqueous solution was extracted with chloroform. After evaporation of the organic solvent, 10.8 g tetraacetyl-rhamnose was obtained, which was melted together with 14 g 4-nitrophenol at 120°C at reduced pressure until the gas formation came to an end. Then, 5 g freshly dehydrated, powdered $ZnCl_2$ was added and the mixture again kept at 120°C until no more acetic acid was evolved. After cooling, the mixture was dissolved in 200 mL chloroform, followed by extraction with 1*M* NaOH until unreacted nitrophenolate was removed. Remaining traces were removed with Dowex 1 × 4 ion exchanger at pH 8.5. For further purification, the mixture was extracted several times with water and dried over $CaCl_2$. After evaporation of the chloroform, the residue was recrystallized several times in methanol after treatment with charcoal, yielding 4.7 g pure acetylated product. The conversion to 4-nitrophenyl-α-L-rhamnoside

was carried out by dissolving the substance in 100 mL methanol and adding 7 mL 0.1*M* sodium methylate. After heating under reflux for 15 min, the solvent was evaporated and the final product recrystallized from ethanol, yielding 1.2 g of colorless needles [mp 179°C $(\alpha)D^{20}$: −144° (methanol)].

## RESULTS AND DISCUSSION

### *Preparation of Immobilized Naringinase*

Pyridino glass beads or pyridino gulsenit (5 g)—prepared according to the Methods section—were suspended in 10 mL dry dioxane and treated with 2 mL cyanogen bromide solution (1 g BrCN/mL absolute dioxane), following a procedure previously described (*3,11*). After 5 min of activation, 40 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 sinter funnel until the filtrate gave no colored reaction with pyridine. Then the activated carriers were incubated with 500 mg naringinase dissolved in 25 mL acetate buffer, pH 4, in a stoppered Erlenmeyer flask. The suspension was kept—with shaking—for 24 h at 37°C. The filtered enzyme immobilisate thus obtained was washed with acetate 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 were stored as wet suspensions at 4°C and remained stable without loss of activity for 1 yr.

The immobilized naringinase thus obtained contained up to 20 mg protein/g dry carrier. The specific activity of the rhamnosidase component was 12.5 nkat/mg protein, the glucosidase activity was 8.0 pkat/mg protein measured with the artificial substrates, 4-nitrophenyl rhamnoside and 4-nitrophenyl glucoside, respectively. When cleaving the natural compounds, the activity varied, depending on the respective substrate (*see* below.)

### *Cleavage of Glycosides with the Help of a Batch Reactor*

#### *Proscillaridin A*

Optimum pH: For the cleavage of the substrate (*see* Fig. 1), 1-mg aliquots of proscillaridin A were suspended with 20 mg immobilized naringinase in 3-mL portions of buffer solutions, with the pH varying between 3 and 10. The various batches, as well as the blanks, containing all the components but the immobilized enzyme, were incubated in stoppered vessels under shaking for 1 h at 37°C, followed by extraction of the product and the unreacted glycosides with 2-mL portions of chloroform. This procedure did not inactivate the immobilized enzyme. The solvent was removed under reduced pressures and the residues dissolved in 1.5

mL 70% ethanol. Then 30-μL portions were assayed quantitatively with the help of HPLC (*see* Methods section). An optimum of pH 3 could be found for the formation of the aglycone scillarenin. Unfortunately, these acidic conditions may lead to slight destruction of the substances during prolonged incubation times. Therefore, a pH of 4 was used in the following procedures. Under this condition no decomposition of the aglycone was observed.

### *Optimal Temperature*

To obtain optimal temperature conditions, several parameters must be taken into consideration. The immobilized enzyme must not be attacked by heat, and no decomposition of substrate and product should occur. On the other hand, the desired product should be formed as quickly as possible. The turnover dependent on the temperature shows a distinct maximum at 80°C when incubated for 1 h, as described above (*see* Fig. 2). Unfortunately, even at lower temperatures the scillarenin formed was already partly decomposed; also, the immobilisate was slightly affected and continued to become denaturated during prolonged incubation times. Therefore, we had to use a temperature not exceeding 40°C. In this range, not only the studied glycosides and their aglycones, but also the immobilisate, remained intact.

