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Studies on the Immobilization of Glucuronidase (Part 2)

Cleavage of Hardly Soluble Substrates in Organic Solvents

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ABSTRACT

Naturally occurring glucuronides and glucosides dissolved in organic solvents can be split with the help of β-glucuronidase (EC 3.2.1.31) immobilized on controlled pore glass. To protect the enzyme against denaturation by the organic solvents and to promote hydrolytic cleavage of substrates, two methods were used: (a) Immobilization via crosslinking with aged glutaraldehyde in presence of bovine serumalbumine; and (b) Adsorption of wet enzyme to the carrier in the presence of organic solvents.

Index Entries: Immobilized glucuronidase; hydrolysis in organic solvents; glucuronides; glucosides.

INTRODUCTION

In many biotechnological processes, it is desirable to use enzymes not only in aq. media but also in water immiscible organic solvents to convert substrates hardly soluble in water. Since most enzymes become denatured by organic solvents, certain devices are necessary to reach this aim. One

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approach, for example, is to use enzymes modified with polyethylene glycol. They are soluble in organic solvents (chlorinated hydrocarbons, toluene, and so on) and exhibit significant enzymatic activity there (*1*). Other approaches are the solubilization of enzymes in apolar solvents via reverse micelles (*2–7*), the suspension of enzymes in organic solvents, and the incubation of enzymes in biphasic systems, which is probably the best known method for the moment (*8,9*).

Unfortunately, not many enzymes withstand these treatments. Using insoluble supports for enzyme catalysis in nonaqueous media might overcome these problems. One proposal was to immobilize the enzymes by simple adsorption to the inert carrier (*4*). The question is, of course, how active are such preparations? It could be shown that chemical reactions that don't need water as a reactant can be significantly accelerated by enzymatic catalysis in organic media (*10*). However, hydrolytic cleavages of substrates dissolved in organic media are hardly described (*11,12*). In this paper, we want to show a possible approach to carry out hydrolytic enzyme reactions in organic solvents with glucuronidase as an example.

β-Glucuronidase (β-D-Glucuronid-glucuron hydrolase EC 3.2.1.31) splits naturally occurring and artificial glucuronides (*13*) into glucuronic acid and aglycones. It accepts also many D-glucosides as substrates (*14*). The soluble enzyme is rather stable in aq. media, but becomes completely denaturated after a short time in the presence of organic solvents unless it is protected by immobilization as will be shown in this paper.

MATERIALS

β-Glucuronidase (β-D-glucuronid-glucuron hydrolase EC 3.2.1.31) from Helix pomatia, controlled pore glass CPG 240-200, 4-nitrophenyl glucoside, 4-nitrophenyl glucuronide, bovine serum albumine (BSA), and convallatoxin were obtained from Sigma, St. Louis; ninhydrin, sodium borohydride, inorganic acids, organic solvents, silica gel plates (60F254), and all buffer substances were purchased from Merck, Darmstadt; glutaraldehyde (25%) was from Fluka, Buchs; Sephadex G 200 was purchased from Pharmacia, Uppsala; and convallosid, scillarenin-β-D-glucosid and scilliphaeosidin-β-D-glucoside were prepared by the Institute of Pharmacognosy, University of Vienna.

METHODS

Immobilization via Crosslinking

Immobilization of the glucuronidase on the surface of controlled pore glass via crosslinking with aged glutaraldehyde was carried out as described in Part 1 of this article (*15*).

Aging of Glutaraldehyde Before Use as a Crosslinking Reagent

In aq. solutions at 8°C and pH 4.5, glutaraldehyde forms several hydrated linear and cyclic modifications besides the monomer. Beyond that, several oligomeric and polymeric modifications are referred in the literature (*16*).

Several methods of aging have been employed: Artificial aging between pH 6 and 8 for several hours at rt or at 80°C did not give the desired results. Only the time consuming method, when commercially available glutaraldehyde (25%) was diluted with water to a concn. of 2.5% and stored at 10°C for at least 3 mo, gave satisfactory results. The progress of the procedures was controlled by spectrophotometric measurements between 190 and 350 nm.