### *Optimal Ratio of Immobilisate and Substrate*

To obtain fixed conditions, aliquots of 1 mg of proscillaridin A, suspended in 3 mL acetate buffer, pH 4, were incubated for 1 h at 37°C with varying amounts of naringinase immobilisate (5–300 mg) and assayed, as described above. The results are given in Fig. 3. Under these conditions, 200 mg of immobilisate gave optimal turnovers of the substrate. However, to obtain nearly quantitative formation of the aglycone, incubation for an additional 15 h was necessary.

### *Cleavage in Organic Solvents*

In looking for optimal working conditions, the behavior of the enzyme immobilisate in organic solvents was studied. Since at least small amounts of water are necessary for the action of our hydrolytic enzyme, 20-mg aliquots of naringinase immobilisate soaked with acetate buffer, pH 4, were incubated with 1 mg proscillarindin A dissolved in 3 mL of the various organic solvents and incubated at 37°C under shaking for 1, 60, and 80 h.

Aliquots (20-μL) of the organic phase of the various batches were assayed for product, as described above. The results are listed in Table 2.

One can see that the best results can be obtained with the most hydrophobic solvents. Solvents miscible with water inactivate the immobilisate. However, the turnover reached after 80 h could not be further increased unless 3 mL acetate buffer, pH 4, were added and the mixtures incubated for an additional 24 h. In that case, up to 95% of the product,

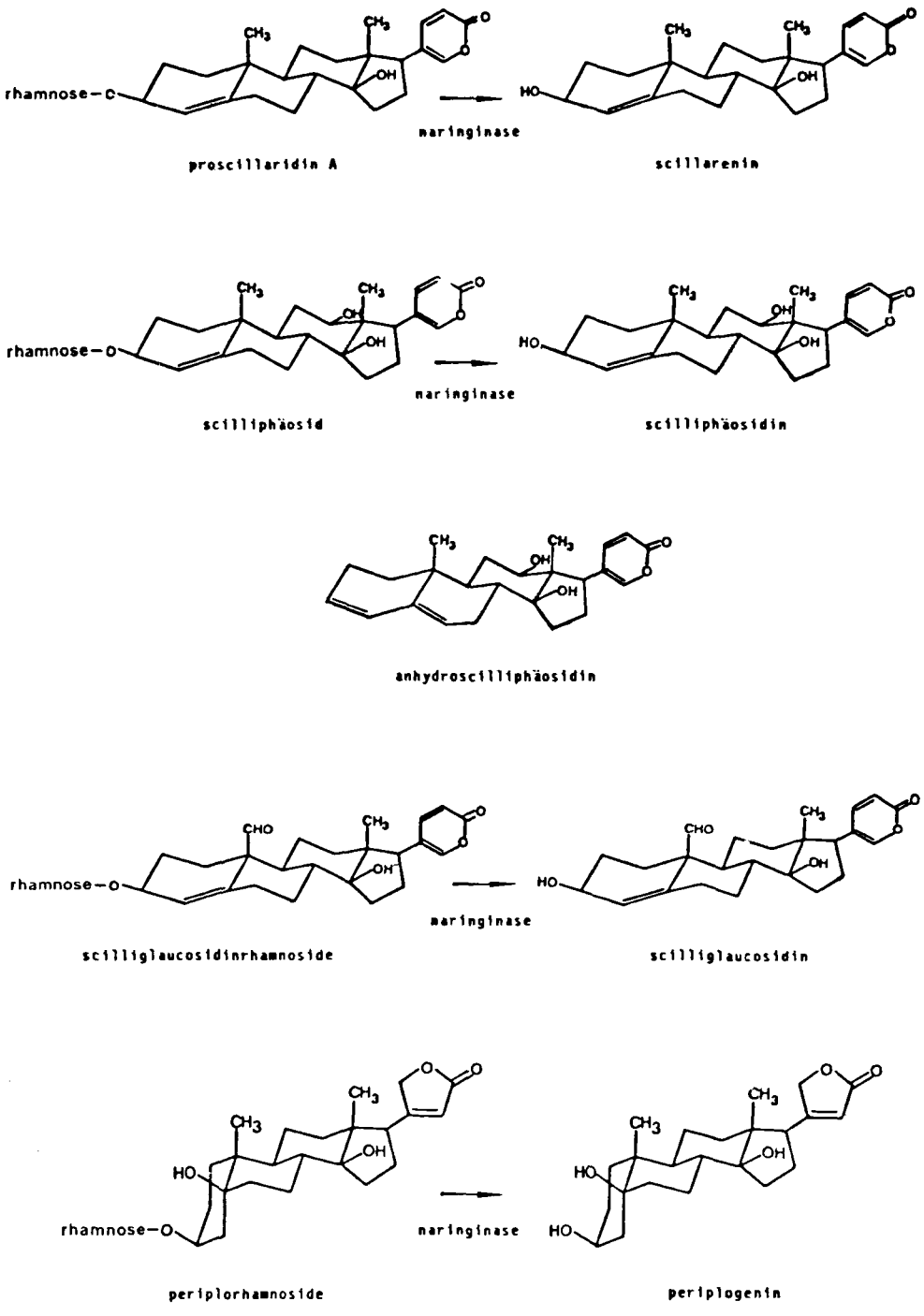


Fig. 1. Cleavage of the glycosides with the help of immobilized naringinase.

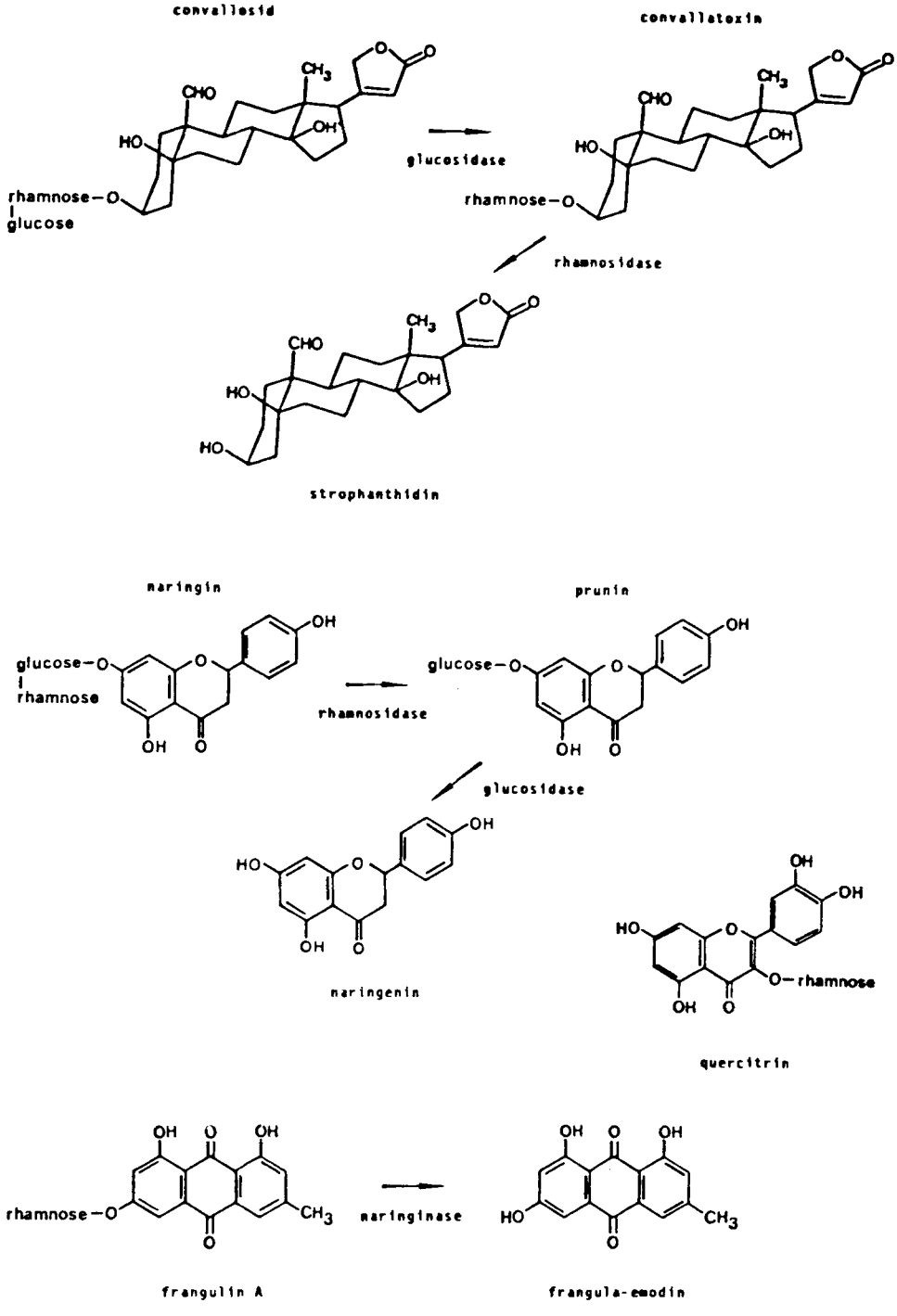


Fig. 1. (*continued*)