Assays for the Products Formed During the Enzymatic Action in Organic Solvents

For the separation, identification, and semiquantitative estimation of all compounds thin layer chromatography on silica gel plates (60F254, Merck) was used. The development of the chromatograms was carried out with several solvent systems:

(a) chloroform/methanol/water = 70:20:3.5
(b) chloroform/methanol/water = 60:40:10
(c) ethylacetate/methanol/water = 81:11:8

For visualization of the spots, the preheated plates (103–105°C) were sprayed with H_2SO_4.

For quantitative determinations HPLC was employed (*17–19*).

Determination of Protein Content

The quantitative determination of immobilized protein was carried out on an amino acid analyzer after acid hydrolysis in 6*M* HCl for 22 h at 110°C or according to Jacobs (*20*).

RESULTS AND DISCUSSION

The aim of these studies was to work out simple procedures to split naturally occurring glucuronides and glucosides, dissolved in organic solvents with the help of the enzyme glucuronidase, for laboratory use. Optimal coupling and working conditions have been worked out in Part 1 of this article (*15*) with the artificial substrates *p*-nitrophenyl glucuronide and *p*-nitrophenyl glucoside. The problem now was to adapt these conditions to the splitting of natural substances.

Working Conditions in Organic Solvents

Immobilizates Obtained by Crosslinking with Dialdehyde in the Presence of BSA

The enzyme coated controlled pore glass was prepared as described in the Methods section. This type of immobilized glucuronidase was used since it gave good results with the artificial substrate under standard conditions (*15*) and did not shrink in the presence of hydrophobic solvents. It has to be pointed out emphatically that for synthesis of the enzyme-immobilizate, 2.5% glutaraldehyde that was aged by storage for at least 3 mo at 4°C had to be used. Only such preparations were able to split natural substances.

Preparations with fresh glutaraldehyde were only capable for splitting the smaller artificial substrates. Also, artificially aged glutaraldehyde solutions (*see* Methods) led to rather poor results. As already pointed out in Part 1 of this article (*15*), even with long-term experiments no leakage could be observed when the immobilizates were stored in 1*M* acetate buffer pH 3.5 at 30°C. Under such conditions, the soluble enzyme and some covalent immobilizates dissociate to a high extent into their subunits, thus loosing their activity.

To study the activity in various hydrophobic organic solvents, aliquots (20 mg) of wet glucuronidase immobilizate were suspended in 4 mL of solvent, containing 1 mg of hexadecyl trimethylammonium bromide as a phase transfer catalyst. Aqueous substrate solution (1 mL) containing 0.5 mg 4-nitrophenyl glucuronide (or -glucoside)/mL and one drop of acetate buffer (1*M*, pH 3.5) were added. The reaction vessel was tightly covered with a screw cap and the mixture incubated for 30 min at 38°C. Subsequently, the organic phase was assayed for the amount of product formed. The results are shown in Table 1.

As can be seen from Table 1, good results could be obtained only with polar hydrophobic solvents and toluene. Since $CHCl_3$ was also an excellent solvent for hydrophobic aglyca of natural compounds, it was used in the following experiments. When stored under these conditions for 2 d, the immobilizate lost 16% of its initial activity that could be fuly restored as described below.

ACTIVITY WITH NATURAL SUBSTRATES: The activity of the immobilizate was tested with the following natural compounds: convallosid—a glycoside with a cardenolid structure, and scillarenin-β-D-glucoside and scilliphaeosidin-β-D-glucoside that are bufadienolides (Fig. 1).

The glycoside (1 mg) was dissolved in 4 mL $CHCl_3$, 20 mg of wet immobilizate were added, and the mixture was kept under shaking at 38°C for 20 h. The small amount of water on the surface of the immobilizate was the only aq. phase in this system. The assay showed that the glycosides could be converted nearly quantitatively to their aglyca under these test conditions.

Table 1
Activity of Glucuronidase Immobilized to CPG via Glutaraldehyde Crosslinking in the Presence of Organic Solvents.

Solvent	Activity, %[a]
Chloroform	117
Isoctane	19
Cyclohexane	9
Toluene	130
Methylene chloride	100

[a] Activity in aq. buffer in the absence of organic solvents is 100%.

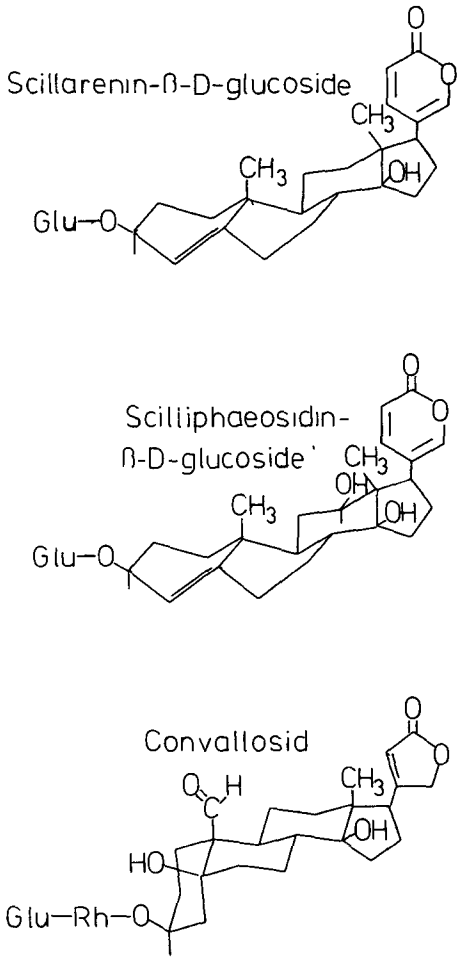

Fig. 1. Natural substrates for immobilized glucuronidase used in this article.

STABILITY IN CHLOROFORM: Aliquots (20 mg) of wet immobilizate were kept in 4 mL $CHCl_3$ in absence of substrate at 38°C under constant shaking. The activity was assayed after 1, 5, 10, 15, 20, 25, 48, 72, and 84 h (*see* Fig. 2) with artificial substrate. As can be seen from the figure, the activity dropped dramatically after 20 h. Substrate present in the reaction mixture stabilized the immobilizate. However, the original activity could be fully restored by keeping the immobilizate in 0.9% NaCl for 24 h. The regenerated immobilizates could be reused many times without loss in activity as long as aging decreased their ability to convert natural substrates to their products. We therefore recommend to use several batches of enzyme immobilizates that can be alternately regenerated, if larger amounts of substrate are to be degraded.

AGING OF THE IMMOBILIZATE: After storage for 1 wk between 4 and 20°C, this type of immobilizate gradually lost its ability to degrade the natural substrates, though it was still fully active with artificial substrate for several months. Treatment of the freshly prepared immobilizates with some aldehydo scavengers ($NaBH_4$, ammonia, or ethanolamine) to remove excess aldehyde groups only led to slight improvements: The immobilizates were still capable for degrading the natural glycosides after 1 mo of storage. However, the turnover at that time was very slow (several days) and the formation of product incomplete, varying between 30 and 40% with all glycosides.