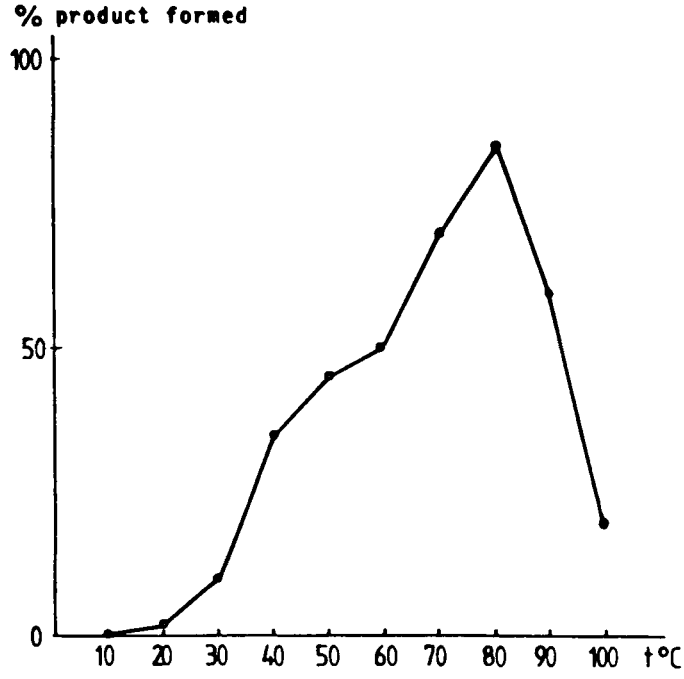


Fig. 2. Temperature effect on the activity of immobilized naringinase with proscillaridin A as a substrate.

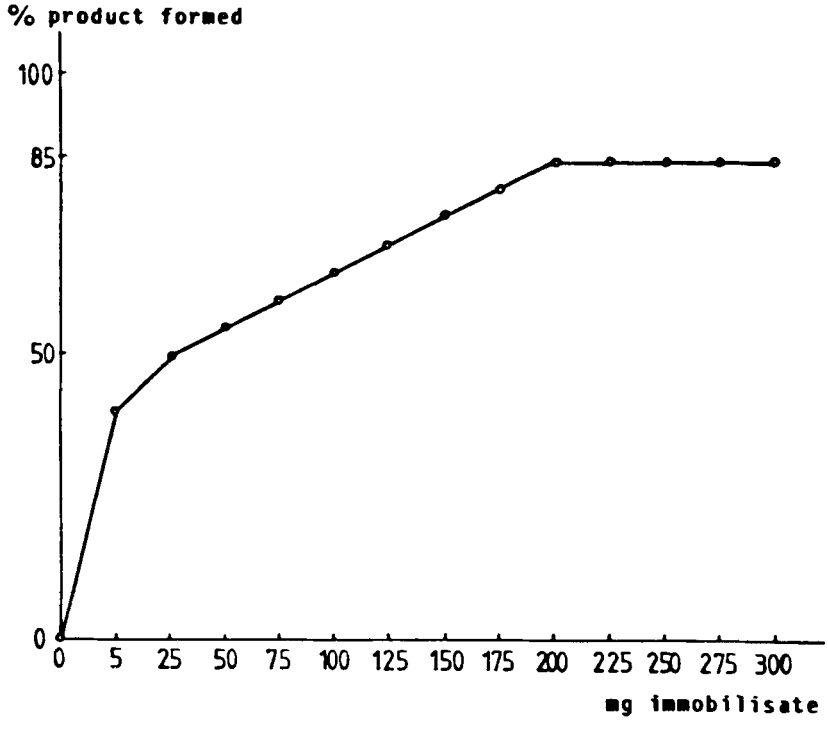


Fig. 3. Determination of the optimal ratio of immobilisate and substrate.

TABLE 2
Formation of Product in Organic Solvents

| Solvent | % Product formed after 1 h | 60 h | 80 h |
|---|---|---|---|
| $CCl_4$ | 15 | 50 | 60 |
| $CHCl_3$ | 5 | 20 | 35 |
| $CH_2Cl_2$ | 10 | 15 | 20 |
| $CHCl_3$ + $CH_3OH$ (1:1) | 0 | 0 | 0 |
| Acetone | 0 | 0 | 0 |
| Cyclohexane | 20 | 25 | 30 |
| Dioxane | 0 | 0 | 0 |
| Tetrahydrofurane | 0 | 0 | 0 |
| Toluene | 10 | 50 | 60 |
| Isopropanol | 0 | 0 | 0 |
| Ethylacetate | 5 | 30 | 30 |

scillarenin (in $CHCl_3$), could be formed. These results show that it is possible, in principle, to employ the enzyme immobilisate in organic, water-immiscible solvents if sufficient water is provided for the hydrolytic action of the naringinase. But this method is time consuming and does not provide optimal conditions.

The influence of urea on the enzymatic action was also tested, with concentrations of 0.5, 1, and 2*M*. These amounts of urea had virtually no influence on the velocity and amount of product formed. So it is advisable to employ the most simple procedure described above, using excess solid substrate suspended only in buffer, since this procedure brings about nearly quantitative formation of the product within a few hours.

### *Scilliphäosid (12-β-hydroxy-proscillaridin A)*

With scilliphäosid it is impossible to obtain the aglycon scilliphäosidin via acid hydrolysis, since chemical splitting results in the formation of anhydroscilliphäosidin (*see* Fig. 1). The enzymatic cleavage of the glycoside is therefore the method of choice. The optimized conditions are: Scilliphäosid (1 mg) suspended in 3 mL acetate buffer, pH 4, together with 50 mg immobilized naringinase. When incubated at 18°C for 3 h, the whole glycoside was converted to its aglycone and could be removed by extraction with chloroform.

### *Scilliglaucodisin-α-L-Rhamnoside*

Optimal conditions: Glycoside (1 gm) suspended with 50 mg enzyme immobilisate in 3 mL acetate buffer, pH 4, resulted in formation of 95% scilliglaucosidin when incubated at 37°C for 2 h.

### *Periplorhamnoside*

Optimal conditions: Quantitative formation of the aglycone after 4 h when treated in the same way as described above.

*Convallatoxin*

Unfortunately, the formation of the aglycone took a lot of time. When incubated in the same manner as mentioned above, 95% of the aglycone strophanthidin was not formed until 20 h. In the same way, both the rhamnosidase and the glucosidase activity of naringinase could be employed to split convallosid (Fig. 1) into its aglycone strophanthidin.

*Naringin*

This flavonoid glycoside is the proper substrate of the naringinase. When incubated in the usual way for 30 min, all substrate is converted into 30% prunin and 70% naringenin. After 2 h, only the aglycone naringenin could be found, which was removed by extraction with ethylacetate.

*Quercitrin*

This 3,3′,4′,5,7-pentahydroxyflavone 3-*O*-α-L-rhamnoside was used to show whether our enzyme is also capable of splitting rhamnosides of the flavonoid type with the sugar moiety located on other than the 7 position. Unfortunately, no conditions could be worked out to attack quercitrin at all. It has to be assumed that the dihydroxyphenyl residue in the 2 position vicinal to the glycosidic bond provides too much steric hindrance.

*Frangulin A*

The 1,6,8-trihydroxy-3-methyl-anthraquinone 6-α-L-rhamnoside can be converted to 98% frangula-emodin under standard conditions, within 4 h. Frangulin B, the corresponding apioside, occurring together with frangulin A, remains completely unattacked under the same conditions.

## CONCLUDING REMARKS

The data given in this paper show clearly that it is possible, with the help of immobilized naringinase, to split even hardly soluble glycosides into their aglycones and sugar component. Moreover, it is possible to avoid large volumes of aqueous buffer solutions or hydrophilic organic solvents to facilitate the solution of the substrates (which very often leads to undesirable denaturation of the enzyme protein). The most simple procedure turned out to give by far the best results. Even though the aglycones formed were removed from the reaction mixture by extraction with organic, water-immiscible solvents, the enzyme was not attacked by this procedure and could be reused many times without loss of activity.

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