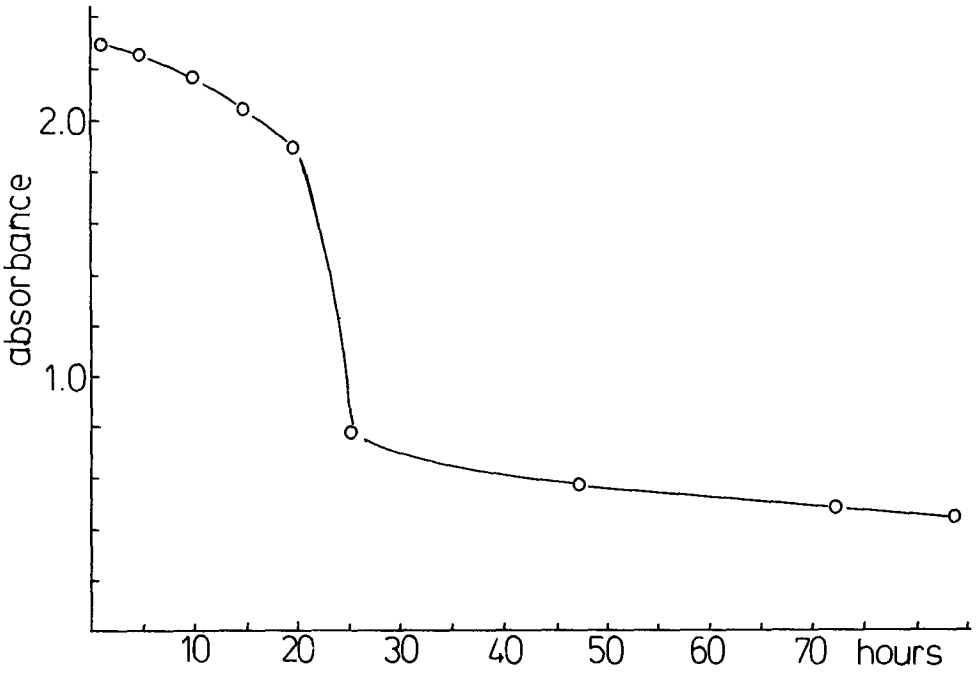

Fig. 2. Activity of the glucuronidase/BSA immobilizate during storage in $CHCl_3$. The change in absorbance/100 mg immobilizate when incubated with 4-nitrophenyl glucuronide for 30 min was measured at 405 nm.

Immobilizates Obtained by Adsorption of Glucuronidase to the Carrier

In looking for a most simple immobilizate to reach our aim, we turned to glucuronidase preparations with only an adsorbed enzyme. To make hydrolytic cleavage of substrates possible in organic solvents, a minimum aq. microenvironment had to be provided for the enzyme. To reach this aim, 100 mg glucuronidase dissolved in 10 mL of an 1% aq. NaCl solution were added to 1 g controlled pore glass. The suspension was degassed for 5 min. After 2 h of constant shaking, 5 mL of chloroform were added. The carrier was allowed to sediment into the lower chloroform layer. After decantation of the aq. layer, the immobilized glucuronidase was stored under chloroform at 4°C for further use. It is recommended to use always freshly prepared immobilizates, since the activity decreased within several weeks. The immobilizates were able to split the natural substrate convalloside dissolved in various organic solvents. The results are shown in Table 2.

The best results were obtained when the wet immobilizates were incubated with the substrate in water immiscible solvents. Addition of a phase transfer catalyst did not improve the results. The amount of product formed after 30 min could not be exceeded by longer incubation times. Only stripping the immobilizate with a mixture of solvent and water (4:1 v/v), under shaking for 10 min, led to slightly further increases of substrate degradation by the immobilized enzyme. Quantitative splitting of the substrate never was observed. With the help of the stripping procedure, water was brought to the microenvironment and glucuronic acid formed by the enzymatic reaction could be removed, thus shifting the chemical equilibrium towards product formation.

Table 2
Activity of Glucuronidase Obtained by Adsorption to the Carrier

Solvent	Yield of product, %
Cyclohexane	66
Toluene	66
Chloroform	50
N-propanol	0
Pyridine	0
Dichloromethane	33
Carbon tetrachloride	66
Dimethyl sulfoxide	0
Tetrahydrofurane	0
Chloroform/methanol (1:1)	0
Ethylacetate	66
Acetone	0
Dioxane	0

Summing up, the results clearly show that it is possible to carry out even hydrolytic enzymatic actions in organic solvents if an aq. microenvironment of the immobilized enzyme can be provided. This microenvironment must be regenerated by stripping to maintain the enzymatic action.

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